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Coordinator: Prof. Francesca Ariani

Comprehensive Evaluation of PD-L1, BCL-2 by Next-Generation Flow Analysis and FISH Abnormalities of Aberrant Plasma Cells in Patients Affected by Smoldering Multiple Myeloma

Disciplinary scientific sector: Blood Disorders

Tutor
Prof. Monica Bocchia

PhD Candidate
Dr. Francesca Bacchiarri

Francesca Bacchiarri

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University of Siena
Dept. of Medical Biotechnologies
Dept. of Medical Sciences, Surgery and Neurosciences
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Abstract

Introduction. Smoldering multiple myeloma (SMM) occupies an intermediate position between what is usually referred to as a pre-malignant condition, monoclonal gammopathy undetermined significance (MGUS) and symptomatic multiple myeloma (MM). SMM has a higher disease burden than MGUS, but does not show end-organ damage or any of the other myeloma-defining events (MDE) observed in MM. In SMM patients tumor circulating plasma cells (CTPC) have been associated with increased risk of progression to Multiple Myeloma (MM).

Aim. The aim is to evaluate by next-generation flow (NGF) the characteristics of PCs in bone marrow (BM), the presence of CTPC, the expression of BCL-2 and PD-L1, correlating the results with FISH analysis for del17p, t(4;14), gain 1q, t(11;14), t(14;16).

Results. From September 2019 to June, 2022 we analyzed 28 patients (M 20; F 8) with a median age of 66 years (40-85). Patients were monitored according to current IMWG guidelines. According to MAYO risk model 5 were at low risk, 17 at intermediate and 6 at high risk of progression. Currently CPCs were not detected at screening, or at subsequent evaluations/last follow up. The most expressed markers were CD56 (89 %), CD27 (92 %), CD81 (71 %), 2 markers were less expressed: CD28 (42 %), CD117 (28 %), CD200 (10 %), CD20 (14 %), CD19 and CD45 (3,5%). CD19 was present in only 1 female patient at intermediate risk who progressed to active myeloma, now under treatment, while CD45 was found in 1 female patient at intermediate risk who maintained a stable disease at last follow-up. Coexistence of markers that are mutually excluded was detected in 10/28 patients (CD27+CD28+) and in 3/28 cases (CD27+CD28+CD81+CD117+) respectively. BCL-2 (MFI) was highly expressed in 11/28 cases (Median 13,4; ≥ 13,5 in 33.3%) while PD-L1 was positive in 7/28 cases (25%). None of the patients had high risk cytogenetic features. At last follow up 4/28 patients (2 M; 2 F) had a progression to multiple myeloma: at diagnosis 3 were at intermediate risk and 1 at high risk, according MAYO progressive model. PFS was in general affected by FLCr (P= 0,0013). Also, an higher percentage of bone marrow plasmacytosis (>30%) can negatively impact on PFS with statistical significance (P = 0,0332). PFS was significantly affected by the expression of BCL-2 (P= 0,094): in the group with BCL-2 negativity mean PFS was 61,778 vs the mean PFS of 90 months in those who were positive. PFS seems to be affected also by PD-L1 levels, even if without statistical significance (P= 0,2986).

Patients at high MAYO risk seem to have an inferior survival, but a statistical significance in the different categories was not determined (P= 0,19). PFS was not statistically affected by CM entity and B2M. All 28 patients are alive at last follow up.

Conclusions. Current standard of care in SMM is still close surveillance, outside of a clinical trial. Our data need further investigations. What confers a positive trend in PFS in the patients that over expressed BCL-2 needs to be further explored. The diversified expression of analyzed markers confirms the high heterogeneity and complexity of the smoldering phase in MM. Research identifying more accurate genomic, clinical, laboratory and/or cytometric markers on PB that would enable us to assign individual risk more precisely is ongoing.

CHAPTER 1

PLASMA CELL NEOPLASMS: MGUS

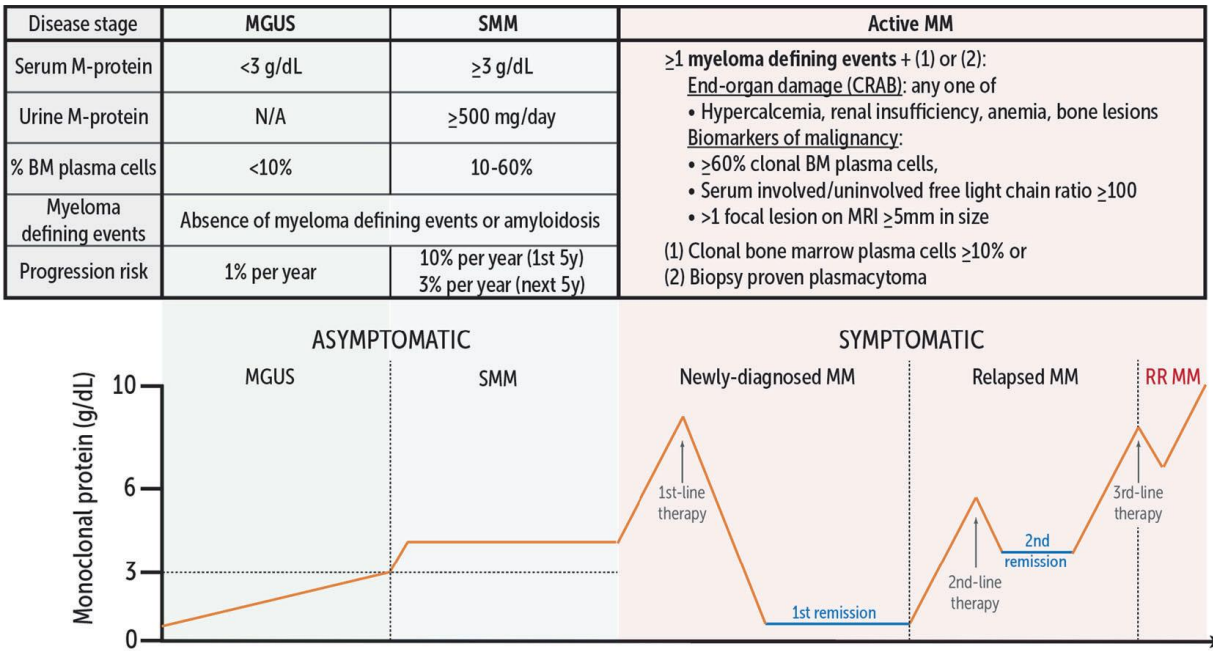
1.1 Introduction

Plasma cells (PC) are mature antibody-producing B cells which reside in the bone marrow (BM) and are essential for maintaining humoral immunity. Plasma cell neoplasms (PCN) are an heterogenous group of end-stage antibody-producing B-cell (i.e., plasma cell) disorders and are classified based on the occurrence of specific symptoms and levels of monoclonal antibodies (most commonly IgG, but can also be IgM, IgA, or very rarely IgD) [*Swerdlow. Blood 2016*].

1.2 Monoclonal gammopathy of undetermined significance (MGUS)

MGUS is defined by a serum monoclonal protein of <3 g/dL (typically non-IgM-type), <10% clonal BM plasma cells, and the absence of end-organ damage ad may progress to another asymptomatic, but more advanced premalignant stage known as smoldering multiple myeloma (SMM) [*Rajkumar, Lancet Oncol.2014;Kyle, N Engl J Med. 2007*].

Fig. 1: Clinical model of disease progression in MM: the IMWG diagnostic criteria. MM arises from an asymptomatic precursor disease. MGUS which is characterized clinically by low tumor burden (M protein<3 g/dL, %BMPC < 10%) and a risk of progression to symptomatic MM of about 1% per year. As tumor burden increases beyond a threshold (M-protein ≥3 g/dL, %BMPC 10–60%), the disease progresses to smoldering multiple myeloma (SMM), another asymptomatic disease state prognostically distinct from MGUS. MM is incurable as patients progressively acquire resistance to therapy progressing through different line of treatment and eventually die. [*Ho Leukemia. 2020*]



The overall prevalence of MGUS is at 2.4% [*Landgren, Leukemia.2014*], with a median age of diagnosis of 70 years, MGUS is predominantly a disease of the elderly, and <2% of patients are <40 years [*Landgren, Blood Cancer* 2017*; Kyle, NEJM 2006*]. The age-adjusted prevalence of MGUS is highest in the black population (0.99%), followed by Mexican Americans (0.55%), and lowest in whites (0.21%) *[Landgren, Blood Cancer* 2017].

MGUS in black patients is associated with lower M-protein levels, higher rate of abnormal FLC ratio, younger mean age distribution, and lower IgM gammopathy prevalence [*Cohen, Am J Med. 1998; Landgren, Mayo Clin Proc. 2007;Landgren, Leukemia. 2009; Weiss, Am J Hematol. 2011].*
Although the prevalence of MGUS is higher in black patients, the rate of progression to MM is the same [*Landgren, Blood. 2006*]. These studies suggest that race and genetic ancestry are important considerations in prognosis, counseling, clinical management, and screening efforts.
MGUS is considered precursors to MM with a 1% risk of progression per year [*Mateos, Cancer Treat Res 2016*].

1.3 Risk factors

Important risk factors in patients developing MGUS and in general MM, include hereditary factors (increased risk of MGUS in first-degree relatives of patients with MGUS) [*Vachon Blood.2009*], male sex (two-folds higher risk) [*Kyle, Res Clin Haematol. 2005*], immunocompromised state (e.g., HIV patients, post-transplant immunosuppression) [*Dezube, AIDS Read. 2004*], occupational exposure to toxins such as asbestos, fertilizers and pesticides, aromatic hydrocarbons, mineral oils, petroleum, and paint [*Landren, Blood 2009*], as well as cigarette smoking [*Landgren, Blood Cancer J.2017*].
Also inflammatory/autoimmune diseases can play an important role: a study of over 4 million male US veterans identified 6687 people with a PC disorder and found that MGUS/MM are associated with broad categories of inflammatory, infectious, and autoimmune disorders with median relative risks of 1.18, 1.29, and 1.15, respectively [*Brown, Blood.2008*].
One retrospective study on veterans found that MM is associated with higher body mass index [*Samanic, Cancer Causes Control.2004*].
In contrast, a retrospective study on 575 Swedish MGUS patients did not find higher rates of MGUS in obese patients but observed an increased progression rate to MM (hazard ratio, HR = 2.66) [*Thordardottir, Blood Adv. 2017*]. A retrospective study of US veterans similarly found that obesity was associated with increased progression. [*Chang SH, J Natl Cancer Inst. 2016*].
In the same cohort, metformin use for at least 4 years among patients with diabetes and MGUS was associated with a lower risk of progression to MM (HR = 0.47) [*Chang, Lancet Haematol. 2015*]. These studies implicate dysregulated metabolism in the development and progression of MGUS and suggest that weight modification and glycemic control may be potential targets for modifying the disease's natural history.

1.4 Environmental exposures

An association between MGUS and radiation exposure was established in a study on 52 525 survivors of the Nagasaki atomic bomb [*Iwanaga, Blood. 2009*]. Although MGUS prevalence in Japan is 2.4% in those >50 years of age [*Iwanaga, Mayo Clin Proc. 2007*], there was a higher prevalence of MGUS in people living within 1.5 km of the bomb hypocenter, compared with those living >3 km away, with a mean prevalence ratio of 1.4. This finding was noted in those who were exposed to a high radiation dose (>0.1 Gy) at an age of ≤20 years. However, this study reported a low rate of progression to MM of 0.7% per year that was independent of radiation exposure.

A prospective study on 555 men working with pesticides (dieldrin, carbon-tetrachloride/carbon disulfide, and chlorothalonil) found a 6.8% MGUS prevalence [*Landgren, Blood 2009*]. Similarly, a study of 479 veterans exposed to the herbicide Agent Orange found an increased risk (odds ratio = 2.37) and prevalence (7.1%) of MGUS [*Landgren, JAMA Oncol. 2015*].

In a 10-year follow-up of 3949 German participants, long-term residential exposure to particulate matter was associated with increased MGUS incidence, and risk increased with larger particle size [*Orban, Environ Int. 2017*]. A recent study on 781 firefighters exposed to airborne carcinogens during the 9/11 World Trade Center attacks found a 7.63% prevalence [*Landgren, JAMA Oncol. 2018*]. More specifically, light-chain MGUS prevalence was more than threefold higher. Air contaminants act as carcinogens and can trigger chronic inflammation, autoimmunity, and, potentially, inflammation-induced oncogenesis, emphasizing a possible immune component in myelomagenesis [*Izbicki, Chest. 2007; Weiden, PLoS One. 2012; Webber, Arthritis Rheumatol. 2015*].

1.5 Familial MGUS and the mode of inheritance

Familial clustering of MM patients has been reported [*Lynch, N Engl J Med. 2008*] and analyses of the largest familial study from the Swedish database reported a 1.7-fold increase in risk of developing MM among first-degree relatives of MM patients [*Landgren, Int J Cancer. 2006*]. That same database, encompassing 4458 MGUS patients and 14 621 first-degree relatives, later revealed that relatives of MGUS patients had a fourfold and 2.9-fold elevated risk for developing MGUS and MM, respectively [*Landgren, Blood. 2009*].

A series of investigations aimed at uncovering the mode of inheritance of MGUS/MM discovered that hyperphosphorylated Paratarg-7 (pP-7), a protein of unknown function, is linked to both familial and nonfamilial MGUS and MM [*Grass, Lancet Oncol. 2009; Grass, Blood. 2011*]. Analyses of 8 families revealed that pP-7 is inherited in a dominant fashion and leads to the development MGUS and MM (odds ratio = 7.9).

Investigators hypothesized that hyperphosphorylation could induce autoimmunity via chronic antigenic stimulation, which in turn may lead to develop a PC disorder. Interestingly, 1 study detected a hyperresponsive B-cell phenotype that was shared by several individuals within families with cases of MGUS/MM [*Steingrímsdóttir, Eur J Haematol. 2011*].

Furthermore, pP-7 was detected in 37% of African Americans, compared with 16.7% in Europeans and 4% in Japanese MGUS/MM patients, suggesting a role for this genetic factor among African Americans [*Zwick, Int J Cancer. 2014*].

Genome-wide association studies (GWASs) were also done to analyze single-nucleotide polymorphisms. Numerous loci were found to influence the risk of developing MGUS and MM, including 3p22.1 (rs1052501), 6p21.33 (rs2285803), 7p15.3 (rs4487645), and 17p11.2 (rs4273077) [*Martino Br J Haematol. 2012; Broderick, Nat Genet.2011; Chubb, Nat Genet. 2013*]. Interestingly, loci 7p15.3 includes CDCA7L, a MYC-interacting gene, making it a potential region for further analysis due to the major role of MYC in driving myeloma [*Maertens, Cell Sci. 2006; Walker, Blood. 2018*].

Another genome-wide association study identified 23 novel loci interactions regulating B-cell receptor, epidermal growth receptor, and cell adhesion–related pathways, which could be related to MGUS development and progression [*Chattopadhyay, Mol Med. 2018*].

1.6 Genomic landscape

1.6.1 Microarrays and next-generation sequencing

Copy-number abnormalities (CNAs), including gains of 1q, 3p, 6p, 9p, 11q, 19p, 19q, and 21q and deletions of 1p, 16q and 22q, can be detected in MGUS, but at a lower frequency (60,6%) compared with MM (100%) [*López-Corral, Leukemia. 2012; Smetana, BioMed Res Int. 2014; Mikulasova, Haematologica. 2017*]. On the other hand, the most common CNAs for IgM MGUS/WM are del6q, +18q, trisomy 4, 5, 12, and monosomy 8 [*Mansoor, Am J Clin Pathol. 2001; Paiva, Blood. 2015*]. However, there seems to be a temporal acquisition of CNAs, some of which are more prevalent at later stages. For example, although del6q is detected in smoldering and symptomatic WM, it is not seen in IgM MGUS, indicating that it may be a secondary event [*Paiva, Blood. 2015; Schop, Cancer Genet Cytogenet. 2006*].

Furthermore, although t(11;14) is uniform across the full non-IgM disease spectrum, t(4;14), t(14;16), and del13q are more common in the SMM and MM stages and may follow a nonrandom natural biological history with 1 chromosomal defect routinely preceding another [Avet-*Loiseau, Cancer Res. 1999; Avet-Loiseau, Blood. 2002; Fonseca, Blood. 2002; Chiecchio, Haematologica.2009; López-Corral, Clin*

Cancer Res. 2011]. Notably, del13q is dependent on the genetic context, whereby it is rare in MGUS and SMM patients with a t(11;14) and t(6;14) compared with MM, whereas it is equally prevalent in all 3 stages in the presence of t(4;14) and t(14;16) [*Chiecchio, Haematologica.2009*].

MM-associated somatic mutations (KRAS, NRAS, DIS3, HIST1H1E, EGR1, and LTB) were detected in few MGUS cases, suggesting a less complex genomic landscape [*Mikulasova, Haematologica. 2017*].

Yet, this may be explained by the low tumor fraction in MGUS, where single-nucleotide variants are more easily missed compared with CNAs. Moreover, MYC translocations and TP53 deletions and mutations were not detected in MGUS, suggesting that these may be drivers of progression. Also, the median number of CNAs was positively correlated with higher risk groups and acquiring somatic mutations. On the other hand, IgM MGUS/WM have a different set of somatic mutations with the most common being the MYD88 L265P mutation, followed by mutations in CXCR4 and KMT2D, in addition to lower frequency mutations including ARID1A, CD79b, MYDBBP1A, NOTCH2, PRDM1, TP53, TRAF3, and TNFAIP3 [*Varettoni, Haematologica. 2017*]. Notably, it was found that most primary translocations result from an aberrant IgH switch recombination event in pre–germinal center B cells leading to increased oncogene expression [*Walker, Blood. 2013; Rasmussen, Haematologica. 2010*].

1.6.2 Gene and microRNA expression profiling

Although DNA studies provided a robust understanding of MM pathogenesis, gene-expression profiling (GEP) studies further characterized disease states. One study reported 52 differentially expressed genes between PCs of patients (MGUS, SMM, and MM) and controls [*Zhan, Blood.2007*]. They identified 4 signatures that classified patients into: MM-like MGUS, non–MM-like MGUS, MGUS-like MM, and non–MGUS-like MM, whereby the MM-like MGUS have an increased risk of progression and the MGUS-like MM have a longer survival. A 70-gene signature (GEP-70) in newly diagnosed MM patients was also found to correlate well with survival and myeloma staging [*Shaughnessy, Blood. 2007*]. A prospective study (SWOG0120) later found that a GEP-70 score more than −0.26 and GEP proliferation index more than −2.73 predicted an increased risk of MGUS/SMM transformation into MM [*Dhodapkar, Blood. 2014*].

One of the most important contributions of GEP was identifying the dysregulation of CCND1 (11q13), CCND3 (6p21), or CCND2 (MAF [16q23] and MAFB [20q11]) as a unifying early event in nonhyperdiploid MGUS [*Fonseca and Debes-Marun CS,Blood 2003; Bergsagel, Blood.2005*].

Expression profiling of 345 microRNAs (miRNAs) in PCs found 41 upregulated and 7 downregulated miRNAs in MGUS compared with normal PCs [*Pichiorri, Proc Natl Acad Sci USA.*

2008] some of which play a role in B- and T-cell differentiation [*Chen, Science. 2004*]. In particular, miRNA-21, -181a, and -106b~25 may involve alterations in the p53 pathway, as they are known to target the p300-CBP–associated factor that acetylates p53 [*Kubiczkova, Haematologica. 2014*].

Moreover, circulating serum miRNA-744, miRNA-130a, miRNA-34a, let-7d, and let-7e were found to be dysregulated in both MGUS and MM, and miRNA-34a and let-7e can particularly distinguish MGUS patients from healthy individuals with a sensitivity of 91.1% and specificity of 96.7% *[Kubiczkova, Haematologica. 2014*].

Additional epigenetic studies found specific genes to be hypermethylated in both MGUS and MM, including p15, p16, p53, DAPK, ARF, SOCS-1, E-cadherin, and hMLH-1 [*Seidl, Cancer. 2004;Martin, Exp Mol Pathol. 2008; Stanganelli, Ann Hematol. 2010; Geraldes, Clin Lymphoma Myeloma Leuk. 2016*], revealing that early MM stages exhibit a similar pattern of tumor-suppressor gene methylation but with a lower methylation index in MGUS.

1.7 Tumor microenviroment

The BM is a collection of cellular (immune, endothelial, adipocytes, mesenchymal stem cells, reticular, and osteolineage cells) and noncellular components, extracellular matrix (ECM), and soluble factors, all of which maintain homeostatic hematopoiesis. Therefore, studies have begun focusing on the BM composition as a permissive microenvironment for clonal selection and progression from MGUS to MM.

1.8 Osteolineage cells

Osteolytic lesions are 1 of the hallmarks of MM and are mainly driven by receptor activator of NF-κB ligand (RANK-L) upregulation and osteoprotegerin (OPG) downregulation in osteoblasts, which activates osteoclasts [*Roodman, Leukemia. 2009*]. Although bone lesions are not observed in MGUS, the RANK-L/OPG and bone fracture risk is already higher [*Politou, Br J Haematol. 2004; Kristinsson, Blood. 2010*]. MM mouse model studies have shown that MM cells can secrete Dickkopf-related protein 1 (DKK1), a Wnt/β-catenin pathway inhibitor, and the antiosteoblastic factors transforming growth factor β and hepatocyte growth factor, which in turn can suppress BMP2 and RUNX2 that induce apoptosis and suppress proliferation and differentiation of osteoprogenitors [*Ducy, Cell.1997; Giuliani, Leuk Lymphoma. 2007; Toscani, Ann N Y Acad Sci. 2015*]. Although these activated pathways are mainly studied in mice experiments, it is still imperative that we elucidate their presence in humans.

1.9 Stromal, endothelial, and mesenchymal stem and progenitor cells

Mesenchymal stem and progenitor cells comprise a major portion of the BM niche and are responsible for regulating adhesion and migration, via VCAM1 and ICAM1, and survival and proliferation of MM cells through direct cell-cell interactions and secreting growth or antiapoptotic factors (insulin-like growth factor 1 [IGF-1], interleukin 6 [IL-6], and CXCL12) [*Roodman, J Bone Miner Res. 2002;Podar, Best Pract Res Clin Haematol. 2007*]. Interestingly, in vitro studies using human cells revealed that these cells are persistently abnormal even in the absence of MM cells, which may explain the nonhealing bone lesions that remain after successfully eradicating the malignant cells [*Reagan, Clin Cancer Res. 2012*].

GEP studies of human stroma, ranging from MGUS and up to relapsed/refractory MM, did show a differentially expressed signature compared with healthy individuals and these included IL-6, DKK1, HOXB [*Todoerti, Exp Hematol. 2010*] and wound healing, tumor necrosis factor α, and hypoxia pathways [*Anguiano, J Clin Oncol.2009*]. The BM is a hypoxic environment and becomes even more hypoxic in the presence of MM cells, thereby inducing endothelial cell neoangiogenesis via expression of IL-17, syndecan 1, hypoxia-inducible factor 1, and vascular endothelial growth factor [*Colla, Leukemia. 2010; Moschetta, Biochim Biophys Acta. 2014*]. This hypoxia is known to drive epithelial to mesenchymal transition of MM cells, decreasing E-cadherin and increasing CXCR4 expression, which promotes myeloma dissemination [*Azab, Blood. 2012*].

Although most of these studies investigated the makeup of myeloma BMs, these changes could have already taken place in the MGUS stage, as other studies have found. For example, fibroblasts were found to begin modifying the BM niche in MGUS, reflected by the gradual increase in the ECM-remodeling proteomic makeup [*Slany, J Proteome Res. 2014*]. Characterizing the proteomic signature of the BM ECM identified a total of 11 proteins in MGUS, compared with healthy controls and MM, including 2 core (proteoglycan 2 and 3) and 9 matrisome-associated proteins such as ficolin 1, cathepsin G, serpins, HRNR, S100A8, and S100A9 [*Glavey, Leukemia. 2017*].

1.10 Immune composition

Evading and suppressing the host immune system is an important step in the progression of MGUS to MM. Usually, natural killer (NK) cells and cytotoxic T lymphocytes are responsible for eliciting an immune response against cancerous cells, however, tumor cells can suppress these anticancer responses. Immune suppression includes loss of antigen presentation, defective immune cell function, depleted myeloma-specific T cells, and increasing immunosuppressive cell types, such as myeloid-derived suppressor cells and regulatory T cells (T_{regs}) [*Pérez -Andres, Cancer. 2006*].

T-cell expansion is observed in both MGUS and MM patients, yet it is more robust when tumor burden is low in MGUS and decreases during progression to MM [*Famularo, J Clin Lab Immunol. 1992; Dhodapkar J Exp Med. 2003*].

Interestingly, although NK-cell expansion is easily discernible in the peripheral blood (PB) and BM of MM patients, their activity is decreased due to a tumor cell–mediated downregulation of NKG2D on NK cells [*von Lilienfeld-Toal, Cancer Immunol Immunother. 2010*].

Increased immune suppression parallels an increase in T-helper 17 (T_h17) cell abundance [*Bettelli, Nature. 2006*], secretion of several cytokines and growth factors [*Mahtouk, BMC Cancer. 2010*], and MM cell induction of T_{reg} expansion via a contact-dependent manner, via inducible costimulator/inducible costimulator ligand [*Feyler, PLoS One. 2012*].

Additionally, stromal cells were shown to inhibit both T- and B-lymphocyte function by activating T_{regs}, leading to a poorer outcome [*Feyler, Br J Haematol. 2009*].

Also, T_{reg} expansion due to MM cell secretion of type 1 interferons was found in the early disease stages of a murine MM transplantable model demonstrated that survival of mice injected with Vk*Myc cells was prolonged when the T_{reg} population was depleted [*Kawano, J Clin Invest. 2018*].

Importantly, the essential role of the immune microenvironment in driving progression was mimicked in genetically humanized $MIS^{(KI)}$TRG6 mice that were transplanted with CD3-depleted mononuclear cells and injected with primary human premalignant and malignant PCs [*Das, Nat Med. 2016*]. The investigators found that injected primary tumor cells of MGUS patients continued to grow progressively.

1.11 From MGUS to MM: a model of clonal evolution

Genetic analysis of MGUS cells have provided evidence that it is a genetically advanced lesion, where in tumor cells carry many of the genetic changes found in MM cells.

Intraclonal heterogeneity is also established early during the MGUS phase. Although the genetic features of MGUS or SMM cells at baseline may predict disease risk, transition to MM involves altered growth of preexisting clones. Interactions of plasma cells with immune cells, bone cells, and others in the bone marrow niche may be key regulators of malignant transformation.

These interactions involve a bidirectional crosstalk leading to both growth-supporting and inhibitory signals (Fig.2). Because MGUS is already a genetically complex lesion, application of new tools for earlier detection should allow delineation of earlier stages, which we term as pre-MGUS.

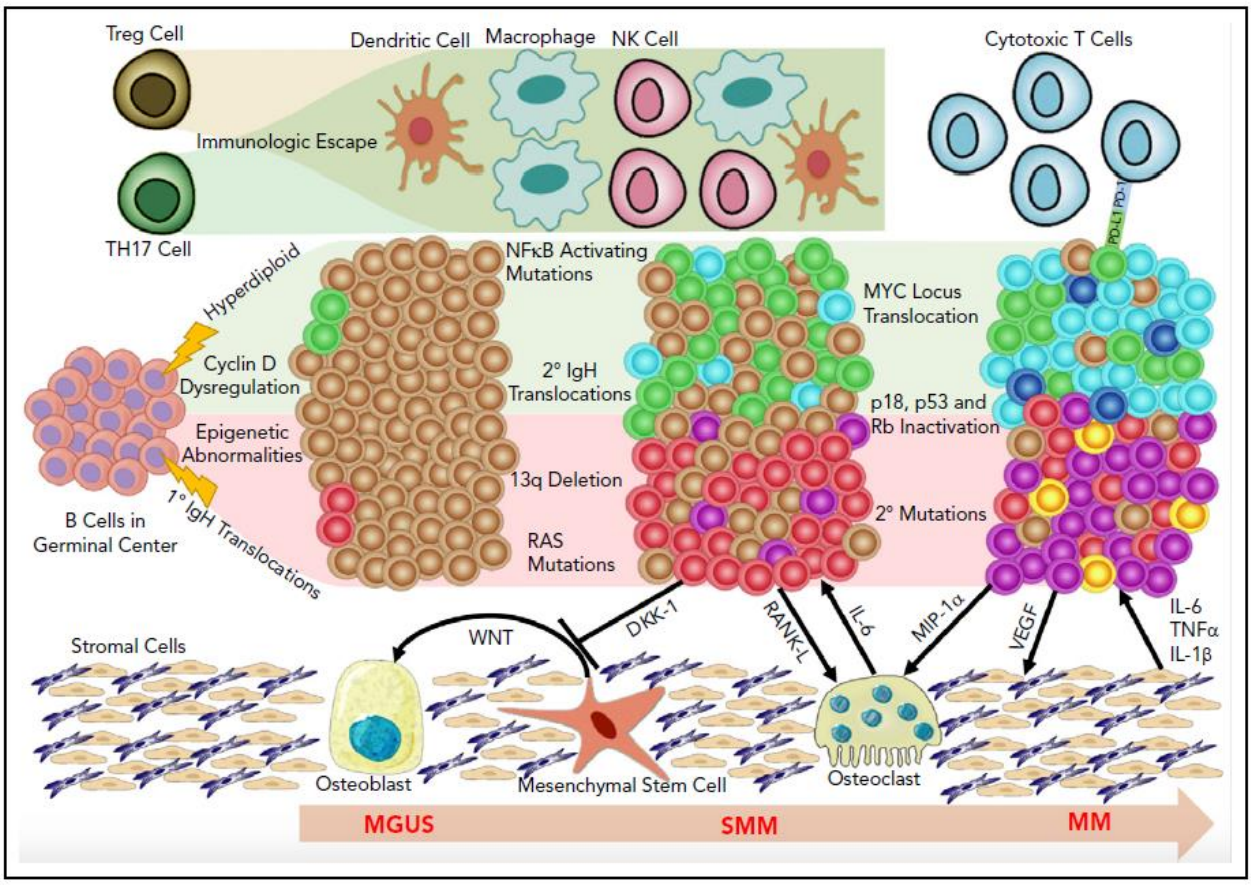


Figure 2: Model of clonal evolution. Myelomagenesis is hypothesized to begin with an initiating event in a germinal center B cell that differentiates into a defected PC carrying chromosomal aberrations and gene-expression and epigenetic signatures that separate it from benign PCs. In the progression from MGUS to SMM, the defected PC clone acquires additional chromosomal aberrations and genetic mutations. This is accompanied by a permissive microenvironment in the BM niche that involves bidirectional crosstalk between the malignant clones and surrounding cells that induces immune suppression and clonal expansion to overt MM. MIP-1a, macrophage inflammatory protein 1a; Rb, retinoblastoma protein; TH17, T helper 17; TNFa, tumor necrosis factor a; VEGF, vascular endothelial growth factor [*Mouhieddine, Blood. 2019*].

1.12 Presentation and clinical consequences of MGUS

MGUS is associated with infections [*Kristinsson, Haematologica 2012*], fractures [*Gregersen, Br J Haematol.2006*], peripheral neuropathy (PN) [*Chaudhry, Mayo Clin Proc.2017*] thromboembolism *[Kristinsson, Blood 2008*], and monoclonal gammopathy of renal significance (MGRS) [*Fermand. Blood. 2013*]. Retrospective studies linking adverse clinical events to MGUS are challenged by lack of clinical testing to prove causal relationship (except for MGRS) and the potential for overestimation of risk given that patients with infection, thromboembolism, or fracture are more likely to seek medical care. Still, there is some rational basis for risk of adverse events in MGUS. Deficient humoral immune responses in MGUS may lead to high rates of infection in this population and MGUS patients have been shown to have reduced antibody response to vaccination [*Prabhala, Blood. 2009*]. Increased hip and spinal fractures are thought to result from altered bone strength and microarchitecture [*Gregersen, Br J Haematol. 2006*] and may be related to elevated RANK-L/OPG ratios [*Politou, Br J Haematol. 2004*]. MGUS patients have decreased bone mineral

density and increased rates of osteoporosis [*Thorsteinsdottir, Blood Adv. 2017*], but with increased bone size and cortical porosity and decreased cortical thickness, suggesting that bone density and strength is altered in a manner distinct from decreased mineralization [*Farr, Blood. 2014*].

PN is found in 10% of MGUS patients, and more commonly in the IgM type [*Chaudhry, Mayo Clin Proc. 2017*].

IgM MGUS is associated with distal acquired demyelinating symmetric neuropathy that presents with sensory ataxia and mild distal motor deficits. Anti–myelin-associated glycoprotein (MAG) antibodies are detected in 50% of these patients [*Gosselin, Ann Neurol.1991*], whereas other PNs may be associated with anti-ganglioside antibodies. There is no proven causal relationship for neuropathy in non-IgM MGUS, so care should be taken to exclude all other potential causes of neuropathy. Rarely, IgG/A MGUS is associated with neuropathy manifesting as a chronic inflammatory demyelinating polyradiculopathy with proximal and distal motor deficits [*Chaudhry, Mayo Clin Proc. 2017*], or axonal neuropathy involving distal extremities that begins with sensory ataxia and progresses slowly to motor weakness [*Rison, BMC Neurol.2016*].

MGRS describes a group of kidney disorders (renal impairment and/or proteinuria) caused by the physiochemical and immunologic properties of deposited monoclonal immunoglobulins in premalignant PC dyscrasias and are diagnosed by renal biopsy [*Leung, Blood. 2012*].

MGRS includes glomerulopathies with immunoglobulin depositions such as those with fibrillar amyloidosis (immunoglobulin light chain [AL], heavy chain [AH], and light and heavy chain [ALH]), microtubular (type I and type II cryoglobulinemias, immunotactoid glomerulopathy), or nonorganized deposits (monoclonal immunoglobulin deposition disease [MIDD]). Proliferative glomerulonephritis with monoclonal immunoglobulin deposits and tubular disorders such as Fanconi syndrome are also types of MGRS [*Fermand, Blood. 2013*].

In a retrospective study of 37 MGRS patients, 22% progressed to end-stage renal disease (ESRD) over an average follow-up of 30.3 months [*Nasr, J Am Soc Nephrol.2009*].

In another retrospective evaluation of 19 MIDD patients, 5-year ESRD-free survival was only 37% [*Heilman, Am J Kidney Dis.1992*].

Notably, for patients who develop ESRD, MGRS often recurs after renal transplant and is associated with allograft loss [*Heilman, Am J Kidney Dis.1992*; *Said, Kidney Int. 2018*].

Importantly, complete hematologic response prior to renal transplant appears to reduce the risk of recurrence and is therefore important for allograft survival [*Sayed, Blood. 2015*]. Thus, it is important to monitor renal function of MGUS patients and maintain a high index of suspicion for MGRS in patients with otherwise unexplained renal dysfunction.

1.13 Management of MGUS

The current standard of care for MGUS is monitoring for progression to enable early detection and intervention. However, growing impetus for investigating early intervention strategies is derived from improved overall survival (OS) and reduced complications in patients who were monitored prior to the diagnosis of MM [*Sigurdardottir, JAMA Oncol. 2015; Bianchi, Blood. 2010*].

In the Mayo Clinic risk-stratification model, progression risk is increased with serum monoclonal protein (>1.5 g/dL), non-IgG disease, and abnormal serum FLC ratios (<0.26 or >1.65) [*Rajkumar, Blood 2005; Kyle N Engl J Med. 2018*].

At time of presentation, MGUS patients should be stratified based on number of risk factors: high-risk patients possess all 3 risk factors and have a 20-year progression risk of 58%; high-intermediate-risk have 2 risk factors and a 37% progression risk; low-intermediate-risk have 1 risk factor and a 21% chance of progression; low-risk have no risk factors and a 5% progression risk [*Rajkumar, Blood 2005; Kyle, N Engl J Med. 2018*].

Alternatively, the PETHEMA Study Group risk-stratified patients using a ratio of abnormal/normal PCs >95% and DNA aneuploidy (hyperdiploidy or hypodiploidy). At 5 years, patients with abnormal PCs >95% and aneuploidy were found to have a 46% risk of progression compared with a 10% risk for patients with 1 risk factor and 2% for patients with none [*Pérez-Persona, Blood. 2007*].

Because the risk of progression varies per patient, the extent and frequency of evaluation is based on individual risk (Table 1).

Table 1. Risk-stratified management of MGUS patients [*Mouhieddine, Blood. 2019*]

All MGUS patients	Risk stratification		Classification	Additional evaluation at diagnosis	Monitoring and evaluation
SPEP, CBC, creatinine	Risk factors for progression :	0 risk factors	Low risk	No additional testing required	Repeat SPEP, CBC, and creatinine in 6 mo and then every 2-3 y if stable, or when symptoms of progression arise
	• M-protein, >1.5 g/dL	1 risk factor	Low-intermediate risk	LDH β2-microglobulin Bone marrow biopsy with FISH IgM MGUS → CT chest and abdomen to evaluate for lymphadenopathy Non-IgM MGUS → skeletal assessment† Light-chain MGUS → NTproBNP, cardiac troponins, urine albumin	If additional testing negative → SPEP, CBC, and creatinine in 6 mo then annually for life if remains stable* If signs of progression → decrease follow-up interval and initiate workup for lymphoplasmacytic malignancy
	• Non-IgG paraprotein (IgA or IgM)	2 risk factors	High-intermediate risk		
	• FLC ratio, <0.26 or >1.65	3 risk factors	High risk		

CBC, complete blood count; FISH, fluorescence in situ hybridization; LDH, lactate dehydrogenase.

*
Include NTproBNP, cardiac troponins, and urine albumin for light-chain disease.

†
Low-dose CT preferred.

At the time of diagnosis, all MGUS patients should have a complete blood count, serum creatinine, and calcium. For high and intermediate-risk patients, including those with IgM MGUS, should be added a baseline lactate dehydrogenase (LDH), β2-microglobulin (B2M), and BM biopsy with fluorescence *in situ* hybridization (FISH) to the initial assessment.

Additionally, a skeletal survey or preferentially a low-dose computed tomography (CT) could be useful for non-IgM, high- and intermediate-risk patients [*Mouhieddine, Blood. 2019*].

IgM patients have a much lower risk of bone involvement and so, like low-risk patients, skeletal assessment is not required in the absence of bone symptoms. However, IgM MGUS patients should undergo CT of the chest/abdomen to evaluate for lymphadenopathy, which could indicate lymphoma or WM. Lastly, due to the risk of AL amyloidosis in light-chain MGUS, these patients should be evaluated with baseline N-terminal pro–B-type natriuretic peptide (NTpro-BNP), cardiac troponins, and urine protein electrophoresis [*Dispenzieri, Lancet.2010; Kumar, J Clin Oncol. 2012*].

If additional workup is within normal limits, a second evaluation for progression is still recommended in 6 months for all patients [*Kyle, Leukemia 2010*]. Patients who have progressive rise in M-protein over consecutive measurements have a higher risk of progression than those with stable M-protein [*Rosiñol, Mayo Clin Proc. 2007*].

Follow-up intervals can then be lengthened toward lifetime annual follow-up for high- and intermediate-risk patients, and every 2 to 3 years, or when symptoms of progression arise, for low-risk patients with stable M-protein, as the risk of progression is highest for the first year after diagnosis and declines thereafter [*Kyle, Leukemia 2010; Bianchi, Blood. 2010*].

MGUS patients with neuropathy should undergo extended evaluation including electromyogram/neuromuscular testing, fat pad biopsy, cryoglobulins, and ganglioside/MAG antibody to rule out amyloidosis, cryoglobulinemia, and neurological disorders. [*Chaudhry, Mayo Clin Proc. 2017; Duston, Arch Intern Med. 1989*].

IV immunoglobulin G (IVIG) and rituximab have been used as first-line management for IgM neuropathy whereas plasmapheresis, IVIG, and steroids have been used in IgG/A neuropathy. [*Chaudhry, Mayo Clin Proc. 2017; Rison, BMC Neurol.2016*].

However, these therapies do not eradicate the paraprotein-producing clone.

There are case reports on the use of high-dose chemotherapy alone136 or followed by autologous stem cell transplant 137 for severe debilitating neuropathy in MGUS, but data for these strategies are limited.

MGUS patients who present with renal impairment should be evaluated for AL amyloidosis and MGRS. After confirming MGRS by kidney biopsy, close cooperation with nephrologists is suggested to determine the optimal treatment strategy based on MGRS subtype, degree of renal

impairment, and risk of progression to ESRD. Renal impairment is usually irreversible as there is no available therapy for clearing monoclonal deposits.

Treatment options usually involve the use of chemotherapies or immunotherapies for targeting the clonal cell population to reduce paraprotein production and preserve renal function [*Fermand, Blood. 2013*].

In 1 case series involving 4 patients with dialysis-dependent MIDD, high-dose melphalan plus autologous stem cell transplant was found to be a safe and effective option resulting in durable responses and allowing subsequent renal transplantation [*Batalini, Biol Blood Marrow Transplant. 2018*].

The lack of treatment approaches that are safe and effective for MGUS-related neuropathy and MGUS with renal impairment highlight an area of need for novel therapeutic agents to be tested specifically in these patients' subgroups.

Currently, daratumumab is being tested in some of these cases but its efficacy is still unknown.

There are no US Food and Drug Administration (FDA)-approved treatments to eradicate MGUS or prevent progression, and patients currently receive therapeutic intervention in the context of a clinical trial only [*Mouhieddine, Blood. 2019*].

The disadvantage of clinical trials is that many patients will not progress to overt malignancy without any therapeutic interventions and are being unnecessarily exposed to potentially toxic therapy. Studies to better understand risks of progression in patients with MGUS and specifically define those at risk of developing MM in their lifetime should be performed so that more selective approaches are used for these patients' populations.

To reduce potential for harm, investigators have considered more innocuous therapeutics for prevention of progression including green tea extract, curcumin [*Golombick, Clin Cancer Res. 2009*], and nonsteroidal anti-inflammatory drugs [*Birmann, Cancer Prev Res (Phila). 2014*]. Currently, when anti-MM therapies are used in prevention trials, the patients at highest risk of progression are targeted. For example, a study evaluating the use of the CD38 antibody daratumumab in high-risk MGUS and smoldering patients is ongoing (Table 2). Even when targeting high-risk patients, the most appropriate trial designs involve minimally toxic interventions, and clinical trial subjects must be fully informed on their individual risk of progression with the risk of therapeutic side effects.

Table 2. Clinical disorders associated with MGUS [*Mouhieddine, Blood. 2019*].

Clinical disorders	Treatment
Monoclonal gammopathies of renal significance	**Reference** Fermand, Blood. 2013
Immunoglobulin light-chain amylodosis (AL)	Stage 1 and II disease: melphalan + dexamethasone If stage III or severe renal dysfunction: cyclophosphamide/bortezomib/dexamethasone
Immunoglobulin heavy-chain amyloidosis (AH)	
Immunoglobulin light and heavy chain (ALH)	
Type 1 cryoglobulinemia	If plasmacytic IgG or IgA: antimyeloma regimens If lymphoplasmacytic IgM: rituximab containing regimen
Type 2 cryoglobulinemia	Rituximab-containing regimen Treat underlying hepatitis C
Immunotactoid glomerulonephropathy (ITG)	Cyclophosphamide/bortezomib/dexamethasone
Monoclonal immunoglobulin deposition disease (MIDD)	Cyclophosphamide/bortezomib/dexamethasone Successful use of autologous stem cell transplant reported
Proliferative glomerulonephritis with monoclonal immunoglobulin deposits (PGNMID)	Cyclophosphamide/bortezomib/dexamethasone
Fanconi syndrome (FS)	Cyclophosphamide/bortezomib/dexamethasone
Paraproteinemic neuropathy	**Reference** Chaudhr, Mayo Clin Proc. 2017
Distal demyelinating symmetric neuropathy with IgM (DADS-M)	IVIG, consider rituximab
IgG/A axonal neuropathy	Plasmapheresis, IVIG, steroids
IgG/A chronic inflammatory demyelinating polyradiculoneuropathy (CIDP)	
Severe and refractory neuropathy	Consider clinical trial or antimyeloma regimens Successful use of autologous stem cell transplant reported

CHAPTER 2

SMOLDERING MULTIPLE MYELOMA AND MULTIPLE MYELOMA

2.1 Smoldering Multiple Myeloma (SMM)

Smoldering multiple myeloma (SMM) occupies an intermediate position between what is usually referred to as a pre-malignant condition, i.e., MGUS and active multiple myeloma. SMM has a higher disease burden than MGUS, but does not show end-organ damage or any of the other myeloma-defining events (MDE) observed in MM [*Kyle, N Engl J Med.1980, Kyle, Br. J. Haematol. 2003;Rajkumar, Lancet Oncol.2014;Caers, The Oncologist 2016;Kunacheewa,Best Pract. Res. Clin. Haematol. 2020*].

First described by Kyle and Greipp in 1980 [*Kyle, Blood. 1980*], SMM is an indolent and asymptomatic clonal proliferation of plasma cells that can progress to MM, is characterized by the absence of specific biomarkers and symptoms, [*Kyle, N Engl J Med. 2007*] but also is differentiated from MGUS by a much higher risk of progression to MM [*Rajkumar, Blood. 2015*]

However, the risk of progression decreases over time: in the first 5 years is 10% per year, then 3% per year for the next 5 years, and 1 % per year thereafter, being similar to MGUS evolving risk. Several studies reported an estimated 25-33% of patients with newly SMM never progress to symptomatic disease, in particular in the first 10 years after diagnosis, and these patients probably have a premalignant state (biological MGUS), even though the clonal bone marrow plasma cells (BMPC) percentage or M protein level is higher than that specified in the clinical definition of MGUS [*Kyle, N Engl J Med.2007; Landgren, JAMA. 2010; Rajkumar, Am J Hematol.2012; Rawstron, Haematologica. 2008*].

A consensus definition for SMM was developed by the International Myeloma Working Group (IMWG) in 2003, 23 years after SMM was first described [*Durie, Hematol J. 2003*].

This delay was largely due to the paucity of therapies that were both effective and well-tolerated in MM, resulting in the recommendation of watchful waiting approach until symptomatic disease developed in patients with SMM [*Landgren Hematol Am Soc Hematol Educ Program.2017*].

The 2003 IMWG criteria defined SMM as either the presence of serum M-protein ≥3 g/dL or ≥10% monoclonal plasma cells within the BM in the absence of end-organ (CRAB criteria) damage [*IMWG, Br J Haematol. 2003*].

In 2010 the IMWG refined the criteria for the diagnosis of SMM/MM [*Rajkumar, Lancet Oncol.2014*]. Specifically, the category of ultra-high risk SMM (defined by serum free light-chain (sFLC) ratio ≥ 100, ≥ 60% BM plasma cells, or > 1 focal BM lesions in the skeleton based on magnetic resonance imaging [MRI]) was recognized to identify patients who carry a risk of progression at 2 years of ≥ 80% and for whom cytoreductive treatment may be warranted to avoid impending organ damage [*Rajkumar, Lancet Oncol.2014*]. Based on the revised IMWG criteria of

2014, ultra-high risk SMM are currently considered to have active MM and treatment is recommended.

SMM is rare, representing 8–14% of the total of MM patients. The median age of onset is 67 years. The incidence is 0.4 cases per 100000 people per year and is higher in Americans of African descent, as reported for MM [*Dispenzieri, Blood Cancer J.2016;Kyle, NEJM 2007*].

The rate of progression from SMM to MM was also found to be higher in the black population compared to whites and also higher in younger SMM patients compared to older patients [*Vuyyala,Clin Lymphoma, Myeloma Leuk.2019*].

2.2 Pathogenesis and Diagnosis of Smoldering Multiple Myeloma

From a biological point of view, SMM shows the initiating events of all monoclonal gammopathies, i.e., recurrent translocations of oncogenes to the immunoglobulin heavy chain (IGH) locus on chromosome 14, or multiple trisomies of odd-numbered chromosomes [*Morgan, Cancer 2012*]. However, these events are required but not sufficient for progression to active

MM. Indeed, progression from SMM to MM is characterized by an ongoing acquisition of additional genomic events, each of which confers a distinct risk.

While the genome of ultra-high risk SMM shows a landscape of mutations and chromosomal abnormalities that is more similar to MM [*Bolli, Nat. Commun. 2018; Bolli, Clin Med. 2021*], abnormalities like del (13q), amp (1q), del (17p) increase in frequency as the disease shifts to active MM [*Avet-Loiseau, Blood 1999*]. Among other genomic events associated with progression there are translocations between the IGH locus and the MYC oncogene [*Bolli, Nat. Commun. 2018*] and accumulation of complex rearrangements [*Bolli,Nat. Commun. 2019*]. Last, the activity of several mutational processes can be tracked over time. Initiating lesions arise from the activity of the DNA activation-induced cytidine deaminase (AID) within the germinal center, while late mutations associated with progression to MM arise from the aberrant activity of the APOBEC (apolipoprotein B mRNA editing catalytic polypeptide-like) family of cytidine deaminases, a mutational process common to many cancers and absent in normal PCs [*Bolli N, Nat. Commun. 2018; Maura F, Leukemia 2018, Maura F, Nat. Commun. 2019*].

Genomics is however not part of routine SMM diagnosis. For this purpose, IMWG criteria are used, taking into account several disease burden measures. Patients with SMM have more than 3 gr/dL of monoclonal component and/or >10% clonal BMPC. In addition, SMM has no evidence of end-organ damage, amyloidosis and MDE [*Rajkumar, Lancet Oncol.2014*]. IMWG recommends SMM is monitored at 2–3 months following initial diagnosis, then every 4–6 months for one year and

every 6–12 months after that if stable [*Kyle. N Engl J Med. 2007*]. However, follow-up intervals also depend on the individual risk of progression, which can be measured by several different approaches.

2.3 Risk factors for progression in SMM

The prognosis of SMM varies considerably, and it is possible to more accurately estimate risk of progression using a variety of prognostic variables.

- M protein concentration

In a Mayo Clinic study Kyle and colleagues found that the size of the serum M protein was a significant risk factor for progression of SMM (P <0.001) [*Kyle, N Engl J Med.2007*].

The median time to progression (TTP) in patients with markedly elevated serum M protein levels (≥4 g/dL) was 18 months compared to 75 months in those with serum M protein levels < 4 g/dL (P< 0,001). Similar results have also been reported by the Spanish Myeloma Group in a study of 93 patients with SMM [*Pérez-Persona, Blood. 2007].*

In light-chain SMM, the risk of progression is higher depending on the level of the urinary M protein. In a study by Kyle and colleagues, the 5-year risk of progression of light-chain SMM was 19% in patients with 24-hour urinary M protein levels of 0.50 to 0.99 g per 24 hours vs 39% in those with urinary M protein levels of ≥1.0 g per 24 hours [*Kyle, Lancet Haematol.2014].*

- M protein type

The type of M protein also influences the risk of progression in SMM. Kyle and colleagues found that TTP is significantly shorter in patients with immunoglobulin (Ig)A M protein compared to IgG M protein (median TTP, 27 vs 75 months, respectively; P=0,004) [*Kyle,N Engl J Med.2007*].

Also, the risk of progression in patients with light-chain SMM was found to be lower, with a median TTP of 159 months; the probability of progression was 28%, 45%, and 56% at 5, 10, and 15 years, respectively [*Kyle RA, Lancet Haematol.2014].*

- Immunoparesis

Suppression of ≥ 1 uninvolved immunoglobulins (immunoparesis) is seen in over 80% of patients; ~50% of patients have suppression of 2 uninvolved immunoglobulin isotypes [*Kyle, N Engl J Med.2007*]. In the Mayo Clinic study of 276 patients with SMM, immunoparesis was a significant risk factor for progression to MM or related disorder [*Kyle, N Engl J Med.2007*].

The median TTP was 159 months in patients with normal levels of uninvolved immunoglobulins,

89 months in those with a reduction in 1 isotype, and 32 months in patients with a reduction in 2 isotypes of uninvolved immunoglobulins (P=0,001). The same effect was also seen in a Spanish study of SMM, in which a decrease in 1 or 2 of the uninvolved immunoglobulins was a significant prognostic parameter in SMM (median TTP, not reached with normal immunoglobulins vs 31 months with a reduction in $\geq$ 1 uninvolved immunoglobulins (P<0,01).
Suppression of uninvolved immunoglobulins has also been found to be a risk factor for progression in light-chain SMM [*Kyle RA, Lancet Haematol.2014*].

- Serum FLC ratio

The serum FLC assay (Freelite, The Binding Site Group, Birmingham, UK) measures free kappa and lambda light chains that circulate unbound to immunoglobulin heavy chains [*Bradwell, Clin Chem. 2001; Katzmann, Clin Chem. 2002; Bradwell, Lancet.2003*]. The normal FLC kappa:lambda ratio is 0,26:1,65. In clonal PC disorders, there is excess production of one FLC type (the clonal component, referred to as the "involved" light chain), which often leads to an abnormal FLC ratio [*Dispenzieri, Leukemia. 2009*].
Dispenzieri and colleagues studied 273 SMM patients seen at the Mayo Clinic from 1970 to 1995 [*Dispenzieri, Blood. 2008*]. An involved/uninvolved FLC ratio of $\geq$8 was a significant risk factor for progression (HR, 2.3; 95% confidence interval, 1.6-3.2; P<0,001). Median TTP was 30 months in patients with an involved/uninvolved FLC ratio of $\geq$8 compared to 110 months for those with an FLC ratio <8. The risk of progression in the first 2 years after diagnosis is ~40% in patients with an involved/uninvolved FLC ratio of $\geq$8.
The risk of progression associated with an abnormal FLC ratio is a continuum [*Dispenzieri, Blood 2008*]. Thus, when the involved/uninvolved FLC ratio rises to $\geq$100, the median TTP is only 15 months, and the 2-year risk of progression approaches 80%. Such patients are now considered to have MM [*Kastritis, Leukemia.2013; Larsen, Leukemia. 2013*].

- Change in M protein level

A key variable that could potentially identify patients with a high risk of progression is change in M protein levels over time. However, such studies have been hampered by the fact that patients with SMM have not been uniformly followed at specified intervals outside of clinical trials. In one study of 53 patients with SMM, those with a progressive rise in M protein (evolving type) had a higher risk of progression compared with those with stable M protein levels [*Rosiñol, Br J Haematol.2003]*. In this study, the evolving type was defined as an increase in the serum M protein level by $\geq$10% on 2 subsequent evaluations.

Patients with an evolving type of SMM had a 65% probability of progression to MM or related disorder in the first 2 years. TTP was 1.3 years in the evolving type vs 3.9 years in the nonevolving type of SMM (P = 0.007). A study by the Southwest Oncology Group found that patients with an M protein level of <3 g/dL that increased to ≥3 g/dL over 3 months was associated with a risk of progression of ~50% at 2 years [*Dhodapkar, Blood. 2014*].

However, in the observation arm of the Spanish trial of SMM, 5 patients with a rise in M protein of ≥25% over 2 subsequent evaluations did not have a significant increase in risk of progression compared with patients without such a rise (2-year risk of progression, 69% vs 75%, respectively).

A rise in M protein level, especially over a short period of time, is of concern, and more data are needed on how best to incorporate such a finding into the management of SMM.

- Extent of bone marrow involvement

The risk of progression in SMM increases with the extent of bone marrow involvement. In the Mayo Clinic study, the median TTP was 117, 26, and 21 months for patients with BMPCs <20%, 20% to 50%, and >50%, respectively (P < 0.001) [*Kyle, NEJM, 2007*]. Subsequent studies show that the risk of progression increases dramatically when the BMPC level is ≥60%, with a 2-year risk of progression of ~90%, and such patients are now considered to have MM [*Rajkumar, N Engl J Med.2011; Kastritis, Leukemia.2013*].

BMPC estimate is done on the bone marrow aspirate or a core biopsy sample, or both, and in the event of a discrepancy, the higher of the 2 values should be used [*Rajkumar, Lancet Oncol. 2014*].

- Immunophenotype

Immunophenotyping with multiparametric flow cytometry is useful in determining prognosis in SMM by accurately distinguishing and quantitating BMPCs that have malignant potential from normal PCs [*Mateos, N Engl J Med.2013*].

Aberrant phenotype is defined by the absence of CD19 and/or CD45 expression, decreased expression of CD38, and overexpression of CD56. In MGUS, a substantial proportion of PCs are polyclonal and exhibit normal immunophenotype, whereas in MM, almost all PCs seen (>95%) are clonal and have an aberrant immunophenotype [*Pérez-Persona Blood.2007; Rawstron, Haematologica 2008*]. In a study of 93 patients with SMM, 60% of patients with SMM have an aberrant immunophenotype similar to MM (>95% PC aberrancy; <5% of the detected PCs are normal) [*Pérez-Persona Blood.2007*]. The risk of progression in such patients was significantly higher as compared with those who had a lower rate of aberrancy in the detected BMPC population

(median TTP, 34 months for patients with ≥95% aberrant PCs vs not reached for patients with <95% aberrant PCs; P < 0.001).

- Tumor genetic abnormalities

The Mayo Clinic group analyzed the prognostic influence of cytogenetic abnormalities in a series of 351 patients with SMM [*Rajkumar, Leukemia. 2013*]. Patients with t(4;14) and/or del(17p) were defined as high-risk SMM. These patients had a significantly shorter median TTP (24 months) compared with patients with trisomies (intermediate risk), other cytogenetic abnormalities including t(11;14) (standard risk), and no cytogenetic abnormalities (low risk). Similar results have also been reported by Neben and colleagues in a study of 249 patients with SMM [*Neben, J Clin Oncol. 2013*].

Dhodapkar and colleagues have assessed the value of gene expression profiling (GEP) signatures in 331 patients with MGUS and SMM [*Dhodapkar, Blood. 2014*]. An increased risk score (> -0.26) based on a 70-gene signature (GEP70) was an independent predictor of the risk of progression to MM. Further studies are needed to determine the incremental value of GEP compared to other, more readily available risk factors discussed earlier.

- Circulating PCs

A high level of circulating tumor PCs (CTPCs) was defined as absolute peripheral blood PCs >5 $<10^6$/L and/or >5% cytoplasmic immunoglobulin–positive PCs per 100 peripheral blood mononuclear cells. Patients with high circulating PCs were significantly more likely to progress to active disease within 2 years compared with patients without high circulating PCs (71% vs 25%, respectively; P = 0.001) [*Rajkumar, Blood 2015*].

- Imaging

MRI is of prognostic value in SMM [*Mariette, Br J Haematol. 1999*]. Moulopoulos and colleagues studied the prognostic value of spinal MRI in 38 patients with newly diagnosed asymptomatic MM [*Moulopoulos, J Clin Oncol. 1995*].

Bone marrow abnormalities were detected in 50% of patients, including diffuse, variegated, and focal changes. Patients with MRI scans showing abnormal bone marrow changes had a median TTP of 16 months vs 43 months in those with normal MRI studies (P < 0,01). Further, median TTP was shorter in patients with focal lesions (6 months) as compared with those who had a diffuse (16 months) or variegated pattern (22 months). In a more recent study of 149 patients with SMM, Hillengass and colleagues detected focal lesions in 28% of patients using whole-body MRI, and the

presence of such lesions was associated with an increased risk of progression to MM [*Hillengass, J Clin Oncol.2010*].

In the same study, the authors also confirmed the adverse prognostic effect of diffuse bone marrow changes detected by MRI (HR, 3.5; P < 0.001). Of importance, in the study by Hillengass and colleagues 15% of patients had >1 focal lesion detected by whole-body MRI. The median TTP in such patients was 13 months, and the 2-year progression rate was 70%. Similar findings have been found in a study reported by Kastritis and colleagues [*Kastritis, Leukemia. 2014*] and in a study by Dhodapkar and colleagues [*Dhodapkar, Blood 2014*]. Patients with >1 focal lesion are now defined as having MM and should not be considered as having SMM [*Rajkumar Lancet Oncol. 2014*].

Data are limited on the role of PET-CT in predicting risk of progression in SMM. However, patients who have focal lesions with increased uptake on PET-CT scans and who have underlying osteolytic destruction are not considered to have SMM; they are defined as having MM [*Rajkumar Lancet Oncol. 2014*].

- PC proliferative rate

A high proliferative rate of clonal PCs is associated with high risk of progression in SMM. Madan and colleagues studied 175 patients with SMM to determine the predictive value of PC proliferative rate measured using a slide-based immunofluorescence method, the PC labeling index (PCLI) [*Madan, Mayo Clin Proc. 2010*]. The median TTP was 1.2 years in patients with a PCLI value ≥1 vs 2.6 years in those with a PCLI value <1 (P<0,001). The PCLI is limited by lack of availability in the clinical setting.

- Risk stratification of SMM

Two models that have been well studied and subsequently validated in a prospective trial include the one proposed by the Mayo Clinic group and another proposed by the Spanish Myeloma Group [*Kyle, N Eng J Med. 2007; Pérez-Persona, Blood 2007*].

The Mayo Clinic model uses the size of the serum M protein and the extent of bone marrow involvement. These 2 variables are used to classify SMM into 3 risk groups: group 1 with serum M protein ≥3 g/dL and ≥10 % BMPCs, group 2 with <3 g/dL M protein and ≥10 % BMPCs, and group 3 with M protein ≥3 g/dL but BMPCs <10%. The median TTP to symptomatic MM is significantly different among the 3 groups: 2, 8, and 19 years, respectively. The probability of progression at 15 years is 87 %, 70 %, and 39 %, respectively. The model developed by the Spanish Myeloma Group uses the presence of 2 risk factors in patients with SMM who have ≥10 % BMPCs: presence of an aberrant PC immunophenotype in >95 % of clonal PCs and immunoparesis (reduction in ≥1

uninvolved immunoglobulins by > 25 % compared to normal)[*Pérez-Persona, Blood 2007*]. Patients with both risk factors have a median TTP of 23 months compared to 73 months when only 1 risk factor is present (either aberrant PCs or immunoparesis) and not reached when neither risk factor is present. In a recent randomized trial, patients were considered high risk if they met either the Mayo Clinic or the Spanish Myeloma Group criteria for high risk SMM [*Mateos, N Eng J Med. 2013*]. The trial showed that patients meeting these criteria had a median TTP of 24 months without therapy, confirming the validity of these criteria.

The Mayo Clinic and Spanish models enable initial risk stratification of SMM that can then be refined using additional prognostic factors. For example, Dispenzieri and colleagues have shown that the prognostic value of the initial Mayo Clinic model can be improved by adding the serum FLC ratio as a variable [*Dispenzieri, Leukemia 2009*]. Each model appears to identify unique patients as high risk, with some, but not complete, overlap [*Cherry BM, Leuk Lymphoma. 2013*].

Revised criteria for high-risk SMM that incorporate the Mayo Clinic and Spanish Myeloma Group criteria, as well as other risk factors that have been well studied and that identify patients with a similar risk of progression (~50% risk of progression within 2 years) are listed in Table 3.

Patients defined as having high-risk SMM using these criteria need close follow-up and are candidates for clinical trials investigating the value of early therapy.

- MAYO Risk model 20/20/20

In 2018, after a large cohort study, the Mayo Clinic group proposed a model based on three risk factors: BMPC > 20%, serum M-protein > 2 g/dL and FLCr > 20 [*Lakshman, Blood Cancer J. 2018*]. In this "20/20/20" model, patients with 0, 1 or ≥2 risk factors are respectively considered at low, intermediate and high-risk of progression. More recently, it has been suggested to include high-risk cytogenetic abnormalities as a fourth risk factor to this model, to further improve its accuracy [*Mateos, Blood Cancer J. 2020*]. This alternative version of the 20/20/20 model arose from another large cohort study which reported that certain cytogenetic abnormalities (i.e., translocation t(4;14), t(14;16), 1q gain, del13q and monosomy 13) are an independent risk factor for progression [*Mateos, Blood Cancer J. 2020*]. To take into account this fourth risk factor, this alternative version of the 20/20/20 model includes a fourth risk category and patients with 0, 1, 2 or ≥3 risk factors are respectively considered at low, intermediate-low, intermediate and high risk of progression.

The 20/20/20 risk stratification model proposed by Lakshman et al. shows promising results and is appealing because the risk factors involved are commonly determined at diagnosis, making its applicability universal [*Tessier, Curr Oncol. 2021*].

However, before being widely adopted, this model needs to be tested in a variety of clinical settings to determine its accuracy and reproducibility.

Table 3: Definition of high-risk SMM

Clonal BMPCs ≥10% and any one or more of the following:
Serum M protein ≥ 3 g/dL
IgA SMM
Immunoparesis with reduction of 2 uninvolved immunoglobulin isotypes
Serum involved/uninvolved FLC ratio ≥ 8 (but < 100)
Progressive increase in M protein level (evolving type of SMM; increase in serum M protein by ≥25% on 2 successive evaluations within a 6-month period)
Clonal BMPCs 50%-60%
Abnormal PC immunophenotype (≥95% of BMPCs are clonal) and reduction of ≥1 uninvolved immunoglobulin isotypes
t(4;14) or del(17p) or 1q gain
Increased circulating PCs
MRI with diffuse abnormalities or 1 focal lesion
PET-CT with focal lesion with increased uptake without underlying osteolytic bone destruction

The term SMM excludes patients without end-organ damage who meet the revised definition of MM; namely, clonal BMPCs ≥60% or serum FLC ratio ≥100 (plus measurable involved FLC level ≥100 mg/L), or >1 focal lesion on MRI scan. The risk factors listed are not meant to be indications for therapy; they are variables associated with a high risk of progression of SMM and identify patients who need close follow-up and consideration for clinical trials.
PET-CT, positron emission tomography with computed tomography.

2.4 Diagnostic evaluation

Baseline studies should include complete blood count, serum creatinine, serum calcium, skeletal survey, serum protein electrophoresis with immunofixation, 24-hour urine protein electrophoresis with immunofixation, and serum FLC assay [*Kyle, Leukemia. 2010*].

Specialized imaging with at least one method such as MRI of the spine and pelvis (ideally whole-body MRI) or 18F-fluorodeoxyglucose PET-CT or low-dose whole body CT is recommended to exclude MM [*Kyle, Leukemia 2010; Bladé, J Clin Oncol. 2010; Rajkumar, Lancet Oncol. 2014*].

Bone marrow examination is required, and should include FISH studies to detect high-risk cytogenetic abnormalities as well as PC immunophenotyping by multiparametric flow cytometry to enable accurate risk stratification.

The M protein, serum FLC levels, complete blood count, calcium, and creatinine should be re-evaluated every 3 to 4 months. In high-risk patients, follow-up should continue indefinitely and include periodic imaging studies to rule out asymptomatic progression. In low-risk patients, follow-up can be reduced to once every 6 months after the first 5 years. In both groups, development of symptoms suggestive of MM or related disorders should be carefully pursued.

In patients with baseline abnormalities on MRI scans, an increase in number and/or size of focal lesions during follow-up has diagnostic and prognostic value [*Merz, Leukemia. 2014*]. Therefore, in patients with MRI scans showing diffuse infiltration, solitary focal lesion, or equivocal lesions,

follow-up examinations in 3 to 6 months are strongly recommended [*Rajkumar, Lancet Oncol. 2014*].

2.5 Management of SMM

Currently, the standard of care for SMM is watchful waiting until the emergence of symptoms or myeloma biomarkers. This approach comes from studies that did not found any advantage in progression-free survival (PFS) or OS by starting treatments at diagnosis [*Riccardi, Br. J. Cancer 2000; Hjorth, Eur. J. Haematol. 1993; He, Cochrane Database Syst. Rev. 2003*].

Therefore, myeloma treatment should be initiated according to the IMWG recommendations [*Rajkumar, Lancet Oncol. 2014*]. These guidelines, outlining so-called MDE, recommend therapy for patients with $\geq$1 of 3 markers of progression: $\geq$ 60% BMPCs, serum FLCr $\geq$100 (this seems to be significant only if urine monoclonal protein is >200 mg/d) ad >1 focal lesion on MRI scan. These criteria are based on retrospective studies that identified these parameters at presentation as leading to high risk of progression.

Patients with standard- or intermediate-risk smoldering MM do not need immediate therapy [*Rajkumar, Lancet Oncol. 2014; Dimopoulos, Hemasphere. 2021*].

Regarding high-risk SMM, which is recently defined by the "20-20-20" rule, in recent studies selecting only patients at high risk of progression, early treatments were associated with survival benefits [*Lancet Oncol. 2016; Brighton, Clin. Cancer Res. 2019; Mateos, Lonial, J. Clin. Oncol. 2020*].

Phase 2 randomized, phase III studies have shown that Lenalidomide plays a significant role in prolonging PFS. In the first study, 119 patients with high-risk SMM (before the introduction of the new criteria for the definition of myeloma) were randomly assigned either to receive treatment with the combination of Lenalidomide plus Dexamethasone (Rd) for 9 cycles followed by Lenalidomide maintenance or to observation [*Rajkumar, Lancet Oncol. 2014*]. At a median follow-up of 75 months, Rd improved both PFS (median PFS not reached versus 23 mo; $P < 0.0001$) and OS compared with observation (HR = 0.43; $P = 0.024$) [*Mateos, N Eng J Med, 2013; Mateos, Lancet Oncol. 2016*].

However, this study was conducted several years ago and enrolled a number of patients who are considered as having MM according to the revised definition. In the second study [*Lonial, J Clin Oncol*. 2020], 182 patients with intermediate- or high-risk SMM were randomly assigned either to receive Lenalidomide monotherapy or to observation. At a median follow-up of 35 months, PFS was longer with Lenalidomide (HR = 0.28; $P = 0.002$); this result was driven mainly by the high-risk SMM group [*Lonial, J Clin Oncol. 2020*]. This study has not reported OS advantage for the

Lenalidomide arm to date. Several phase II studies using Daratumumab (Dara) monotherapy [*Landgren, Leukemia. 2020*], Isatuximab (Isa) monotherapy or other Rd-based regimens (with Elotuzumab [EloRd], or with Ixazomib) have shown encouraging results.

All the above data suggest that high-risk SMM patients should be encouraged to participate in randomized phase III trials to reveal the best treatment that offers OS advantage. To date, no treatment has been approved for SMM.

There is, therefore, a growing interest in identifying high risk patients to explore therapeutic options in this specific subgroup. Many efforts have been made, throughout the last years, to develop a risk stratification model able to predict the risk of progression of SMM, however, none has yet been adopted internationally. As concluding remarks, the current standard of care in SMM is close surveillance, outside of clinical trials, irrespective of risk status, and whenever possible, participation in clinical trials is highly encouraged.

2.6 Multiple myeloma (MM)

MM is characterized by clonal expansion of terminally-differentiated plasma cells producing monoclonal component, and a variable clinical course [*Boyle, Haematologica.2014; Swerdlow, Blood 2016*].

It accounts for 1% of all cancers and 10% of all hematologic malignancies, making it the second most common blood cancer behind non-Hodgkin lymphoma (NHL).

Epidemiological studies estimate the worldwide 5-year prevalence of MM at ~230000 patients [*Cid, Pharmacoepidemiol Drug Saf.2016*], with an incidence in Europe is 4,5-6/100000/year [*Dimopoulos, Hemasphere. 2021*].

Predominantly a disease of the elderly, the median age of patients at diagnosis of MM is 66–70 years, with 37% of patients under 65 years and 0,02–0,3% under 30 years [*Bianchi, Cancer J Clin. 2014; Kazandjian, Semin Oncol. 2016*]. Similar to its precursor states (MGUS and SMM), there is marked racial disparity in the incidence and age of onset of MM, which suggests that racial heterogeneity is present early in myeloma tumorigenesis and carries through disease progression [*Waxman, Blood.2010; Landgren, Blood Cancer J. 2017*]. Specifically, African-Americans are twice as likely to have MM compared to their white counterparts and this disparity is even greater in the under 40-year-old age group (more than threefold excess risk) [*Durie, Hematol J. 2003*].

The median age of diagnosis of MM is 4 years younger for blacks (66 years) compared with whites (70 years) [*Durie, Hematol J. 2003*]. The risk of progression to MM from MGUS, however, is the same between blacks and whites [*Landgren, Blood.2006*]. The increased incidence and younger age

of diagnosis of MM in blacks therefore likely reflects the higher prevalence of MGUS [*Landgren, Mayo Clin Proc.2007; Greenberg, Leukemia. 2012; Landgren, Leukemia.2014*].

2.7 Diagnosis of MM

The monoclonal proliferation of plasma cells results in the production of monoclonal antibody and end-organ damage [*Jurczyszyn, Encycl. Biomed. Gerontol. 2019*]. This can damage bone marrow, resulting in cytopenia [*Colmone, Science 2008*]. The accumulation of the monoclonal antibody, Bence–Jones proteins, can precipitate in the urine resulting in kidney damage (usually type 2 renal tubular acidosis) and renal failure, and it can be seen in two-thirds of MM cases [*Colmone, Science 2008*]. Multiple myeloma also activates osteoclasts in the bones through the Multiple myeloma also activates osteoclasts in the bones through the nuclear factor kappa-B ligand (RANKL), resulting in the destruction of bone via lytic lesions that predispose to pain, fractures, and mobility issues, and hypercalcemia. In fact, the hallmark end-organ damage of MM is referred to as "CRAB" symptoms: hypercalcemia, renal involvement, anemia, and bone lesions [*Michels, Am. Fam. Physician 2017*].

Therefore patients affected by MM often present with CRAB symptoms, while rarely a monoclonal component can be an accidental finding during routine blood exams, like those patients with suspected MGUS/SMM, who are by definition asymptomatic.

When MM is suspected, blood and urine electrophoresis should be performed to look for the monoclonal light-chain secreted by the neoplasm.

Blood levels of IgG, IgM, and IgA can identify the isoform of light-chain produced. If levels are elevated but under 3 g/dL, the disease can be classified as SM or MGUS instead of MM.

A sFLC assay is more sensitive than urine (where the light-chain protein is called a Bence–Jones protein). Serum albumin and β2microglobulin from peripheral blood are also valuable for diagnosing and staging the disease. The definitive diagnosis requires a bone marrow biopsy with greater than 10% clonal plasma cells, or the presence of a plasmacytoma elsewhere. End-organ damage is necessary to distinguish from SM. Renal indices should be evaluated to a glomerular filtration rate, usually calculated by creatinine levels, that can be used to establish renal insufficiency.

Imaging, such as CT (without contrast dye due to renal damage), MRI, and PET scans, is used to uncover lytic bone lesions. If patients are unable to undergo these imaging procedures, a skeletal survey is used instead. Eighty percent of patients have some skeletal lesions, fractures, or osteopenia at the time of diagnosis [*Rajkumar, Lancet Oncol.2014; Rajkumar, Mayo Clin. Proc. 2016*]. The revised diagnostic criteria by the International Myeloma Working Group Diagnostic

Criteria for MM and related Plasma Cell disorders are shown in Table 4 [*Rajkumar, Am. Soc. Clin. Oncol. Educ. Book 2016; Moreau, Ann Oncol. 2017*].

These tests can allow for the differential diagnosis between MM, SMM and MGUS.

The diagnosis of MM requires ≥10% clonal BM plasma cells or biopsy proven bony or extra-medullary plasmacytoma and any of the following MDE:

• Evidence of end-organ damage (the so-called CRAB criteria: hypercalcemia, renal insufficiency, anaemia or bone lesions) that is felt to be related to the underlying plasma cell disorder.

Of note, renal insufficiency can be defined not only by creatinine:

> 2 mg/dL but also by creatinine clearance < 40 mL/min [measured by validated equations such as the Modification of Diet in Renal Disease (MDRD) or Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)]

Moreover, lytic lesions can also be defined by CT and not only by conventional X-ray.

• Any biomarkers of malignancy:

- ≥ 60% clonal BM plasma cells
- Involved/uninvolved serum FLC ratio ≥ 100
- > 1 focal lesion on MRI studies (each focal lesion must be ≥ 5mm in size).

Table 4: The revised diagnostic criteria by the International Myeloma Working Group Diagnostic Criteria for MM and related Plasma Cell disorders. CRAB: Hypercalcemia, Renal failure, Anemia, Bone pain; FLC: free light chains; MGUS: monoclonal gammopathy of undetermined significance [*Padala, Med. Sci. 2021*]

Disorder	Diagnostic Criteria
SMOLDERING MULTIPLE MYELOMA	Two criteria must be met: (1) Serum monoclonal protein (IgG or IgA) 3 gm/dL or more, or urinary monoclonal protein 500 mg or more per 24 h and/or clonal bone marrow plasma cells 10–60% (2) No evidence of myeloma-defining events or amyloidosis
MULTIPLE MYELOMA	Two criteria must be met: (1) Clonal bone marrow plasma cells more than 10% or biopsy-proven bony or extramedullary plasmacytoma Plus, one or more of the following myeloma-defining events: (1) Presence of CRAB criteria which can be attributed to the underlying plasma cell proliferative disorder, specifically: - Hypercalcemia: serum calcium >11 mg/dL - Renal insufficiency: creatinine clearance <40 mL/min or serum creatinine >2 mg/dL - Anemia: hemoglobin <10 g/dL or >2 g/dL below the lower limit of normal - Bone lesions: one or more osteolytic lesions on skeletal radiography, CT, or PET-CT (2) Clonal bone marrow plasma cell percentage 60% or more (3) Involved/uninvolved serum FLC ratio of 100 or more (involved FLC level must be 100 mg/L or more) (4) More than one focal lesion on MRI studies (at least 5 mm in size)
IgM MGUS	Three criteria must be met: (1) Serum IgM monoclonal protein less than 3 gm/dL (2) Less than 10% bone marrow lymphoplasmacytic infiltration (3) No evidence of anemia, constitutional symptoms, hyperviscosity, lymphadenopathy, or hepatosplenomegaly secondary to underlying lymphoproliferative disorder
LIGHT-CHAIN MGUS	All the following criteria must be met: (1) Abnormal FLC ratio (Less than 0.26 or more than 1.65) (2) Increased level of the appropriate involved light chain (increased kappa FLC when the ratio is more than 1.65, and lambda FLC in patients if the ratio is less than 0.26) (3) Absence of immunoglobulin heavy-chain expression on immunofixation (4) No evidence of end-organ damage that can be attributed to the plasma cell proliferative disorder (5) Clonal bone marrow plasma cells less than 10% (6) Urinary monoclonal protein less than 500 mg/24 h

2.8 Staging and risk assessment in MM

The course of MM is highly variable, and the clinical behavior is remarkably heterogeneous. Many studies have identified prognostic factors capable of predicting this heterogeneity in survival: serum β2microglobulin, albumin, C-reactive protein and LDH.

The two main staging systems used for MM are the international staging system (ISS) and the Durie–Salmon system (DSS). The ISS stratifies cases into three stages: Stage I for those with a B2M less than 3.5 mg/L and albumin > 3.5 g/dL, Stage III for those with a B2M greater than 5.5 mg/L, and Stage II for all those in between [*Rajkumar, Lancet Oncol. 2014*].

The revised ISS adds prognostic information such as LDH levels and chromosomal abnormalities detected via FISH. In an international clinical trial with 3060 participants, for those with stage I disease, OS and PFS at five years was 82% and 55%, respectively. Median PFS was 66 months, and OS was not reached. For Stage II disease, these values were 62% and 36%, 42 months and 83 months, respectively. For Stage III disease, these values were 40%, 24%—29 months and 43 months, respectively. Further risk stratification can be made based on chromosomal translocation, many of which involve the IgH locus on chromosome 14 (14q32) [*Rajkumar, Am. Soc. Clin. Oncol. Educ. Book 2016*].

With the advent of new therapies, the overall survival of the disease has increased, and with improving survival, there was a need for re-staging the disease for early diagnosis, treatment, and preventing end-organ damage [*Rajkumar, Am. Soc. Clin. Oncol. Educ. Book 2016*].

Until recently, MM was defined by the presence of a clonal process that correlates with end-organ involvement (presence of CRAB features). In patients with the absence of CRAB criteria, three biomarkers were included in the diagnostic criteria by the IMWG in 2014 [*Rajkumar, Am. Soc. Clin. Oncol. Educ. Book 2016*].

In addition, the recommendations were to include CT and PET-CT to diagnose the involvement of bones. A new staging system has been developed that incorporates high risk cytogenetic abnormalities in addition to standard laboratory markers of prognosis [*Rajkumar, Am. Soc. Clin. Oncol. Educ. Book 2016*].

Both the ISS and DSS systems assess the tumor burden, but neither ISS nor DSS takes into consideration the biology of the disease, which determines the overall survival [*Greipp, J. Clin. Oncol. 2005; Hari, Leukemia 2009; Rajkumar, Am. Soc. Clin. Oncol. Educ. Book 2016*].

The revised international staging system (R-ISS) combines elements of tumor burden (ISS) and disease biology [*Palumbo, J. Clin. Oncol. 2015*]. It was developed based on a study of 11 international trials.

The 5 year survival rates among the patients with Stage I, II, and III R-ISS were 82%, 62%, and 40%, respectively [*Rajkumar, Am. Soc. Clin. Oncol. Educ. Book 2016*].

In table 5 are summarized the several cytogenetic abnormalities can occur, in particular some can identify high-risk disease.

Table 5: Primary and secondary genetic events that can be identified by FISH [*Sonneveld, Blood.2016*]

Primary genetic events			Secondary genetic events		
IgH translocation	**Gene(s)**	**Frequency (%)**	**Deletion**	**Gene(s)**	**Frequency (%)**
t(4;14)	*FGFR3/MMSET*	15	1p	*CDKN2C, FAF1, FAM46C*	30
t(6;14)	*CCND3*	4	6q		33
t(11;14)	*CCND1*	20	8p		25
t(14;16)	*MAF*	4	13	*RB1, DIS3*	44
t(14;20)	*MAFB*	1	11q	*BIRC2/BIRC3*	7
			14q	*TRAF3*	38
			16q	*WWOX, CYLD*	35
			17p	*TP53*	7
Hyperdiploidy			Gain		
Trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, 21	NA	50	1q	*CKS1B, ANP32E*	40

IgH translocations. In MM, primary events are chromosome translocations involving the immunoglobulin heavy chain (IgH) locus and hyperdiploidy, with multiple copies of odd-numbered chromosomes (Table 5) [*Munshi NC, Clin Cancer Res. 2011*]. IgH translocations are observed in 40% of cases. Frequently involved partner chromosomes/loci are 4p16 (FGFR3/MMSET) (12%-15%), 11q13 (CCND1) (15%-20%), 16q23 (MAF) (3%), 6p21 (CCND3) (,5%), and 20q11 (MAFB) (1%) [*Kuehl WM, Nat Rev Cancer. 2002*].

Translocation t(4;14) leads to deregulation of fibroblast growth factor receptor 3 (FGFR3) and multiple myeloma SET domain (MMSET) [*Neben K, Haematologica. 2010; Boyd KD, Leukemia. 2012*].

Because FGFR3 is not expressed in one third of patients with t(4;14), the target gene is most likely MMSET [*Fonseca R, Blood. 2003*]. t(4;14) is associated with impaired PFS/OS [*Gertz, Blood. 2005*]. Importantly, bortezomib seems to improve the negative prognostic impact of t(4;14) [*Barlogie, Blood. 2008; Cavo M, Lancet. 2010; Sonneveld, J Clin Oncol. 2012*; *Sonneveld, J Clin Oncol. 2013*]. Prolonged survival was reported in t(4;14) treated with high-dose therapy (HDT) and tandem autologous stem cell transplant (ASCT) [*Moreau P, Leukemia. 2007; Shaughnessy JD Jr, Blood. 2007*]. SNP arrays showed the heterogeneous adverse impact of t(4;14) related to concomitant CA [*Hebraud, Blood.2015*].

Translocation t(14;16) results in deregulation of the c-MAF proto-oncogene and predicts poor outcome. An Intergroupe Francophone de Myélome (IFM) analysis showed no adverse impact of t(14;16), possibly because 60% of patients received a double ASCT [*Avet-Loiseau, Blood.2011*].

Translocation t(14;20) results in deregulation of the MAFB oncogene and confers a poor prognosis [*Boyd, Leukemia. 2012*].

Translocation t(11;14) results in upregulation of cyclinD1 and was identified as favorable in some studies, whereas it had no impact in others [G*ertz, Blood. 2005; Fonseca, Blood. 2002; Avet-Loiseau, Blood. 2007*]. This translocation is associated with CD20 expression and a lymphoplasmocytic morphology. In general t(6;14), t(11;14), gain(5q), and hyperdiploidy do not confer poor prognosis.

Genomic imbalance. Hyperdiploidy, which occurs in ~ 50% of NDMM, is associated with improved PFS/OS [*Neben, Haematologica. 2010; Avet-Loiseau, Blood, 2007*]. In the MRC IX trial, coexisting hyperdiploidy did not abrogate the poor prognosis of adverse CA [*Pawlyn Blood 2015*]. In contrast, in a retrospective analysis, PFS of patients with t(4;14) was negatively affected by del(1p32), del22q, and >30 structural CA, whereas del(6q) worsened PFS and del(1p32) worsened OS, and >8 numerical changes improved OS in del (17p) [*Hebraud, Blood 2015*].

Modern techniques (GWAS) identify additional CNV above karyotypic hyperdiploidy [*Walker, Blood. 2010*].

Del(13q) predicts impaired PFS/OS when detected by karyotyping [*Chiecchio, Leukemia 2006*]. The adverse impact of del(13q) by FISH is associated with del(17p) and t(4;14). del(13q) as single CA does not confer poor survival [*Neben, Haematologica. 2010; Boyd, Leukemia. 2012; Avet-Loiseau, Blood 2007*].

Del(17p) or ***del(17)*** has a negative impact on PFS/OS. Deletion of TP53 induces clonal immortalization and survival of tumor cells [*Teoh, Leukemia. 2014*].

Gain(1q) frequently coincides with del(1p32), which confers poor prognosis [*Hebraud, Blood. 2015; Boyd, Clin Cancer Res. 2011;Chang, Bone Marrow Transplant. 2010; Hebraud Leukemia. 2014*].. Patients with $\geq$3 copies of 1q have a worse treatment outcome, reflecting a dosage effect of genes such as CKS1B [*Boyd, Leukemia. 2012*].

Hypoploidy is regarded as a poor prognostic CA. It is currently unclear which minimum percentage of cells carrying del(17p) is required for an adverse prognosis or whether this varies with the choice of therapy and stage of disease. Minimal percentages of 20% and 60% have been recommended for del(17p) [*Boyd, Leukemia. 2012, Hebraud, Blood.2015*].

The prognostic impact of CA may vary from diagnosis to (refractory) disease because of the selection of subclonal disease [*Walker, J Clin Oncol. 2015*]

Multiple adverse CA. Among patients with an adverse IgH translocation 62% have gain(1q) compared with 32.4% in controls. [*Boyd, Leukemia. 2012*].

The frequency of del(17p) is similar in patients without adverse IgH translocations. Among patients with an IgH translocation and/or gain1q or del(17p), 20% shared ≥2 CA. When CA occurred in isolation, each lesion had a similar impact on OS.

The triple combination of an adverse IGH translocation, gain(1q), and del(17p) was associated with a median OS of 9.1 months, demonstrating the progressive impact of co-segregation of multiple adverse CA on OS [*Boyd, Leukemia. 2012*]. The IFM showed that in 110 patients displaying either t(4;14) or del(17p), 25 had both abnormalities. In patients with t(4;14), PFS was worse with concomitant del(1p32), del(22q), and/or > 30 structural changes, whereas del(13q14), del(1p32), and higher number of CA shortened OS. In patients with del(17p), del(6q)reduced PFS, whereas gain15 and del14 had a protective effect. Del(1p32) shortened OS, whereas >8 numerical changes improved OS [*Hebraud, Blood.2015*].

Good combined with adverse CA. Gain of 5(q31) improved outcome with hyperdiploid MM [*Avet-Loiseau, J Clin Oncol.2009*]. Among patients with hyperdiploidy, trisomies 3 and 5 confer a favorable prognosis [*Hebraud, Blood.2015*]. Presence of trisomies in patients with t(4;14), t(14;16), t(14;20), or TP53 deletion in MM reduced their adverse impact [*Kumar, Blood. 2012*].

2.9 Cytogenetic risk classifications

The definition of HR disease is subject to diagnostic and treatment options. With median PFS and OS of transplant-eligible (TE) patients approaching 4 and 10 years, most investigators consider HR disease as OS <3 years, with ultra-HR disease having a survival <2 years.

Risk classifications are based on FISH. IMWG proposed a model of HR MM defined as at least one of the following: del17p, t(4;14), or t(14;16) determined by FISH [*Fonseca, Leukemia. 2009*].The Mayo Clinic classification added hypodiploidy and t(14;20) to the definition of HR MM [*Stewart, Leukemia. 2007*]. Later classifications attempted to separate MM into several risk groups.

In MRC IX, 3 groups were identified (ie, favorable risk [FR: no adverse IgH translocation, del(17p), or gain(1q]), intermediate risk (IR: 1 adverse CA), and HR (>1 Adverse CA).

Median PFS/OS of patients with FR, IR, or HR was 23.5, 17.8, and 11.7 months and 60.6, 41.9, and 21.7 months, respectively [*Boyd, Leukemia. 2012*].

Ultra-HR was defined as ≥3 CA (2%; median OS, 9 months). These classifications may change with treatment modalities. An example is t(4;14),which may be IR rather than HR when novel agents are given.15,45,46 IMWG stated that HR MM should include t(4;14), t(14;16), or del(17p) [*Chng, Leukemia.2014*].

Table 6: Cytogenetic risk features [*Sonneveld, Blood.2016*]

	High-risk	Standard-risk
Cytogenetic abnormality	FISH: t(4;14), t(14;16), t(14;20), del(17/17p), gain(1q) Nonhyperdiploid karyotype Karyotype del(13) GEP: high-risk signature	All others including: FISH: t(11;14), t(6;14)

Risk classifications based on FISH and ISS. The combination of ISS with HR CA reflects tumor mass, patient condition, and genetics. IMWG showed that t(4;14) and/or del(17p) separates 2 groups with different event-free survival (EFS) and OS within each ISS stage and combining t(4;14) and del(17p) with ISS stage improved prognostic staging [*Avet-Loiseau, Leukemia. 2013*].
Neben et al combined ISS with t(4;14) or del(17p) [*Neben, Haematologica. 2010*].
Median PFS after ASCT were 2.7, 2.0, and 1.2 years for the FR group (ISS I, no HRCA), IR(ISS I and HR CA or ISS II/III without HR CA), and HR (ISS II/III and HR CA). Five-year OS were 72%, 62%, and 41%, respectively.

Risk classifications based on FISH, ISS, and lactodehydrogenase.
A meta-analysis of randomized trials in NDMM confirmed that combining ISS, serum LDH, and FISH identifies 4 risk groups including a very HR population (5%-8%). Patients with ISS stage III, elevated LDH, and t(4;14) or del(17p) have a 2-year OS of only 54.6% [*Moreau, J Clin Oncol. 2014*]. More recently, the revised ISS was defined, incorporating HR FISH (t(4;14), t(14;16), and del(17p) with ISS and LDH [*Palumbo, J Clin Oncol. 2015*].

mSMART. The Mayo Stratification of Myeloma and Risk-Adapted Therapy (mSMART) criteria use a combination of FISH, plasma cell labeling index, and GEP as tools to identify 3 risk categories (standard risk [SR], IR, HR) for prognostication of patients with NDMM [*Mikhael, Mayo Clin Proc.2013*]. Patients can be stratified for different therapeutic approaches [*Chng, Leukemia. 2014*].

Consensus statement. Translocations t(4;14), t(14;16), t(14;20), and del(17/17p) and any nonhyperdiploid karyotype are HR cytogenetics in NDMM regardless of treatment. Gain(1q) is associated with del(1p) carrying poor risk [*Sonneveld, Blood. 2016*].
Combinations of $\geq$3 CA confer ultra-HR with <2 years survival. Routine testing should include t(4;14) and del(17p). Clinical classifications may combine these lesions with ISS, serum LDH, or HR gene expression signatures. CA may differ in first and later relapse because of clonal evolution, which may influence the effect of salvage treatment.

CHAPTER 3

PATHWAYS ALTERED IN MM AND DETECTION OF PERIPHERAL PLASMA CELLS

3.1 Pathways altered in MM patients: BCL-2

Several pathways can be expressed by myeloma cells. A lot of studies have evaluated the upregulation of BCL-2 family expression that confers resistance to apoptosis in myeloma cells.

Apoptosis plays a key role in protection against genomic instability and maintaining tissue homeostasis, and also shapes humoral immune responses. During generation of an antibody response, multiple rounds of B-cell expansion and selection take place in germinal centers (GC) before high antigen affinity memory B-cells and long-lived plasma cells are produced. These processes are tightly regulated by the intrinsic apoptosis pathway, and malignant transformation throughout and following the GC reaction is often characterized by apoptosis resistance. Expression of pro-survival BCL-2 family protein MCL-1 is essential for survival of malignant PC in multiple myeloma. In addition, BCL-2 and BCL-XL contribute to apoptosis resistance [*Slomp, Front. Oncol.2018*].

MCL-1, BCL-2, and BCL-XL expression is induced and maintained by signals from the bone marrow microenvironment, but overexpression can also result from genetic lesions. Since MM PC depend on these proteins for survival, inhibiting pro-survival BCL-2 proteins using novel and highly specific BH3-mimetic inhibitors is a promising strategy for treatment [*Slomp, Front. Oncol.2018*].

The BCL-2 protein family consists of pro-survival BCL-2-like proteins (BCL-2, BCL-B, BCL-W, BCL-XL, BFL-1/A1, and MCL-1), pro-apoptotic BH3-only proteins (initiators), and pro-apoptotic effectors BAX, BAK [*Czabotar, Nat Rev Mol Cell Biol. 2014*], and possibly BOK [*Haschka, FEBS J. 2017; Zheng, Cell Rep. 2018; Ke, Cell 2018*].

Cytotoxic stimuli such as DNA damage, chemotherapeutic agents, or cytokine deprivation promote upregulation of BH3-only proteins, which inhibit pro-survival BCL-2 family members [*Czabotar, Nat Rev Mol Cell Biol. 2014*]. In addition, post-translational modification of BH3-only proteins can affect their stability, activity, and subcellular localization [*Lomonosova, Oncogene 2008*].

BH3-only proteins vary in their affinities for different pro-survival proteins. For instance, BAD only binds with high affinity to BCL-2, BCL-XL, and BCL-W, while NOXA selectively inhibits MCL-1 and BFL-1/A1. BIM, PUMA, and BID have high affinity for all pro-survival proteins [*Chen, Mol Cell 2005; Happo, J Cell Sci. 2012*]. If all available pro-survival proteins are sequestered by BH3-only proteins, BAX and BAK can disrupt the mitochondrial outer membrane, leading to cytochrome C release, caspase activation, and execution of apoptosis [*Wong, J Exp Clin Cancer Res. 2011*].

In addition, some BH3-only proteins, including BIM, PUMA, and BID, can directly bind to BAX or BAK and induce conformational changes that contribute to BAX/BAK activation [*Letai, Cancer Cell 2002; Llambi, Mol Cell 2011; Czabotar, Cell 2013*].

Regulation of apoptosis is essential for generation and selection of high-affinity PC, and malignant transformation of cells in this process often coincides with defects in apoptosis.

3.2 The BCL-2 Family in Multiple Myeloma

MCL-1 protein expression is increased in newly diagnosed MM compared to healthy PC, and protein levels are even higher at relapse [*Wuillème-Toumi, Leukemia 2004*]. In addition, overexpression of MCL-1 is associated with shorter patient survival [*Wuillème-Toumi, Leukemia 2004*]. Using RNA interference lethality screening in cell lines, MCL-1 was also identified as one of the most important and selective survival genes for MM [*Tiedemann, Cancer Res.2012*].

In subsets of MM cell lines and patient samples, BCL-2 and BCL-XL expression is also high [*Punnoose, Mol Cancer Ther. 2016*], suggesting that these three proteins may act redundantly in preventing apoptosis. Since expression of both pro-survival and pro-apoptotic BCL-2 family members is heterogeneous, and the interplay between them is complex and dynamic, dependence on MCL-1, BCL-2, and BCL-XL is likely to differ between patients [*Bodet, J. Cancer 2010; Morales, Blood 2011; Punnoose, Mol Cancer Ther. 2016*].

Signals and cellular processes that may lead to overexpression of MCL-1, BCL-2, and BCL-XL in MM are indicated in Figure 3.

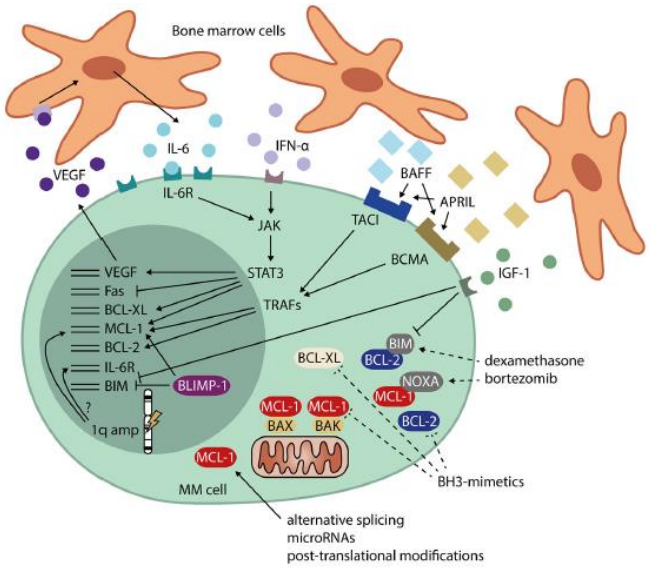


Figure 3: Signals and cellular processes that mediate apoptosis resistance in MM. MM cells receive signals from the bone marrow microenvironment that stimulate their survival. These signals include IL-6 and IFN-α, leading to JAK/STAT signaling and expression of MCL-1, BCL-XL, and VEGF. VEGF, in turn, promotes IL-6 production by neighboring cells. Other signals from the bone marrow microenvironment include BAFF and APRIL, which signal via TRAFs and induce expression of MCL-1 and BCL-2. IGF-1 signaling downregulates BIM, transcriptionally as well as post-translationally. MM cells also have high expression of the PC transcriptional regulator BLIMP-1, which promotes MCL-1 and represses BIM expression. Amplification of the 1q chromosome arm often occurs in MM. The genes for both MCL-1 and the IL-6 receptor (IL-6R) are present on this locus, possibly leading to overexpression in 1q-amplified MM. In addition to transcriptional regulation, MCL-1 is heavily regulated post-transcriptionally, which may contribute to the high MCL-1 protein levels found in MM. Dashed lines represent methods for interference in apoptosis resistance by MM drugs dexamethasone and bortezomib, and by BH3-mimetics. APRIL, a proliferation-inducing ligand; BAFF, B-cell activating factor; BCMA, B-cell maturation antigen; BH-3, BCL-2 homolog 3; BLIMP-1, B lymphocyte-induced maturation protein 1; IFN-α, interferon alpha; IGF-1, insulin-like growth factor 1; IL-6, interleukin 6; IL-6R, interleukin 6 receptor; JAK, janus kinase; STAT3, signal transducer and activator of transcription 3; TACI, transmembrane activator and calcium-modulating ligand interactor; TRAF, TNF receptor-associated factor; VEGF, vascular endothelial growth factor [*Slomp and Peperzak, Frontiers in Oncology, 2018*]

3.2.1 Survival Signals From the BM Microenvironment

MM cells reside in the BM, where they interact with extracellular matrix proteins and cells from the BM microenvironment, which include stromal cells, osteoblasts, osteoclasts, endothelial cells, fibroblasts, adipocytes, and cells of hematopoietic origin [*Cimmino, Proc Natl Acad Sci USA 2005*]. MM cells promote neighboring cells to produce IL-6 [*Dankbar, Blood 2009*], which induces JAK/STAT3 signaling in MM, leading to transcription of MCL-1 and BCL-XL [*Puthier. Br. J. Haematol. 1999; Puthier, Eur J Immunol. 1999; Jourdan, Oncogene 2003; Gupta, Blood 2017*]. MCL-1 expression in MM can also be IL-6-independent *[Zhang, Oncogene 2003*], or occur via other signals from the BM microenvironment [*Gouill, Cell Cycle 2004*].

For instance, signaling through BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand), whose levels are increased in MM patients compared to healthy controls, induces expression of both MCL-1 and BCL-2 and promotes PC survival [*Moreaux Blood 2004; Peperzak, Nat Immunol. 2013*]. Other survival signals from the bone marrow environment include interferon α (IFN-α), which induces MCL-1 in a STAT3-dependent manner [*Jourdan Cell Death Differ. 2000*], and IGF-1, which downregulates expression of BIM [*Bruyne, Blood 2010*].

3.2.2 Genetic Lesions

MM is characterized by recurrent chromosomal aberrations, some of which may be linked to apoptosis pathways. Translocations or chromosomal amplifications and gains involving 18q are rare in MM [*Pratt, J Clin Pathol Mol Pathol. 2002*], suggesting that *BCL2* overexpression is not a key event in malignant transformation. No other genetic lesions in MM have directly been correlated to overexpression of a BCL-2 family member. Nevertheless, gain or amplification of 1q21, the chromosome region containing the *MCL1* gene, occurs in approximately 40% of MM cases and correlates with poor disease prognosis [*Shah, Leukemia 2017*].

Notably, *IL6R*, the gene encoding the IL-6 receptor, is also located on 1q21, as are several other candidate drivers of high-risk disease [*Pawlyn, Nat Rev Cancer 2017*].

T(4;14), which is present in 10–15% of MM patients [*Shah, Leukemia 2017*], may lead to disruption and subsequent overexpression of *FGFR3*, which is considered an oncogene [*Pratt, J Clin Pathol Mol Pathol. 2002*]. In a murine IL-6-dependent hybridoma cell line, FGFR3 was shown to signal through STAT3 and substitute IL-6 signaling, leading to increased BCL-XL expression and decreased apoptosis [*Plowright, Blood 2000*].

Correspondingly, specific tyrosine kinase inhibitors with known anti-FGFR3 activity induced apoptosis in t(4;14)-positive cell lines [*Grand, Leukemia 2004*].

3.2.3 MCL-1 Stabilization

Unlike for BCL-2 and BCL-XL [*Punnoose, Mol Cancer Ther. 2016*], transcriptional activity of *MCL1* does not directly correlate to protein levels. MCL-1 is unique within the BCL-2 family because it has a large N-terminal domain that allows for post-translational modification [*Kozopas, Proc Natl Acad Sci USA 1993; Yang, J Cell Biol. 1995*]. Proteasomal degradation of MCL-1 occurs upon phosphorylation and subsequent poly-ubiquitination of this N-terminal region.

Kinases associated with phosphorylation of MCL-1 include JNK, GSK-3, and ERK-1 [*Thomas, FEBS Lett. 2010*]. Ubiquitin ligases Mule, $SCF^{\beta-TrCP}$, SCF^{Fbw7}, and APC/C^{Cdc20} were shown to target MCL-1 for proteasomal degradation after recognizing specific phosphorylated residues [*Mojsa, Cell 2014*]. This process can be reversed by deubiquitinases, such as USP9X [*Schwickart, Nature 2010*]. The contribution of these kinases and ubiquitin modifiers to MCL-1 regulation in MM is currently unknown. If the key players in MCL-1 regulation can be identified for MM, these MCL-1-modifying proteins may be interesting targets for therapeutic intervention.

3.2.4 Overcoming Apoptosis Resistance: BCL-2 Proteins as Therapeutic Targets in MM

As apoptosis resistance in B-cell malignancies often results from overexpression of pro-survival BCL-2 family proteins, inhibiting these proteins is a promising strategy for development of targeted therapeutics. Several BCL-2 family inhibitors, also named BH3-mimetics because of their structural and functional resemblance to the BH3 domain of BH3-only proteins, are currently in clinical development. BCL-2 inhibitor Venetoclax is the first BH3-mimetic approved by the Food and Drug Administration. It was approved in 2016 for treatment of CLL with a 17p deletion [*Zhu, Drug Des Devel Ther. 2017*]. Additionally, Venetoclax was tested in phase I clinical trials with relapsed and refractory MM patients, where monotherapy was particularly effective when the t(11;14) translocation was present [*Kumar, Blood 2017*].

T(11;14) is associated with an increased *BCL2*/*MCL1* mRNA ratio, but the mechanism behind this is unknown [*Touzeau, Leukemia 2014*].

When MM patients were treated with Venetoclax in combination with conventional MM drugs bortezomib (a proteasome inhibitor) and dexamethasone, it was well tolerated and the response rate was highest in patients with high *BCL2* expression [*Moreau, Blood 2017*]. Experiments in cell lines even indicate more-than-additive effects when Venetoclax is combined with proteasome inhibitor carfilzomib or dexamethasone, due to upregulation of NOXA and BIM, respectively [*Matulis, Leukemia 2016*]. If conventional treatment increases availability of BH3-only proteins and their distribution toward pro-survival target proteins, this may increase sensitivity to BH3-mimetic drugs.

While the results of MM treatment with Venetoclax underline the potential of using BH3-mimetics in MM, they also suggest that Venetoclax may only be effective in a subset of patients, namely those who have relatively high BCL-2 and relatively low MCL-1. Based on *in vitro* and xenograft experiments, MCL-1 is often shown to be essential for MM survival and its generally high expression may confer resistance to Venetoclax [*Punnoose, Mol Cancer Ther. 2016; Gong, Blood 2016; Bose, Leuk Lymphoma 2017*]. Therefore, MCL-1 itself is a very promising therapeutic target in MM, and multiple MCL-1 inhibitors are currently under development [*Chen, Expert Opin Ther Pat. 2017*]. MCL-1 inhibitor S63845 efficiently kills MM and other MCL-1-dependent cancer cell lines [*Kotschy, Nature 2016*].

Its derivate S64315/MIK665 is currently being tested in phase I clinical trials by Servier for acute myeloid leukemia and myelodysplastic syndrome (NCT02979366), and by Novartis for MM and DLBCL (NCT02992483). In addition, clinical testing in MM patients has started with MCL-1 inhibitors developed by Amgen, named AMG 176 and AMG 397 (NCT02675452 and NCT03465540, respectively) [*Caenepeel,Cancer Discov.2018*], and by AstraZeneca, named AZD5991 (NCT03218683) [*Hird, Cancer Res. 2017*].

Simultaneous targeting of multiple BCL-2 family proteins may be a solution to resistance in case of redundancy between MCL-1, BCL-2, and BCL-XL in MM. Before the development of Venetoclax, BH3-mimetics with broader protein specificity have been studied, such as Navitoclax [*Opferman, FEBS J. 2016]*.

Navitoclax (ABT-263) mimics the selectivity of BAD, thereby inhibiting only BCL-2, BCL-XL, and BCL-W. When tested in CLL patients, results were promising, but dose-limiting thrombocytopenia was observed as a result of BCL-XL inhibition [*Tse, Cancer Res. 2008; Mason, Cell 2007; Wilson, Lancet Oncol. 2010*].

This led to the development of BCL-2-selective BH3-mimetic Venetoclax [*Souers, Nat Med. 2013*]. Other putative BCL-2 family inhibitors with broad target specificity, such as Obatoclax (GX15-070), were shown to function partly or completely in a BAX/BAK-independent manner, and are therefore no longer considered BH3-mimetics [*Vogler, Cell Death Differ. 2009*].

The results with Navitoclax indicate that potential side-effects of BCL-2 family inhibitors may be dose-limiting, and that combined inhibition of BCL-2 family members may only be possible if the concentration of each specific inhibitor remains below the threshold of toxicity.

MCL-1 is not only essential for B-cells and PC, it is also essential in other cell types, including hematopoietic stem cells [*Opferman, Science 2005*], cardiomyocytes [*Thomas, Genes Dev. 2013*], and neural precursor cells [*Malone, Mol Cell Neurosci. 2012*].

In contrast to healthy cells, increased expression of pro-apoptotic molecules ("priming") renders malignant cells more susceptible to apoptosis upon inactivation of pro-survival proteins [*Certo, Cancer Cell 2006*].

Since MCL-1 is the most dominant pro-survival protein in MM, its inhibition leads to release of a large proportion of pro-apoptotic proteins present in MM cells, thereby promoting apoptosis induction. In mice, MCL-1 inhibitor S63845 was tolerated well at concentrations that killed cancer cells [*Kotschy, Nature 2016*], even when murine Mcl-1 was replaced by its human ortholog, thereby increasing inhibitor sensitivity of all cells [*Brennan, Blood 2018*]. This may yield a therapeutic window for targeting MCL-1, especially if MCL-1 inhibitors are combined with existing treatments that increase pro-apoptotic protein expression.

High expression of pro-survival BCL-2 family proteins contributes to outgrowth and drug resistance of malignant B-cell clones. While beneficial for cell survival, addiction to high levels of specific pro-survival BCL-2 proteins also makes cells vulnerable to BCL-2 family inhibition using BH3-mimetic drugs. MM is characterized by high expression of MCL-1, and overexpression of BCL-2 and BCL-XL is observed in subsets of patients. Constitutive overexpression of these pro-survival proteins in MM results from a range of microenvironmental signals and different genetic

lesions. This complex regulation of MCL-1, BCL-2, and BCL-XL offers multiple direct and indirect targets for therapeutic intervention. Recent development of BH3-mimetic drugs, that specifically target MCL-1, BCL-2, or BCL-XL, may contribute to overcoming apoptosis resistance and improving treatment for MM. Studies about the expression of BCL-2 in patients affected by smoldering multiple myeloma are still lacking.

3.3 Pathways altered in MM patients: PD-1/PD-L1

3.3.1 Targeting immune checkpoints

Immune checkpoints, a plethora of inhibitory or stimulatory pathways, are encoded in the immune system and are essential for self-tolerance and also for the modulation of physiological immune responses. The processes of activation, maturation and expansion of T lymphocytes, and inhibition of their apoptosis are supported by stimulatory checkpoints and their ligands (e.g., CD137/CD137L, CD28/CD80, and CD86, CD27/CD70, CD40/CD40L, OX40/OXO4OL, GITR/GITRL, ICOS/ICOSL), while an opposite effect is elicited by inhibitory checkpoints with their ligands (PD-1/PD-L1 and PD-L2, CTLA-4/CD80 and CD86, A2AR/adenosine, KIR/MHC class I, LAG3/MHC class II) [*Jelinek, Immunology 2017*]. They are crucial against the activation of autoimmunity and for the protection against damage of tissue when the immune system is activated against an infection under normal circumstances [*Pardoll, Nat Rev Cancer 2012*].

Nevertheless, there is a possibility that tumor cells may become invisible to the host's immune system when they start to express ligands of checkpoint receptors on their surface and thus abuse and hijack these native pathways [*Armand, Blood 2015*].

Inhibitory immune checkpoint blockade with blocking mAbs (immune checkpoint inhibitors) has consequently emerged as a novel option for cancer treatment. Indeed, checkpoint inhibitors are now a conventional part of the treatment of numerous types of solid tumors (melanoma, non-small cell lung cancer, renal cell carcinoma, head and neck carcinoma) and Hodgkin's lymphoma. [*Brahmer, N Engl J Med.2015; Ansell, N Engl J Med. 2015; Larkin, N Engl J Med. 2015; Motzer, N Engl J Med. 2015*].

3.3.2 Role of PD-1/PD-L1 pathways

There are two chief, well-described inhibitory pathways: cytotoxic T-lymphocyte associated protein 4 (CTLA-4, CD152) as a checkpoint receptor and its cognate ligands B7–1 (CD80) and B7–2

(CD86), and programmed-death 1 (PD-1, CD279) receptor with its two ligands PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC).

PD-1, a 288 amino acid type I transmembrane protein, is a part of the CD28 receptor family and is expressed on antigen-activated and exhausted T and B cells [*Ishida, EMBO J.*1992]. Two ligands, PD-L1 and PD-L2, are expressed on antigen binding cells (macrophages and dendritic cells) as well as on a subset of activated B lymphocytes and microvascular endothelial cells.

Furthermore, there has been a detection of a constitutive level of PD-L1 expression on the cells of various tissues (heart, lung, liver, pancreatic islet cells, astrocytes, etc.) [*Freeman, J Exp Med. 2000; Rodig, Eur J Immunol.*2003].

Engagement of the PD-1 receptor with its ligands PD-L1 or PD-L2 prompts the temporary down-regulation of T cell function, namely decreased T cell proliferation, cytokine production and cytotoxicity and increased susceptibility to apoptosis [*Freeman, N Exp Med 2000; Parry, Mol Cell Biol. 2005; Armand, Blood 2015*]. This cascade leads to T cell exhaustion and immune escape which is originally well known in chronic viral infections such as hepatitis B, hepatitis C or HIV, where these changes protect the host from an excessive immune response [*Day, Nature 2006; Boni, Virol 2007*].

Not only T cells, but tumor-associated macrophages and NK cells too are involved in the PD-1/PD-L1 pathway [*Gordon, Nature Blood 2010; Benson, Blood 2014*]. Many solid and hematologic tumors use this strategy (overexpression of PD-L1 or 2) to escape from the host's immune surveillance. The recognition of this immune evasion mechanism has led to the development of therapeutic mAbs directed against receptors or ligands involved in this pathway.

3.3.3 Expression of PD-L1 on Myeloma plasma cells

There are discrepancies between many research groups concerning PD-L1 expression on plasma cells. It has been demonstrated by several studies that PD-L1 expression is limited to PCs of MM patients, and is absent on those of healthy donors (HD) [*Liu, Blood 2007; Tamura, Leukemia 2013; Ray Leukemia 2015; Görgün, Clin Cancer Res. 2015*].

Likewise, PD-L1 expression was reported to be higher on PCs in MM and SMM than in MGUS [*Liu, Blood 2007;Tamura, Leukemia 2013; Ray Leukemia 2015; Görgün, Clin Cancer Res. 2015; Dhodapkar, Blood 2015*].

Nevertheless, these investigators used different methodologies, different cut-offs of positivity, different gating strategies, and their results varied in the percentage of PD-L1 positive PCs [*Liu, Blood 2007*], HD (n = 20, range, 0.1%–2.7%; median: 1%), MGUS (n = 42, range, 0–48%; median:

2.05%), MM (n = 82, range, 0–92%; median: 23%) [*Görgün, Clin Cancer Res. 2015*], newly diagnosed multiple myeloma [NDMM] (n = 6, mean = 16.5%), relapsed/refractory multiple myeloma [RRMM] (n = 10, mean = 26.6%), HD (n = 3, mean = 4.7%), Tamura et al. did not report exact results, Ray et al. use MFI scale without units. As PD-L1 has a uniquely unimodal and homogenous pattern of expression, it is probably more exact to compare its MFI (mean fluorescent intensity) than the percentage of positive cells [*Paiva, Leukemia 2015*] used comparison of MFI as well as gating of only clonal PCs and not total PCs and they, in fact, found no difference in PD-L1 expression between NDMM, MGUS and HD. These results were confirmed by a later study [*Kelly, Leukemia 2018*].

Interestingly, they confirmed a statistically higher expression of PD-L1 on clonal PCs from MRD positive myeloma patients compare with HD as well as higher PD-L1 expression on clonal PCs compare with total PCs in MGUS and MRD positive MM patients.

The study by Dhodapkar et al. has also shown that PD-L1 expression on malignant PCs was associated with an increased risk of progression from SMM to MM [*Dhodapkar, Blood 2015*].

These studies confirm that it is not currently completely clear that PD-L1 is overexpressed on pathological PCs compare with normal PCs.

3.3.4 Expression of PD-L1 on immune cell subsets

A crucial role in regulating the response of T cells against tumors is played by dendritic cells (DCs). The BM of MM patients was found to have increased levels of plasmocytoid DCs (pDCs) [*Chauhan, Cancer Cell 2019*].

The over-expression of PD-L1 on pDCs in MM patients has been demonstrated by several authors [*Ray, Leukemia 2015; Sponaas, PLoS ONE 2015*].

Numerous studies have demonstrated that there is an overexpression of PD-1 on CD4+ and CD8+ T cells from MM patients compared to HD [*Rosenblatt, J Immunother Hagerstown Md 1997.2011; Görgün, Clin Cancer Res.2015*].

Paiva et al. have shown a significant surge in PD-1 expression on CD4+ and CD8+ T cells only in relapsed or relapsed/refractory MM (RRMM) and MRD positive MM patients [*Paiva, Leukemia 2015*].

PD-1 absence on normal CD56+CD3- NK cells from HD has been confirmed by all published studies. A markedly increased expression of PD-1 on NK cells from MM patients compared to HD was reported by the Benson et al. and Görgün et al. studies; whereas Paiva et al. found no difference between them [*Benson, Blood 2010; Görgün, Clin Cancer Res.2015; Paiva, Leukemia 2015*].

3.3.5 PD-1/PD-L1 inhibitors in multiple myeloma

The treatment of cancer, especially of various types of solid tumors, has been revolutionized by the blockade of the PD-1/PD-L1 pathway by immune checkpoint inhibitors. Their success amongst hematologic malignancies, however, has been limited so far to the treatment of classic Hodgkin's lymphoma, which portrays a typical overexpression of PD-1 ligands (PD-L1, PD-L2) as a consequence of changes in chromosome 9p24.1 [*Jelinek, Front Immunol. 2018*].

Their current application in multiple myeloma is rather uncertain, as discordant results have been reported by distinct research groups concerning especially the expression of PD-1/PD-L1 molecules on malignant plasma cells or on the responsible immune effector cell populations, respectively.

In MM it seems that an approach based on combination treatment might be appropriate as unsatisfactory results have been yielded by monotherapy with PD-1/PD-L1 inhibitors. Immunomodulatory drugs, which are the current cornerstone of MM treatment, are the most logical partners as they possess many possibly synergistic effects. Nevertheless, the initially optimistic results have become disappointing due to the excessive and unpredictable toxicity of the combination of Pembrolizumab with Lenalidomide or Pomalidomide [*Jelinek, Front Immunol. 2018*].

The FDA has suspended or put on hold several phase 3 trials in relapsed as well as in newly diagnosed myeloma patients. There are also other potentially synergistic and promising combinations, such as the anti-CD38 monoclonal antibody Daratumumab, irradiation, etc.

Not only the effective partner but also the correct timing of the initiation of the PD-1/PD-L1 inhibitors treatment seems to be of utmost importance.

Currently, the role of PD-1/PD-L1 plasma cells expression and a potential area of treatment in smoldering myeloma remains uncertain.

3.4 Circulating plasma cells in plasma cell neoplasms

Despite BM is the most frequently involved tissue in PCN [*Billadeau, Blood 1992; Witzig, Blood 1996; Röllig, Lancet 2015*], and a close interaction with the BM microenvironment is required for long-term persistence of normal plasma cells and tumor PC [*Morgan, Cancer 2012; Pérez-Andrés, Leukemia 2005; Moschetta, Curr. Osteoporos. Rep. 2017*], previous studies have recurrently shown involvement of PB in a substantial fraction of patients [*Billadeau Blood 1992, Billadeau Blood 1996; Rawstron, Br. J. Haematol. 1997; Kumar, J. Clin. Oncol. 2005; Gonsalves, Br. J. Haematol. 2014; An, Ann. Hematol. 2015; Periago, Am. J. Hematol. 2016*].

However, the frequency of PB involvement depends on the sensitivity of the methods used and the specific diagnostic subtype of PCN [*Billadeau Blood 1992, Billadeau Blood 1996; Kumar, J. Clin.*

Oncol. 2005; Gonsalves, Br. J. Haematol. 2014; An, Ann. Hematol. 2015; Periago, Am. J. Hematol. 2016].

Thus, PB involvement by CTPC increases from MGUS -19 to 37%- to MM -50 to 75% [*Billadeau Blood 1996; Rawstron, Br. J. Haematol. 1997; Paiva, Blood 2013; Bae, Cytom. B. Clin. Cytom 2016*], and PCL (100%) [*De Larrea, Leukemia 2013; Kyle, N. Engl. J. Med. 2018*], depending on whether immunocytochemistry or conventional 4–8-color flow cytometry are used, respectively.

Despite such variability and the relatively low sensitivity of the methods used so far, the presence of CTPC in PB of newly diagnosed MGUS and SMM patients has been associated with an increased risk of progression to MM [*Witzig Cytometry 1996; Kumar, J. Clin. Oncol. 2005; Nowakowski, Blood 2005; Paiva, Blood 2013; De Larrea, Leukemia 2013; Bianchi, Leukemia 2013; Gonsalves, Br. J. Haematol. 2014; Bae, Cytom. B. Clin.Cytom 2016*], and within MM with an adverse outcome [*Witzig, Blood 1996; Nowakowski, Blood 2005*], both when evaluated at diagnosis and after therapy [*Dingli, Blood. 2006; Chakraborty, Blood Cancer J. 2016; Chakraborty, Haematologica 2017*].

In the last years, a next-generation flow cytometry (NGF) approach has been established for high-sensitive minimal residual disease (MRD) monitoring in the BM of MM patients, after therapy [*Flores-Montero, Leukemia 2017; Kumar, Lancet Oncol 2016*].

In 2018 Sanoja-Flores et al applied for the first time NGF technique for the detection of very low levels of PB involvement by CTPC in newly diagnosed PCN patients (MGUS, SMM, MM), exploring its potential prognostic impact [*Sanoja-Flores, Blood Cancer Journal 2018*].

From the phenotypic point of view, although PB CTPC showed a similar profile to that of BM PC, they displayed significantly lower ($P < 0.05$) expression levels of the CD38, CD138, CD81, CD56, CD27, and Vs38c maturation-associated markers, together with CD117 and to a lesser extent also the Ki67-proliferation marker ($P = 0.11$), supporting a more immature and less proliferative immunophenotype for paired PB vs. BM PCs.

Other maturation-associated PC markers displayed either a tendency towards lower (CD20, $P = 0.14$; and CD19, $P = 0.06$), or similar expression levels -CD45 ($p = 0.47$) and Sm/CyIg light chains (SmIgκ/λ, $P = 0.68$; CyIgκ, $P = 0.9$; CyIgλ, $P = 0.7$)- in BM vs. PB TPC.

Overall, their results showed an up to ~2-fold increased frequency of cases presenting with CTPC in PB by NGF vs. both immunocytochemistry and conventional flow cytometry, among MGUS (59% vs.19–37%) [*Billadeau Blood 1996; Paiva, Blood 2013*], SMM (100% vs. 15–50%) [*Paiva, Cytom. Part B Clin.Cytom. 2010; Bianchi, Leukemia 2013*] and MM (100% vs. 50–73%) [*Billadeau Blood 1996; Paiva, Blood 2013; Bae, Cytom. B. Clin. Cytom 2016*].

In contrast, only a small percentage of SP and macrofocal MM had detectable CTPC in PB.

Altogether, these results confirm and extend on previous observations indicating that the presence of CTPC in PB is usually associated with systemic disease (i.e., MGUS, SMM and MM), higher numbers of PB CTPC within patients with systemic disease reflecting a more malignant clinical behavior [*Witzig, Cancer 1993; Billadeau Blood 1996; An, Ann. Hematol. 2015*], while it is a rare finding among tissue-localized PC tumors (e.g., SP and Macrofocal MM) [*Witzig, Blood 1996*].

In line with these findings, the overall number of PB CTPC as assessed by NGF also increased progressively from SP and macrofocal MM to MGUS, SMM, and MM, the number instead of the presence vs. absence of CTPC providing an accurate discrimination between MGUS and MM in the great majority of patients.

Also, would support further evaluation of the benefit of including PB CTPC counts in new minimally invasive (i.e., PB-based) diagnostic algorithms, to distinguish between MGUS and MM; alternatively, it might be used as a prognostic factor in both diseases.

Previous studies based on less sensitive approaches indicated that the presence of CTPC in PB and/or their number, are both associated with an increased risk of transformation of MGUS to MM [*Kumar, J. Clin. Oncol. 2005; Periago Am. J. Hematol 2013*] and the outcome of SMM and MM, when assessed both at diagnosis [*Bianchi Leukemia 2013; Gonsalves, Leukemia 2014; An, Ann. Hematol. 2015; Vagnoni, Br. J. Haematol. 2015; Gonsalves, Leukemia 2017*], and after therapy [*Dingli, Blood. 2006; Chakraborty, Blood Cancer J. 2016; Chakraborty, Haematologica 2017*].

Despite the still relatively limited number of patients investigated per diagnostic category and the short median follow-up, our results confirm and extend on these findings. Thus, MGUS showing higher numbers of PB CTPC displayed shorter TTP to MM, while in SMM, the prognostic impact of the number of CTPC in PB appears to be more limited and independent from both the Mayo Clinic and the Spanish scoring systems.

Nevertheless, the data on SMM should be interpreted with caution due to the limited number of these cases. Most interestingly, CTPC counts in MM within the range of MGUS patients were associated for the first time here, with a significantly longer PFS and OS, independently of response to therapy evaluated according to both the CR and MRD status.

Therefore, within MM, the presence of high number of CTPC seems to represent a strong adverse prognostic factor, very low numbers of CTPC in PB at diagnosis (i.e., similar to those observed in MGUS), potentially contributing to the identification of those few MM cases that show a good long-term outcome, even when they do not reach BM MRD-negativity or CR.

The persistence/presence of CTPC in MM patients who had undergone therapy, might be used as a surrogate marker of BM MRD-positivity.

Confirmation of these findings deserves further prospective studies in large series of patients with longer follow-up.

The precise biological significance of the presence and the levels of CTPC in PB of MGUS and MM patients, still remains largely unknown [*Paiva, Leukemia 2011; Paiva, Blood. 2013*]. Classically, the presence of CTPC in PB of MM has been viewed as a sign of dissemination of BM TPC into the circulation leading to distinct tissue-homing patterns and the formation of new PC tumors at distant (e.g., extramedullary) sites [*Billadeau, Blood 1996; Ghobrial, Blood.2012; Paiva, Blood. 2013*].

However, compared to BM PC, PB CTPC display features of more quiescent cells with greater resistance to chemotherapeutic agents and higher potential for self-renewal, together with a potentially more immature phenotype [*Billadeau, Blood 1996; Kumar, Am. J. Hematol. 2004; Paiva, Blood. 2013; Mishima, Cell Rep. 2017*], suggesting that PB CTPC might constitute (and behave as) true MM stem cells [*Dela Cruz, Am. J. Blood Res. 2011; Ghobrial, Blood. 2012; Johnsen, Haematologica 2016*].

Sanoia-Flores et.al [*Sanoja-Flores, Blood Cancer J. 2018*] confirmed that PB CTPC from MM and MGUS patients are immunophenotypically more immature than their BM counterpart, as reflected by the expression of significantly lower levels of markers that are typically acquired by PC during migration from secondary lymphoid tissues to the BM, such as CD38 and CD138 [*Jego, Blood1999; Medina, Blood 2002; Perez-Andres, Cytom. Part B Clin. Cytom. 2010; Caraux, Haematologica, 2010; Flores-Montero, Cytom. Part B Clin. Cytom. 2016*], and adhesion molecules that anchor PC to stromal structures such as CD56, CD81, and CD117 [*Mateo, J. Clin. Oncol. 2008; Caraux, Haematologica 2010; Schmidt-Hieber, Haematologica, 2011; Márk, Hematol. Oncol, 2017*]. Also, PB CTPC display lower expression levels of activation/differentiation-associated antigens such as CD27 [*Márk, Hematol. Oncol 2017*] and Vs38c, a rough endoplasmic reticulum protein directly linked to a high rate of protein (i.e., Ig) synthesis and secretion [*Turley, J. Clin. Pathol.1994; Banham, J. Clin. Pathol. 1997; Medina, Blood 2002*].

PB CTPC detected in this large series of patients also tended to show lower levels of expression (vs. BMPC) of the Ki67-proliferation associated marker [*Kumar, Am. J. Hematol. 2002; Ghobrial, Blood 2012; Paiva, Blood 2013; Röllig, Lancet. 2015*].

In contrast, they did not found significant differences as regards the phenotypic profile of PB vs. BM TPC for other maturation-associated antigens previously described to be aberrantly expressed by TPC in MM and MGUS patients, such as CD19, CD20, CD45 and sm/cyIg [*Paiva, Cytom. Part B Clin. Cytom. 2010; Flores-Montero, Cytom. Part B Clin. Cytom. 2016*].

This might be due to the fact that the pattern of expression of these markers could more closely reflect tumor phenotypes potentially associated with specific genetic lesions -i.e., CD20 expression in cases carrying t(11;14) [*An, Leuk. Res. 2013*] than actual maturation-associated phenotypes.

Despite all the above, CTPC in MM might also correspond to an admixture of immature (i.e., potential stem cell-like) TPC and more mature (i.e., BM derived) myeloma PC.

In any case, if this whole concept about the greater immaturity and stem cellness of PB vs. BM TPC holds true, PB CTPC in MM and MGUS might play a key role in disease dissemination throughout the BM (and in a subset of MM patients, also to extramedullary sites), at the same time they would be unable to appropriately home in the BM when niches are (almost) full and occupied by long-living (more mature) and growing tumor PCs. Altogether, this might also contribute to explain, at least in part, the non-linear (significant) correlation here reported between the PB and BM TPC burden. Similarly, it might also contribute to explain the unexpectedly higher number of circulating NPC in PB in more advanced vs. earlier stages of the disease (i.e., MM vs. MGUS), despite an almost complete depletion of their normal long-living BMPC counterpart is frequently observed in association with low serum non-involved immunoglobulin levels in MM, but not in MGUS.

In such case, progressive unspecific blockade of both TPC and (recently produced short-lived) NPC into the BM, due to the lack of (empty) PC niches occupied by TPC would occur together with a parallel increase in PB of both CTPC and NPC. The long-living nature of CTPC would provide an advantage to this PC population over short-lived NPC (recently produced in lymphoid tissues). This would lead to selective accumulation of TPC in the BM with progressive depletion of normal long-living PC in BM. As a consequence, abnormally low serum antibody production and immunoparesis would emerge as a hallmark of advanced disease [*Pérez-Persona, Blood 2007; Bladé, J. Clin. Oncol. 2010*].

The presence of CTPC in PB as assessed by NGF is a hallmark of both SMM and MM and a highly frequent finding among MGUS, while absent in most SP and macrofocal MM cases. Higher numbers of CTPC in PB were strongly associated with features of malignant disease, providing a powerful minimally-invasive blood test to discriminate between MGUS and MM at diagnosis and to identify both MGUS cases at high-risk of progression to MM, and a small subset of MM patients with low number of CTPC (within the range of MGUS cases) that display a significantly longer survival despite not achieving BM MRD negativity or CR. Currently, studies clarifying the role of CTPC in SMM are needed.

CHAPTER 4:
MATERIALS AND METHODS

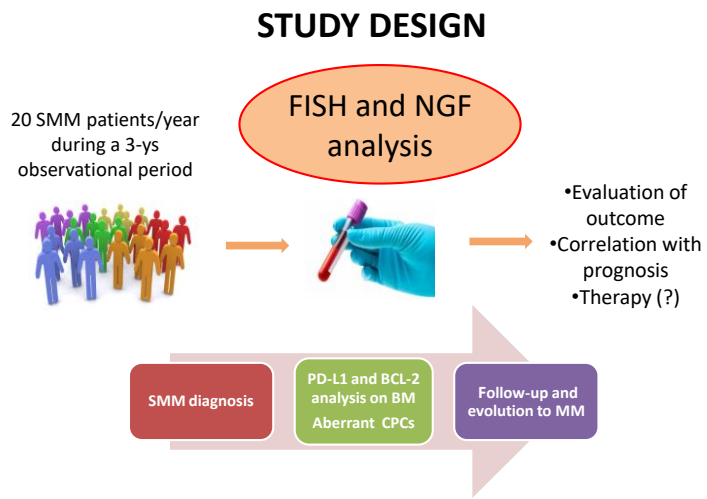
4.1 Aim of the study

The aim of the present study is to evaluate by next-generation flow (NGF) and to correlate the characteristics of PCs in the BM with the expression of BCL-2 and PD-L1, and to evaluate the presence of CPCs and correlate the results with FISH analysis for del17p, t(4;14), gain 1q, t(11;14), t(14;16) in patients affected by smoldering multiple myeloma. All these data are added to the current SMM evaluation clinical risk (Mayo Clinic and IMWG). During periodic follow up the patients underwent to evaluation of outcome (stability of disease or progression to MM) (Fig.4).

4.2 Patients and Methods

Patients with a SMM diagnosis and with a monoclonal component >1.5 g/l and /or abnormal serum FLCr, and /or increasing monoclonal component in 6 months and consecutively presented to the Hematology Division, University of Siena, at Policlinico Le Scotte, Azienda Ospedaliera Universitaria Senese, Siena, were enrolled. Also patients with a recent diagnosis of SMM in the last 12 months were searched in the local database. At diagnosis complete blood count, peripheral smear, renal function, calcemia, LDH, β2microglobulin, total proteins with electrophoresis, immunoglobulin and free light chains dosage with Bence Jones proteinuria were done. Bone examination consisted in bone marrow biopsy, bone marrow aspirate, flow cytometry and FISH samples. Patients also undergone radiologic examination (X-Ray or total body CT-Scan). SMM Patients were classified according to the IMWG criteria, then Mayo risk model was applied for risk stratification. SMM that progressed to MM were both uniformly treated according to current guidelines. Written informed consent was given by each individual prior to entering the study according to the Declaration of Helsinki, and the study was approved by the local ethics committees. None of the samples received was inadequate for further staining and processing.

Fig.4: Study design



4.3 Flow cytometry

Evaluation of Minimal Residual Disease was done by Next Generation Flow (Fig. 4 A), following protocol developed by the Euroflow-consortium [*Paiva, Blood 2016*].

NGF is a highly standardized and sensitive technique, based on the use of two 8-color pre-titrated tubes, BD OneFlow^{TM} PCST (BD Biosciences) and BD OneFlow^{TM} PCD (BD Biosciences), both containing all the markers needed to discriminate normal PCs and pathologic PCs. PCST contains a set of backbone markers (CD38, CD138, CD19, and CD45) and additional markers to characterize pathologic PCs (CD56, B2, CD28, CD27, CD117, CD81) plus the antibodies for the Ig light chains k and λ. PCD contains, instead, the backbone markers CD38, CD138, CD19, and CD45 and CD27, CD28, CD81 and CD117.

Additionally, we evaluated the expression of markers CD200, CD20 (Figure A tube 3), Isotype control and PD-L1 (Figure 5A tube 4 e 5), Isotype control and BCL-2 (Figure 5A tube 6-7). Moreover, Peripheral blood also was studied with markers expressed by abnormal myeloma cells. CD138 CD38 and CD45 were used to identify bone marrow and peripheral PCs. (Fig. 5B).

In Figure A the panel of markers evaluated by next generation flow cytometry on bone marrow samples is shown. Peripheral blood also was studied with markers expressed by abnormal myeloma cells (Fig.5B). CD138 CD38 and CD45 were used to identify bone marrow and peripheral PCs. Activation markers were evaluated (CD27, CD28, CD38, CD45, CD200), adhesion molecules (CD56/NCAM, CD81, CD138), BCR signaling molecules (CD19, CD20) and also markers recurrently reported to be absent on normal PC populations like CD117.

Fig.5: Antigenic markers evaluated in BM and PB.

A

BONE MARROW ASPIRATE

FITC	PE	PERCPCy5.5	PECy7	APC	APC-H7	V450	V500
CD38	CD56	B2-micro	CD19	KAPPA	LAMBDA	CD45	CD138
CD38	CD28	CD27	CD19	CD117	CD81	CD45	CD138
	CD200	CD19	CD38	CD56	CD20	CD138	CD45
			CTR		CD38	CD138	CD45
			PD-L1		CD38	CD138	CD45
	CTR				CD38	CD138	CD45
	BCL-2				CD38	CD138	CD45

B

PERIPHERAL BLOOD

FITC	PE	PERCPCy5.5	PECy7	APC	APC-H7	V450	V500
					CD38	CD138	CD45

4.4 FISH analysis

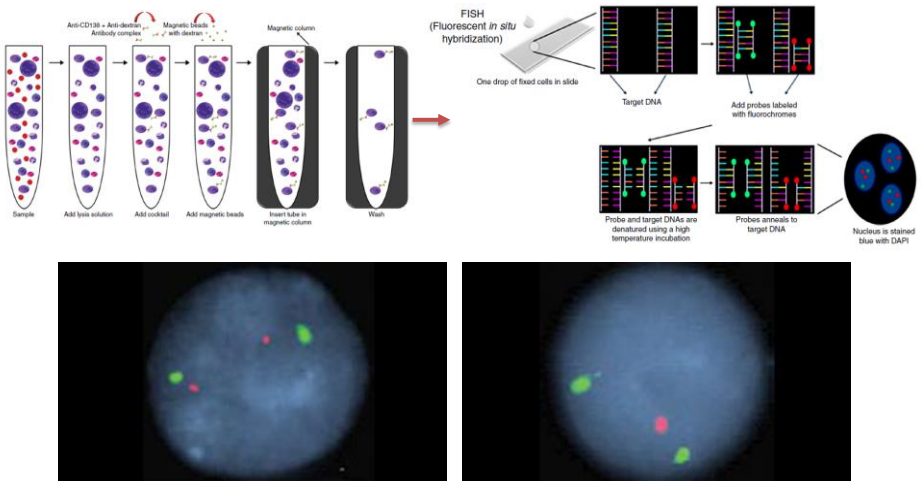
FISH analysis was performed in patients with at least 20% of bone marrow plasma cells. FISH is done in interphase cells: the procedure requires plasma cell separation through microbeads, then incubation is carried out with specific probes (after a series of steps the fixed cells are put on several slides and probes labeled with fluorochromes added), as represented in figure 6.

Harvest procedure: Three milliliters of hypotonic potassium chloride solution (KCl– 0.075 mol/L) were added to the MACS enrichment sample and incubated for 16 min at 37◦C. After this time, 1 mL of fixative solution (Carnoy's solution: 3:1 methanol/acetic acid) was added and the tube was centrifuged at 1500 rpm for 8 min. The supernatant was discarded; material was re-suspended in3 mL of Carnoy's solution and centrifuged again at 1500 rpm for 8 min. The latter procedure was repeated two more times and the resulting pellet was used in the iFISH procedure (Figure 6).

Hybridization. The pellet obtained in the last step was re-suspended in600–1000 L of Carnoy's solution and centrifuged in a Cytospin centrifuge at 1000 rpm for 5 min (100–200 L of the material for each slide). According to the manufactures' protocol, slides were pretreated in the following solutions: 2× SSC, 70%ethanol, 85% ethanol and 100% ethanol for 2 min at room temperature. Ten microliters of Cytocell Aquarius®probe was applied on a slide and cover slipped. This was done with each different probe. Co-denaturation of the probe and target DNA was performed on a hot plate at 75◦C for 5 min. Slides were incubated for 12–16 h in a humid chamber for hybridization and then were washed in 0.4× SSC solution at 42◦C for 2 min followed by a second wash (2× SSC + 0.1% NP40) at room temperature for 1 min. Nuclei were counterstained with 10 L of DAPI II (Cytocell Aquarius®) and cover slipped. Slides were stored at 4◦C for 10 min.

Probe panel selection and scoring Five probes (Cytocell Aquarius®) were selected: Amplification1q dual color; Deletion 13q (*RB1*) dual color; Deletion 17p13.1(*P53*) dual color; t(4;14) *IGH/FGFR3* dual color, dual fusion and t(14;16) IGH/MAF dual color, dual fusion. iFISH analysis of probe hybridization was performed with a 100× objective fluorescence microscope (Zeiss, Germany) with single and triple emission filters.

Fig.6 Interphase fluorescence *in situ* hybridization procedure



a) A normal vysis probe for chromosome 17; b) Vysis probe showing 17p delection

4.5 Statistical analysis

OS and PFS were calculated using the Kaplan-Meier method. The log-rank test was used to compare survival curves. Categorical variables were compared by chi-square or Fisher exact tests when appropriate. Covariates were: BM plasmacytosis (< 30% or > 30%), B2M (< 3,5 or > 3,5 mg/L), FLCr (< 20 or > 20), CM (< 2 or > 2 g/dL), MAYO risk (High, Intermediate, Low), BCL-2 and PD-L1 overexpression.

Statistical analyses were performed using MedCalc software (Med-Calc Software, Broekstraat, Mariakerke, Belgium). Significance was set at P =0,05.

CHAPTER 5

RESULTS

5.1 Results

Patients and clinical characteristics. From September 2019 to June, 2022 we analyzed 28 patients (M 20; F 8) with a median age of 66 years (40-85). A total of 44 cases were initially selected and 16 resulted in screening failure due to diagnosis of lymphoplasmacytic lymphoma (1 case), MGUS (8 cases), MM (7 cases). Median follow-up was 18 months (5-32) and all patients were alive at last study evaluation. Patients were monitored according to current IMWG guidelines. At screening, blood cell count was normal, serum FLCr was abnormal in 21/28 patients, BJ proteinuria and skeletal imaging were negative in all patients. FISH was abnormal only in 1 patients (chromosome 4 monosomy).

According to MAYO risk model 5 were at low risk, 17 at intermediate and 6 at high risk of progression. 11 patients had a IgG kappa SMM, 7 IgG lambda, 7 IgA lambda, 2 IgA kappa, 1 IgM kappa+IgG lambda. Currently CPCs were not detected at screening, or at subsequent evaluations/last follow up.

General patients characteristics are summarized in table 7.

Table 7: General characteristics

Patients characteristics	Results
Age (years), median	66 (40-85)
Hb (g/dL), median	14,1 (12-16,3)
WBC (x 10^9/L), median	6,37 (5-11,7)
PLTs (x10^9/L), median	229 (116-442)
Serum M protein (g/dL), median	1,75 (0,72-3,9)
Kappa/lambda ratio, normal/abnormal	7/21
BM PC (%), median	20 (15-50)
Previous MGUS	13 (46%)
HCV+ **Cardiac disease** **Hypertension** **Other cancer** **Chronic infections** **Reumatic disease** **Hemocromatosis**	4 4 9 4 2 1 1

NGF-Flow and FISH results. The most expressed markers were CD56 (89 %), CD27 (92 %), CD81 (71 %), 2 markers were less expressed: CD28 (42 %), CD117 (28 %), CD200 (10 %), CD20 (14 %), CD19 and CD45 (3,5%) (Table 8).

CD19 was present in only 1 female patient at intermediate risk who progressed to active myeloma, now under treatment, while CD45 was found in 1 female patient at intermediate risk who maintained a stable disease at last follow-up.

Coexistence of markers that are mutually excluded was detected in 10/28 patients (CD27+CD28+) and in 3/28 cases (CD27+CD28+CD81+CD117+) respectively.

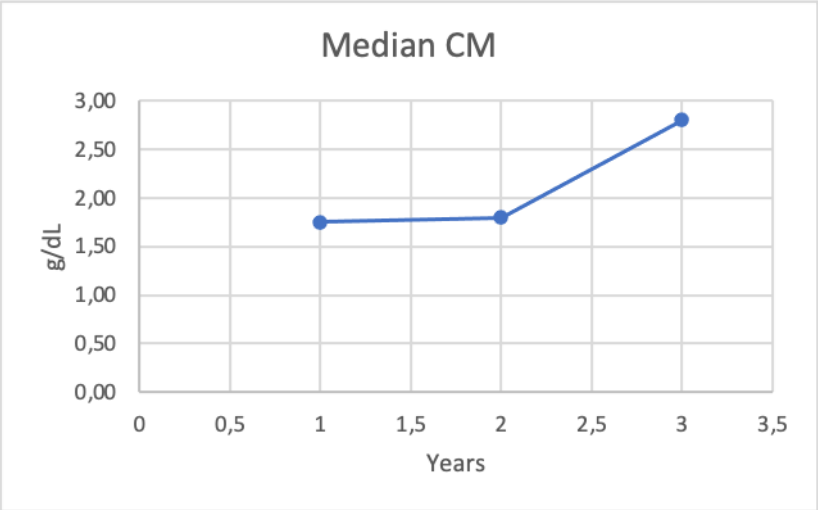
BCL-2 (MFI) was highly expressed in 11/28 cases (Median 13,4; ≥ 13,5 in 33.3%) while PD-L1 was positive in 7/28 cases (Median 25 %).

Table 8: NGF evaluation in 28 patients

Cytometry evaluation	Results
k/L	15 kappa, 13 lambda
CD200	3 (10%)
CD56	25 (89%)
CD19	1 (3,5%)
CD20	4 (14 %)
CD27	26 (92 %)
CD28	12 (42 %)
CD117	14 (28 %)
CD81	20 (71 %)
CD45	Medium intensity in 1 case
PD-L1	7 (25%)
BCL-2	11 (39%; median MFI 13,4)

Median serum M component was 1,75 g/dL at screening, with a progressive increase during time: 1,8 g/dL at intermediate analysis (at 18 months) and 2,8 g/dL at last follow-up (Table 9).

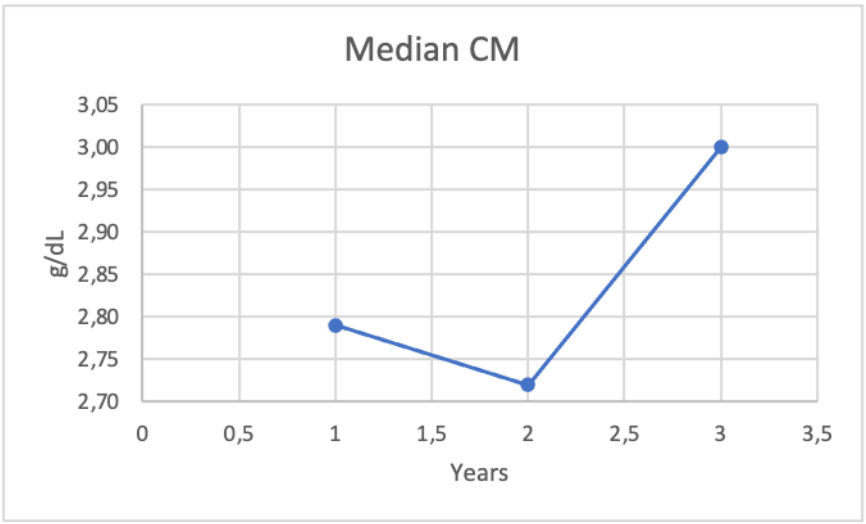
Table 9: Median M protein increase over time in 28 patients



FISH was not informative in 14 cases due to bone marrow plasmacytosis <20%, while in 9 patients was negative and in 1 case was abnormal (1 signal for chromosome 4, compatible with a FGFR3 monosomy or deletion).

Overall, 6 patients had a high-risk group according MAYO stratification and were respectively IgG lambda (2), IgA lambda (3), IgG kappa (1). Among them 3 had a previous MGUS, and 2 a previous SMM diagnosis. Serum M protein at screening and during follow-up was greater than the general group (Fig.8). Median blood cell count, skeletal imaging and BJ proteinuria were also normal at screening. In this group FISH was normal in all cases. At last follow up 1 patient, with a previous MGUS, had a progression to active myeloma, and he's receiving treatment with Daratumumab-Lenalidomide-Dexamethasone with stable response at cycle 5.

Table 10: Medium M protein increase over time in high risk group



In high-risk patients CD27 was the most expressed marker, while CD200, CD28 and CD117 were less expressed; CD56 and CD81 were present in half patients (Table 11). PD-L1 was expressed in 1 patient (64%) and BCL-2 in 4 (median MFI 13,75; range: 1,7-24,2).

Table 11: Cytometry evaluation in high-risk group

Cytometry evaluation	Results
k/L	1 K, 5 L
CD200	1/6
CD56	3/6
CD19	Negative in all cases
CD20	Negative in all cases
CD27	5/6
CD28	2/6
CD117	2/6
CD81	3/6
CD45	Negative in all cases
PD-L1	Expressed in 1 case
BCL-2	4/6 (median MFI 13,75)

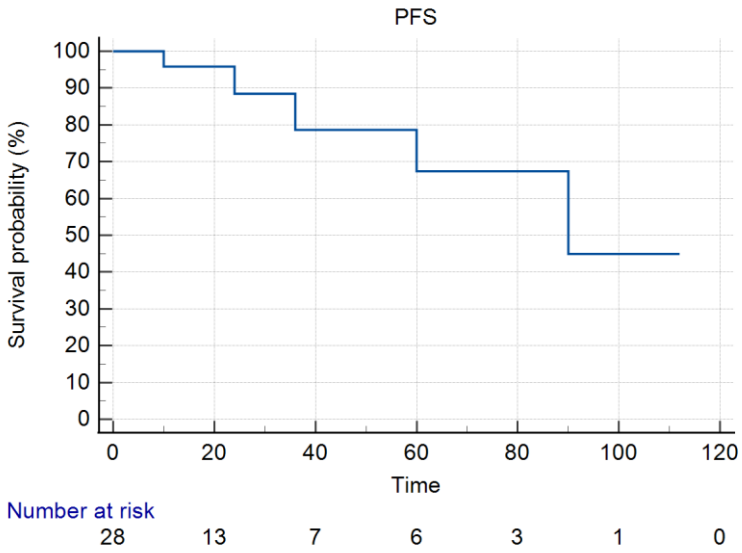
At last follow up 4/28 patients (2 M; 2 F) had a progression to multiple myeloma: at diagnosis 3 were at intermediate risk and 1 at high risk, according MAYO progressive model (Table 12).
2 patients had a previous MGUS and 2 a diagnosis of SMM (from 8 and 7 years, respectively). At last follow-up all patients had a normal FISH result, 1 patient received therapy with Bortezomib-Thalidomide-Dexametasone obtaining PR and she's waiting for autologous stem cell transplant, 2 are receiving Daratumumab-Lenalidomide-Dexamethasone and 1 who recently progressed, is about to start therapy.

Table 12: NGF evaluation in patients progressed to MM:

Cytometry evaluation	Results
k/L	3 K, 1 L
CD200	Negative in all cases
CD56	3/4
CD19	Poorly expressed, 1/4
CD20	Negative in all cases
CD27	3/4
CD28	3/4
CD117	3/4
CD81	2/4
CD45	Negative in all cases
PD-L1	Expressed in 1 case
BCL-2	High expression in 1 case (MFI 13,3)

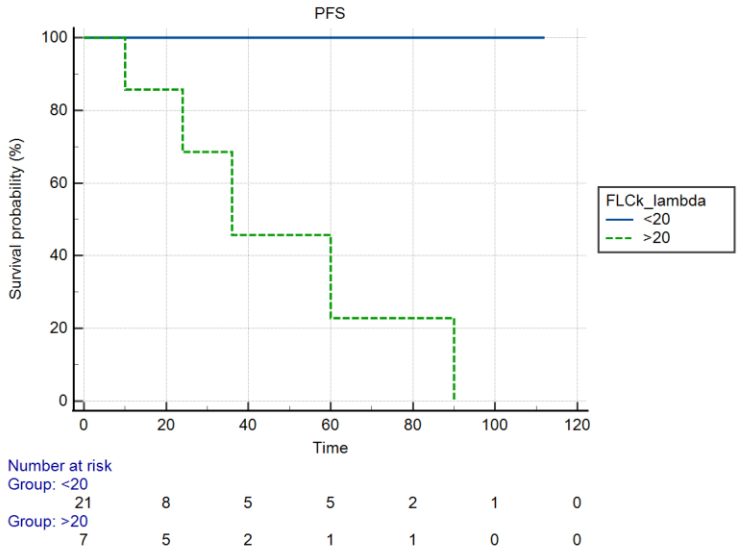
Figure 7 shows the overall PFS in our population (1-112 months).

Fig.7 PFS in all patients



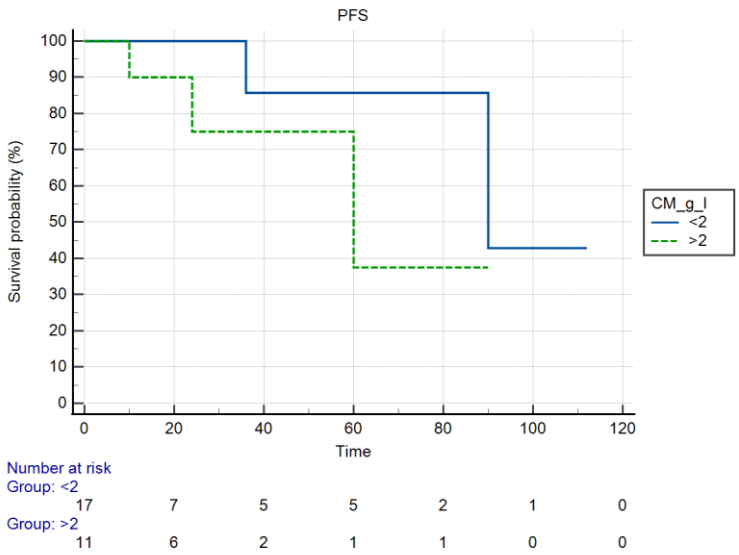
PFS was in general affected by FLCr (Fig.8). In the group of patients with FLCr > 20 the PFS was inferior with statistical significance (P= 0,0013; 0 cases with FLCr <20 and 5 cases with FLCr >20).

Fig.8 PFS and FLC ratio



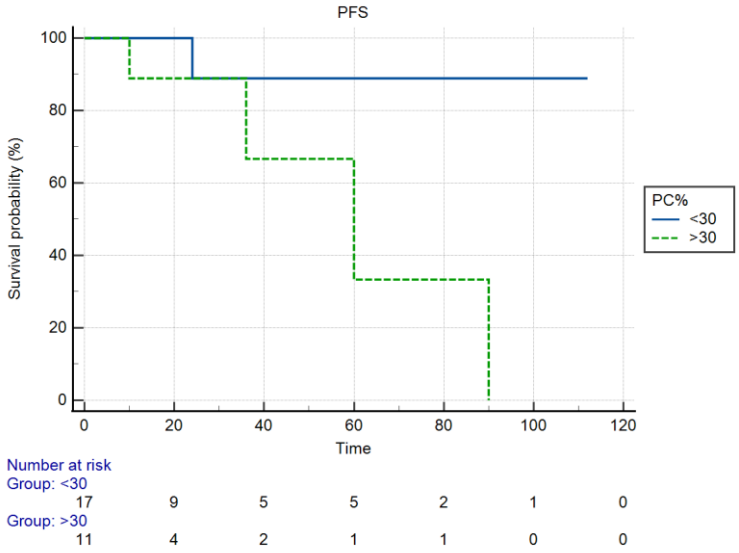
Also PFS seems to be negatively affected by the entity of CM (> 2 g/dL), even if we didn't demonstrate a statistical significance (P = 0,2202).

Fig.9 PFS and CM (g/dL)



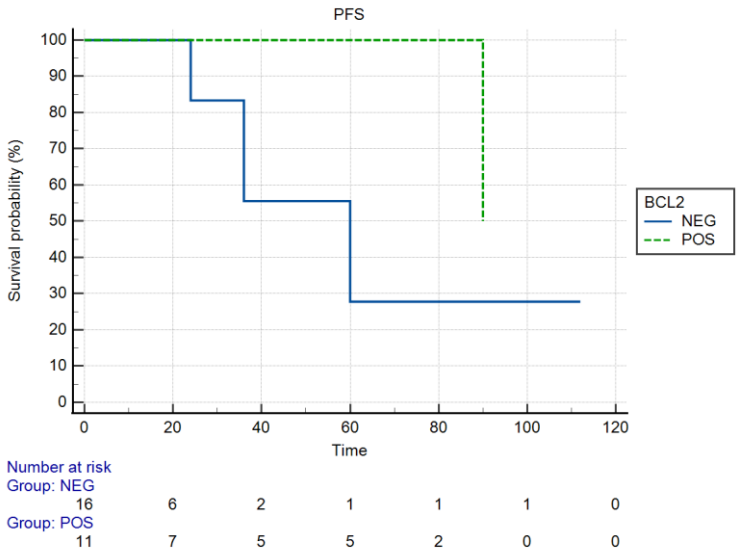
The figure 9 shows that an higher percentage of bone marrow plasmacytosis (>30%) can negatively impact on PFS with statistical significance (P = 0,0332). The mean PFS was 102,222 months in patients with <30% of PC, while in the group with bone marrow plasma cells >30% the survival was nearly half, being of 59,111 months (Fig.10).

Fig.10 PFS and plasmacytosis



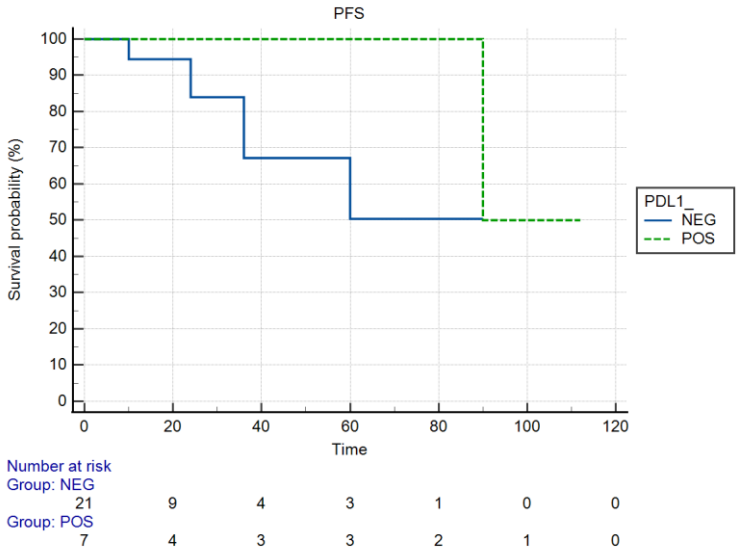
PFS was significantly affected by the expression of BCL-2 (P= 0,094): in the group with BCL-2 negativity mean PFS was 61,778 vs the mean PFS of 90 months in the group with BCL-2 positivity.

Fig.11 PFS and BCL-2



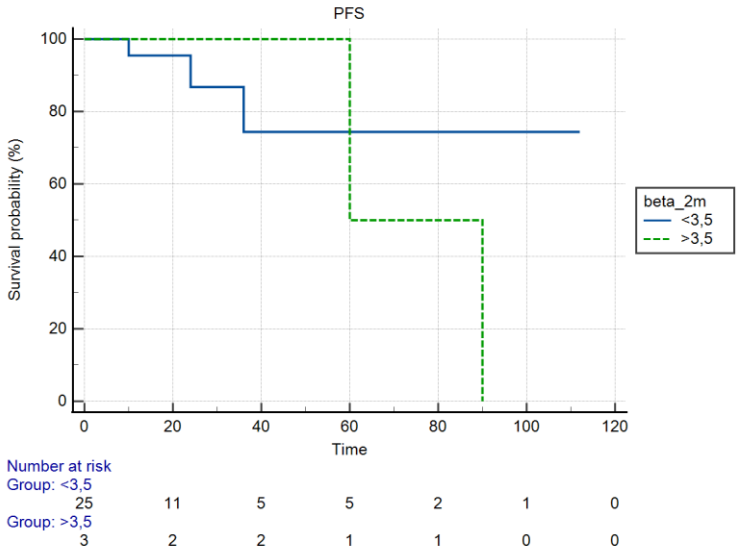
PFS seems to be affected also by PD-L1 levels, even if without statistical significance (P= 0,2986). Patients had a mean PFS of 64,526 months in the negative group vs the mean PFS of 101 months in the positive group (Fig.12).

Fig.12 PFS and PD-L1



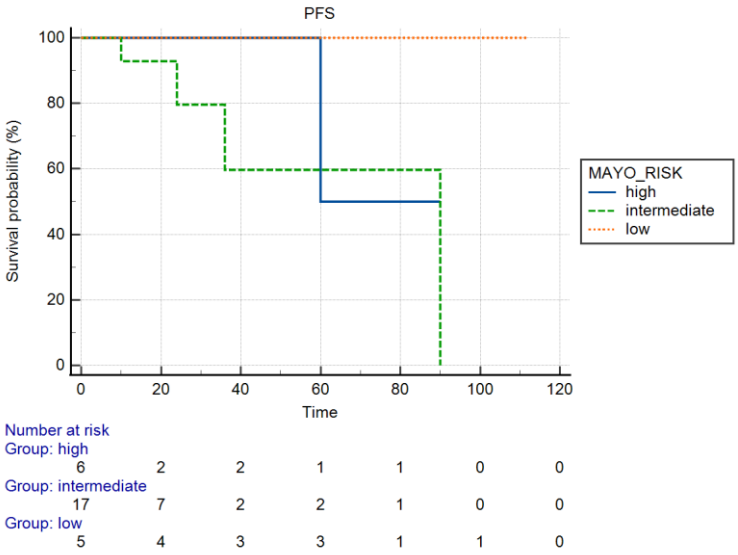
Without statistical significance (P= 0,3046), PFS was inferior in the group with normal (<3,5 mg/L) β2microglobulin (90,306 vs 75 months, figure 13).

Fig. 13 PFS and B2M (mg/L)



Finally, patients at high risk MAYO had an inferior survival with 1 case in HR patients, 4 in IR, 0 in LR (Fig.14). As expected, the low risk category had the better PFS (P= 0,19).

Fig.14 PFS and MAYO stratification



CHAPTER 6

DISCUSSION AND CONCLUSIONS

6.1 Discussion

In the last years, myeloma research was enriched by the description of new pathways, in particular PD1/PD-L1 and BCL-2 axis lead to new drugs development. The results were not always satisfactory and clinical trials about PD1/PD-L1 inhibitors in MM have been stopped for an excessive number of adverse events and number of deaths.

However, the importance PD1/PD-L1 and BCL-2 pathways and then the potential use of selective therapies had not yet fully described in smoldering patients.

In our study the characterization of CTPC was not superimposable. New approaches have been developed that allow for the highly-sensitive detection of CTPC in patients with PCN. From a clinical point of view, the detection of CTPC provides useful and relevant information for the differential diagnosis and prognostic stratification of patients with plasma cell neoplasms at diagnosis. At the same time, it enables more frequent, minimally invasive monitoring of both newly-diagnosed MGUS and treated MM patients. Overall, the presence of CTPC is associated with disseminated disease at diagnosis, as well as a higher risk of malignant transformation of MGUS and a poorer outcome (i.e., decreased PFS and/or OS rates) in both newly-diagnosed SMM and treated MM, even though the detection and cytometric characterization of CTPC seems to differ among the studies.

The current study has the limitation of a small sample of patients with a short follow up, however for the first time we tried to examine the presence of CTPC, to correlate it with cytometric markers (in particular BCL-2 and PD-L1) and to correlate it with FISH results and clinical risk markers.

As regards to bone marrow plasma cells infiltration, we confirmed with statistical significance that the amount of bone marrow disease burden directly affects the PFS: patients with > 30% plasmacytosis had an inferior survival (P = 0,0332).

BCL-2 (MFI) was highly expressed in the BM of a relevant fraction of patients (11/28, 39%; median MFI 13,4), while PD-L1 was less present (7/28, 25%). PFS seems to be related to BCL-2 expression: the patients who had it elevated seemed to have a better PFS (P= 0,0940). These data need further investigations. For example, at present we know that BCL-2 and PD-L1 can confer a survival advantage in the tumor cells, therefore what confers a positive trend in PFS in the patients that over expressed BCL-2 needs to be further explored.

The overexpression of BCL-2 can confer a pro-survival advantage, but it also makes cells vulnerable to BCL-2 family inhibition using BH3-mimetic drugs. MM is characterized by high expression of MCL-1, and overexpression of BCL-2 and BCL-XL is observed in subsets of patients. Constitutive overexpression of these pro-survival proteins in MM results from a range of microenvironmental signals and different genetic lesions. Also, MCL-1 protein expression is

increased in newly diagnosed MM compared to healthy PC, and protein levels are even higher at relapse. In addition, overexpression of MCL-1 is associated with shorter patient survival.

Since expression of both pro-survival and pro-apoptotic BCL-2 family members is heterogeneous, and the interplay between them is complex and dynamic, dependence on MCL-1, BCL-2, and BCL-XL is likely to differ between patients [*Slomp, Front. Oncol.2018*].

This complex regulation of MCL-1, BCL-2, and BCL-XL offers multiple direct and indirect targets for therapeutic intervention. However, we don't know the exact pathway in SMM, and the importance for progression to symptomatic MM. Also, in our patients the expression of MCL1 was not examined and in the future this could be of interest.

The positive influence of BCL-2 on survival in SMM patients has to be defined by larger studies and in our group this could be related to the absence of t(11;14).

Indeed, genetic lesions confer different prognosis. A Cytogenetic study in SMM led by Rajkumar et al. [*Rajkumar, Leukemia 2013*] related cytogenetic aberrations to PFS and TTP to symptomatic MM. Interestingly, PFS was significantly shorter in patients with the high risk cytogenetic marker t(4;14) compared to patients with the standard-risk t(11;14), with a median 28 versus 55 months, respectively (P = 0,025).

These data could be important in the future, because of the possibility to tailor therapy based on genetic data, for example, to evaluate the efficacy of targeted therapy with the BCL-2 inhibitor Venetoclax in preventing progression for patients with t(11;14).

As regards to FISH results, none of the patients had high risk cytogenetic features, and only in 1 case FGFR3 monosomy/delection was detected. These data can also explain the low progression rate even in a small sample of cases.

In bone marrow all 28 patients expressed variability at NGF results: the most expressed markers were adhesion molecules (CD56 and CD81), activation markers like CD27, with a small expression of CD28, CD200. Also BCR signaling Molecules like CD19 and CD20 were less frequently detected. Compared to active myeloma, these data show a great heterogeneity even in the early smoldering phase. Indeed, CD19 was present in only 1 female patient at intermediate risk who progressed to active myeloma, still under treatment, while CD45 was found in 1 female patient at intermediate risk who maintained a stable disease at last follow-up.

Our patients had normal blood count, calcemia, and Bence-Jones proteinuria were negative.

As described in literature, hypertension, cardiac disease, HCV positivity and other cancer are frequently detected, but correlations with development of smoldering myeloma cannot be defined in our group.

We also collected data over time about FLCr, B2M, CM, founding that their entity could affect PFS. In particular, the value of FCLr >20 was related to a worst PFS with statistical significance (P= 0,0013).

These data, together with the amount of bone marrow plasmacytosis are 2 important parameters that characterized the MAYO risk score and the more recent IMWG score which are the most validated and used methods to calculate prognosis in SMM patients.

The importance of FLCr is also confirmed by the current IMWG treatment guidelines, because an FLCr > 100 is one of MDE for initiating treatment.

However, the decision of starting therapy in SMM patients, who are by definition "asymptomatic" had been a debated issue in the scientific community for a long time.

Recently, Vaxman and Gertz, gave a personal opinion about not starting treatment in 2 different SMM cases, and decided to maintain a close surveillance, even in 2 cases of patients with a high risk of progression at the time of diagnosis (FLCr >100), obtaining positive results [*Vaxman, Blood 2022*]. Many experts would have advocated starting therapy many years earlier.

As regards to B2M and CM, their entity can be very important for the diagnosis and monitoring of patients, even in the absence of a statistical significance on PFS due to the small group of patients.

Median serum M component had a modest increase during follow-up, these data can explain the tendency to progress to active MM as natural course of some SMM patients. According to MAYO risk model we had a small fraction of patients at high risk, while the majority was intermediate.

In the high-risk MAYO group serum M protein was at screening and during follow-up greater than the general group. In all 28 patients MAYO risk model, as expected, correlated to PFS, that tends to be lower as the risk class increases, even if we couldn't demonstrate a statistical significance.

However, in our small group of patients, high risk group was not correlated to a particular immunophenotype. Of note, all 28 patients are alive at last follow up.

6.2 Conclusions

In these preliminary results we demonstrated absence of CTPCs, variable expression of PD-L1, high BCL-2, and FISH negativity in almost all patients: this could be explained by less genetic instability and disease severity compared to active MM cases. The diversified expression of analyzed markers confirms the high heterogeneity and complexity of the smoldering phase in MM. The significant impact of BCL-2 in our small group of patients deserves further studies on larger series of patients. In future the presence of CTPCs, the expression of BCL-2 and PD-L1 could be better evaluated.

The current standard of care in SMM is still close surveillance, outside of a clinical trial, irrespective of risk status, but participation in clinical trials is highly encouraged, whenever possible. Two large randomized clinical trials have demonstrated benefit for early intervention in high-risk SMM, but the definition of high-risk is different among clinical trials and the discordance between those definitions is high. The data published so far does not justify lowering the treatment threshold. Therefore, before recommending therapy to this heterogeneous patient population, more data needs to be published to uniformly select patients who might benefit from intervention, and these models should include dynamic parameters.
Research identifying more accurate genomic, clinical, laboratory and/or cytometric markers on PB that would enable us to assign individual risk more precisely is ongoing.

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