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Titolo Tesi

TOWARDS A MULTIMODAL RISK STRATIFICATION MODEL IN SMOLDERING MULTIPLE MYELOMA

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ABSTRACT

Smoldering multiple myeloma (SMM) is a biologically heterogeneous condition ranging from indolent to highly progressive disease. Accurate risk stratification is essential to guide monitoring and identify candidates for early therapeutic intervention.

We evaluated serum BCMA (sBCMA) levels, SKY92 gene expression profiling (GEP), cytogenetic abnormalities detected by conventional and virtual FISH, and whole-body diffusion-weighted MRI (DWI–WB–MRI), analyzing their association with disease progression (PFS). Given the potential impact of treatment on prognostic value, treated and untreated patients were analyzed separately too.

DWI–WB–MRI detected abnormal marrow infiltration in 31% of patients, occurring significantly more frequently in patients who progressed (86% vs. 21%; p = 0.0119). Normal MRI findings correlated with longer PFS (HR = 0.2753; p = 0.0102) and retained significance in untreated patients (HR = 0.1535; p = 0.0014).

Among patients evaluated for sBCMA, higher levels were observed in those who progressed, with a non-significant trend toward longer PFS in patients with lower values. SKY92 identified high-risk profiles more frequently in progressing patients, also showing a non-significant trend toward improved PFS in low-risk profiles. Virtual FISH enabled evaluation of additional patients and detected del(17p) in cases with normal standard FISH. Moreover patients without abnormalities, by either standard or virtual FISH ,showed slightly better, non-significant PFS.

Overall, DWI–WB–MRI emerged as the strongest predictor of progression, while sBCMA, SKY92, and cytogenetic findings provided complementary prognostic information. Virtual FISH improved genomic resolution and identified additional high-risk lesions. Differences in MRI-based outcomes between treated and untreated patients may further suggest a potential impact of early intervention in SMM. Despite the limitations of small sample size and short follow-up, these results support a multimodal risk stratification approach

integrating imaging, molecular, and serological markers, which warrants validation in larger prospective cohorts.

INTRODUCTION

Monoclonal gammopathies are a heterogeneous group of disorders characterized by clonal proliferation of plasma cells or B lymphocytes producing a single monoclonal immunoglobulin (M protein) detectable in serum or urine. In particular plasmacells originate from post–germinal center lymphocytes that regain proliferative capacity and home back to the bone marrow. Through a multistep process, these plasmacells progressively accumulate and expand, ultimately driving the transformation of MGUS into smoldering multiple myeloma (SMM) and overt Multiple Myeloma (MM), or related disorders [1].

The most common clinical entity within this group of disorders is Monoclonal Gammopathy of Undetermined Significance (MGUS) that is a pre-malignant plasmacell dyscrasia. It is characterized by a low level of M protein (less than 30 g/l) , fewer than 10% plasmacells in bone marrow and absence of clinical symptom linked to gammopathy. The prevalence of MGUS increases with age, reaching approximately 3–5% in individuals over 50 years old, and up to 7–10% in those over 80 years. Epidemiological studies show a higher prevalence in men compared to women and in individuals of African descent compared to Caucasians, while it is lower in Asian populations. MGUS is an asymptomatic and biologically benign precursor state that confers an average annual risk of progression of approximately 1% to multiple myeloma, AL amyloidosis, or other lymphoplasmacytic malignancies [1], [2], [3], [4].

The high prevalence of MGUS in the elderly population, together with its potential progression to aggressive hematologic malignancies such as multiple myeloma, underscores the importance of long-term clinical surveillance.

Smoldering multiple myeloma represents an asymptomatic intermediate stage between MGUS and symptomatic multiple myeloma, characterized by an increased clonal plasmacell burden without evidence of end-organ damage (CRAB features: hypercalcemia, renal insufficiency, anemia, or bone lesions) or active myeloma-defining

biomarkers, according to the updated IMWG criteria. In particular, the diagnostic criteria for SMM include:

- Serum monoclonal protein ≥3 g/dL and/or
- Bone marrow clonal plasma cell infiltration between 10% and 60%
- Absence of myeloma-related symptoms or organ damage (CRAB or SLiM criteria).

With the introduction of the "SLiM-CRAB" criteria in 2014, patients presenting with ≥60% bone marrow plasma cells, an involved/uninvolved free light chain ratio ≥100, or >1 focal lesion ≥5 mm on whole-body MRI are now classified as having active multiple myeloma rather than SMM [5].

SMM accounts for approximately 15% of all monoclonal gammopathy diagnoses, with an estimated incidence of 0.9 cases per 100,000 persons per year. The median age at diagnosis is approximately 67 years, with a slight male predominance [6].

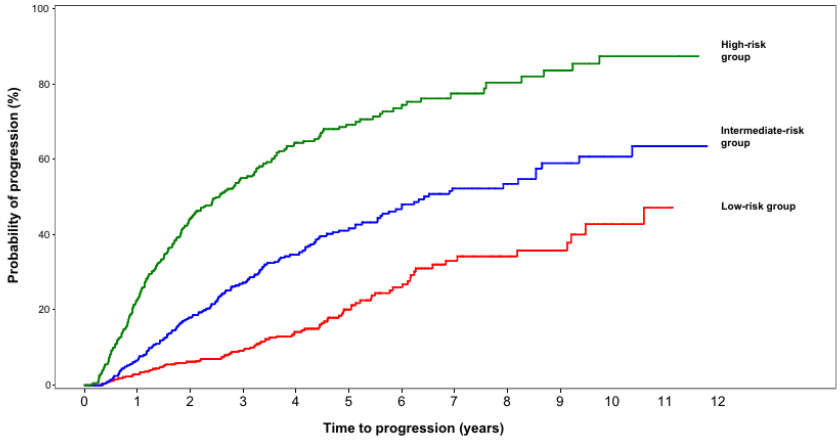
SMM carries a significantly higher risk of progression compared with MGUS. Historically, the risk of progression to symptomatic multiple myeloma has been estimated at approximately 10% per year during the first five years, 3% per year over the subsequent five years, and 1% per year thereafter. However, some patients exhibit markedly higher progression risks, defining the category of "high-risk SMM" [3], [7], [8].

Current risk stratification for SMM, as proposed by the Mayo Clinic and the IMWG, includes serum monoclonal protein level, the percentage of plasmacells in the bone marrow, and the free light chain (FLC) ratio. In particular, the Mayo Clinic model, proposed in 2018 ("20-2-20 model"), incorporates three independent risk factors:

1. Bone marrow plasma cells >20%
2. Serum M protein >2 g/dL
3. FLC ratio >20

The presence of these risk factors stratifies patients into 3 distinct risk categories (Fig.1)[9], [10].

- 0 factors: Low risk (6% progression at 2 years)
- 1 factor: Intermediate risk (18% progression at 2 years)
- 2-3 factors: High risk (44% progression at 2 years)



Risk Stratification groups	Number of risk factors	Hazard Ratio (95% CI)	Risk of progression (2 years)	# of patients
Low-Risk	0	Reference	6.2%	522 (38.3%)
Intermediate	1	2.99 (1.97 – 4.54)	17.9%	445 (32.7%)
High	2-3	9.02 (6.15 – 13.2)	44.2%	396 (29.1%)

Figure 1. IMWG risk for SMM [10]

Ultimately, SMM is an asymptomatic form of myeloma, but it is not simply a biological intermediate stage between MGUS and MM. In particular, SMM is a heterogeneous, clinically defined condition, with approximately two-thirds of patients exhibiting clinical behavior similar to MGUS (pre-malignancy) and approximately one-third displaying behavior akin to MM (biological malignancy) [11].

Currently, in Italy, the standard of care for SMM remains observation, with treatment deferred until the development of symptomatic disease, while therapy within clinical trials is reserved for patients with high-risk disease. Indeed, recent randomized trials have suggested that early intervention can improve progression-free survival, sparking a debate on early therapeutic strategies in selected patient subgroups.

In particular, in the QuiRedex trial, high-risk SMM were randomized to receive either early treatment with lenalidomide plus dexamethasone or observation. The trial demonstrated that early intervention with lenalidomide and dexamethasone in patients with high-risk smoldering myeloma significantly prolongs both time to progression to active myeloma and overall survival, with follow-up extending up to 12 years [12].

Similarly, in the AQUILA study, patients with high-risk SMM were randomized to receive subcutaneous daratumumab monotherapy or active monitoring, and daratumumab was associated with a significantly lower risk of progression to active myeloma or death, as well as improved overall survival, compared with observation [13].

Collectively, these observations highlight MGUS and SMM as biologically stable yet genetically dynamic conditions, providing a critical window for studying the early events underlying plasmacell neoplasia and guiding risk-adapted monitoring and management strategies.

BACKGROUND AND PROJECT RATIONALE

Over the past ten years, new scientific evidence has identified numerous additional risk factors potentially associated with SMM progression, which have not been incorporated into the current IMWG risk stratification for SMM. This project aims to evaluate the significance of cytogenetic and molecular alterations, new-generation imaging techniques—particularly whole-body MRI (WB-MRI)—and serum BCMA levels in predicting the risk of progression from smoldering multiple myeloma (SMM) to active multiple myeloma (MM).

Ultimately, these data could be integrated into conventional scoring systems to improve the prediction of progression risk. Enhanced risk stratification may facilitate closer monitoring and timely therapeutic interventions, potentially reducing clinical complications, the associated burden of care, and, in the long term, altering the natural history of multiple myeloma.

CYTOGENETIC ALTERATIONS IN SMOLDERING MULTIPLE MYELOMA

The development of multiple myeloma is classically considered a multistep process characterized by the progressive acquisition of genetic abnormalities. Among these, immunoglobulin heavy chain translocations and hyperdiploidy represent common initiating events that give rise to monoclonal gammopathy of undetermined significance (MGUS). Subsequent accumulation of additional mutations leads to SMM; however, these alterations alone are insufficient to drive transformation to overt MM, as MGUS and SMM patients frequently harbor such genetic lesions while remaining asymptomatic [14] .

Different studies have demonstrated the impact of cytogenetic abnormalities on the risk of progression in smoldering multiple myeloma [15].

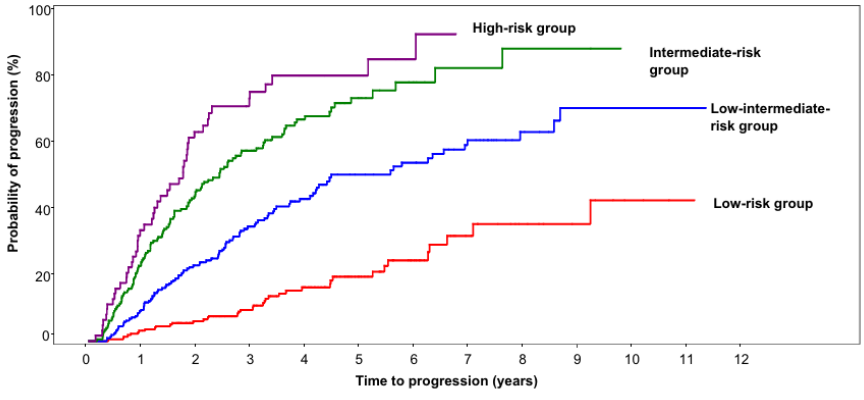
FISH (Fluorescence In Situ Hybridization) is a technology that uses labeled nucleotide probes, known as DNA probes, to identify specific regions of a chromosome, that is, particular DNA sequences. Unlike conventional karyotyping, which requires dividing

cells, FISH can be performed on interphase nuclei, thereby increasing its sensitivity for detecting clonal aberrations.

The most common FISH abnormalities in SMM and MM include hyperdiploidy (trisomies of odd-numbered chromosomes); deletion 17p (TP53 deletion); Gain or amplification of 1q21 and IgH translocations including t(11;14)(q13;q32) CCND1/IgH, t(4;14)(p16;q32) FGFR3/IgH, t(14;16)(q32;q23) MAF/IgH **[**14**]**.

Recognizing the prognostic relevance of cytogenetic abnormalities, in 2020 the International Myeloma Working Group (IMWG) incorporated these markers into the 2/20/20 risk stratification model. This model integrates high-risk cytogenetic features, including t(4;14), t(14;16), +1q, and/or del(13q), to improve the identification of patients with high-risk SMM [10]. Specifically, the 2/20/20 model stratifies patients into 4 distinct risk categories (Fig. 2).

- 0 factors: Low risk (6% progression at 2 years)
- 1 factor: Low-Intermediate risk (23% progression at 2 years)
- 2 factors: Intermediate risk (46% progression at 2 years)
- 3-4 factors: Hgh risk (63% progression at 2 years)



Risk Stratification groups	Number of risk factors	Hazard Ratio (95% CI)	Risk of progression (2 years)	# of patients
Low	0	Reference	6.0%	225 (32.7%)
Low-intermediate	1	4.16 (2.26 – 7.67)	22.8%	224 (32.5%)
Intermediate	2	9.82 (5.46 – 17.7)	45.5%	177 (25.7%)
High	3-4	15.5 (8.23 – 29.0)	63.1%	63 (9.1%)

Figure 2. IMWG risk for SMM considering cytogenetics [10]

GENE EXPRESSION PROFILE (GEP) IN SMOLDERING MULTIPLE MYELOMA

Next-generation sequencing (NGS) is a high-throughput technology that determines the sequence of DNA or RNA to investigate genetic variations associated with disease. Unlike traditional Sanger sequencing by capillary electrophoresis , which sequences one DNA fragment at a time, NGS can simultaneously sequence millions of DNA strands. This capability allows for the parallel interrogation of hundreds to thousands of genes across multiple samples, as well as the comprehensive identification and analysis of diverse genomic features within a single sequencing run.

Consequently, NGS has revolutionized the molecular characterization of plasmacell disorders by enabling the comprehensive detection of somatic mutations, copy number variations (CNVs), translocations, and mutational signatures, while also confirming the presence of intraclonal heterogeneity at all stages of plasma cell dyscrasia progression.

Recent studies have demonstrated the presence of intraclonal heterogeneity across all stages of plasma cell dyscrasia evolution, suggesting that disease progression may be driven by inter-subclonal competition and the outgrowth of the fittest subclones [16], [17].

Two distinct patterns of progression have been described: a‘static progression model’, in which the subclonal architecture is preserved as the disease advances to symptomatic Multiple myeloma, implying that progression merely reflects the time required to accumulate a sufficient disease burden; and a ‘spontaneous evolution model’, characterized by dynamic changes in subclonal composition. The latter represents a compelling example of spontaneous Darwinian evolution, where the subclonal composition of smoldering MM shifts in the absence of treatment-induced selective pressure, due to the stochastic acquisition of additional mutations conferring a proliferative advantage to one subclone [18].

Moreover different gene expression signatures have been indentified in multiple myeloma and have have emerged as valuable biomarkers for predicting the risk of clonal evolution and the progression of plasmacells toward overt MM [19].

GEP70 (UAMS70) is a gene expression signature consisting of 70 genes, developed at the University of Arkansas for Medical Sciences (UAMS). It was originally created to stratify prognostic risk in active multiple myeloma (MM) by identifying patients with high risk of progression and poor survival. It is based on the expression of genes involved in proliferation, cell cycle regulation, cell adhesion, metabolism, and other tumor aggressiveness pathways [20].

GEP 70 signature was studied in smoldering myeloma too and it was demostrated that high-risk GEP70 identified patients with worse median time to progression (TTP) respect to low–risk (Fig.3)[21].

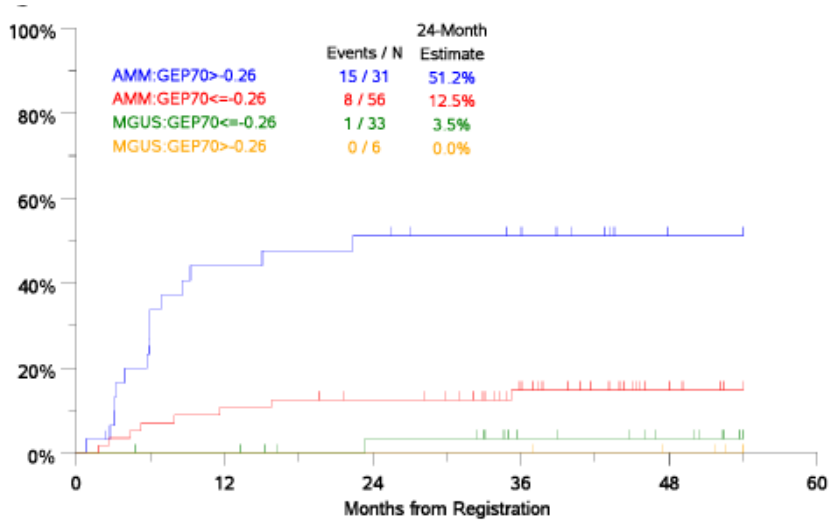


Figure 3. PFS risk by GEP 70 [21]

A prospective analysis identified a four-gene signature (including RRM2, DTL, and ASPM) capable of predicting the need for treatment within 2 years in patients with smoldering multiple myeloma (SMM). This signature, termed GEP4, showed superior performance compared with the previous GEP70 model, with an estimated 2-year progression probability of 85.7% versus 49% [22].

Finally, SKY92 is a gene expression profiling (GEP) assay that classifies multiple myeloma (MM) patients as either high-risk or standard-risk. The 92 genes included in the panel are involved in key cancer-related pathways, such as cell cycle regulation, apoptosis inhibition, DNA repair, MYC activation, and chromosomal instability. The test has been independently validated in 16 patient cohorts comprising over 3,300 individuals with MM, establishing it as a robust prognostic tool for symptomatic disease [23], [24].

Nevertheless there are no published clinical studies validating SKY92 in smoldering multiple myeloma (SMM) and its utility in this setting needs further research. Unfortunately, evaluating BMPC in SMM can be challenging because of the low level of marrow infiltration and the possible peripheral blood contamination of the aspirate [25]. However, the polytypic-PC score did not emerge as an independent variable in the multivariate models when GEP variables were included, suggesting that, despite the potential caveat of contaminating normal PCs, the GEP by purified CD138+ PCs can provide powerful information regarding the risk of disease progression in SMM[21] . Further analyses integrating genetic profiling of tumor cells with the biology of the tumor microenvironment may further refine our ability to predict the risk of progression to clinical MM [26], [27].

WHOLE BODY MRI IN SMOLDERING MULTIPLE MYELOMA

Advances in morphological and functional imaging have enabled superior detection of early bone disease, bone marrow infiltration, and paramedullary as well as extramedullary involvement in multiple myeloma. The two functional imaging modalities that are most widely used and standardized are ^18F-fluorodeoxyglucose positron emission tomography/computed tomography (^18F-FDG PET/CT) and whole-body magnetic resonance imaging with diffusion-weighted imaging (WB-DWI-MRI).

IMWG recommends WB-MRI for all patients with a suspected diagnosis of SMM or MM as well as in case of suspected relapse because of its superior sensitivity and utility in identifying myeloma-defining events. Indeed, according to the International Myeloma

Working Group (IMWG) criteria, the presence of >1 focal lesions on MRI has became a myeloma-defining event requiring treatment initiation [5].

In particular 3T Whole-body MRI with diffusion-weighted imaging (WB-DWI-MRI) enables comprehensive assessment of the axial and appendicular skeleton without ionising radiation. It is particularly sensitive in detecting focal lesions (≥5 mm) and diffuse marrow infiltration patterns. It combines high quality morphological images with "functional" information. Diffusion-weighted imaging (DWI) is based upon measuring the random Brownian motion of water molecules in extracellular space within a tissue [28]. It's used as a core sequence of WB-MRI protocols for disease assessment because of its sensitiveness to tissue cellularity and cell viability offering excellent lesion-to-background contrast and quantification of the degree of water motion by calculation of the apparent diffusion coefficient (ADC) map. In particular changes in ADC can reflect variations in cellularity [29], [30] . DWI and ADC are used respectively as qualitative and quantitative indicators of disease presence. Moreover any changes in bone marrow cellularity, water content reflect in changes in ADC values (increase),T2 signal ,dixon-type pulse sequences T1 and relative FF (rFF) map. The combination of anatomical sequences and at least two "functional" quantitative sequences makes DW-MRI a multiparametric imaging technique [31]. Any WB-MRI protocol should include DWI to improve diagnostic sensibility, as well as ADC and FF imaging, in conjunction with all other sequences, to maximize specificity in reporting.

Interpretation of imaging studies of myeloma patients is often challenging[31]. In the field of plasmacell disorders, application of the 'Guidelines for Acquisition, Interpretation, and Reporting of Whole-Body MRI in Myeloma Myeloma Response Assessment and Diagnosis System (MY-RADS)' is fundamental for standardization. MR images show patterns of myeloma disease: normal, focal (focal lesions superior or equal to 5mm), diffuse, focal on diffuse, micronodular (focal lesions inferior to 5 mm) and paramedullary/extramedullary tissue. Additionally, MY-RADS provides a mean for assessing response to treatment using Response Assessment Criteria (RAC), a Likert scale with five points including ADC

evaluation with morphological information. Specifically, RAC 1 and 2 correspond to therapeutic response, RAC 3 to disease stability, and RAC 4 and 5 to disease progression. A structured report has been proposed for WB-MRI [32], [33].

^{18}fluorodeoxyglucose (^{18}F-FDG) PET/CT combines metabolic and anatomical imaging, enabling detection of hypermetabolic lesions suggestive of active disease. Therefore it is mandatory for confirming a suspected diagnosis of solitary plasmacytoma and for distinguishing between smouldering and symptomatic multiple myeloma when WB-MRI cannot be performed [34].

Multiple studies have demonstrated role and prognostic significance of MRI findings in symptomatic Multiple Myeloma through evalutations combining 3T Whole-body MRI with diffusion-weighted imaging (WB-DWI-MRI)and ^{18}F-FDG PET/CT [35], [36], [37].

However, in the context of SMM, there remains a paucity of studies assessing the prognostic value of WB-DWI-MRI. Current evidence suggests that advanced imaging techniques may provide valuable information for risk stratification, not only by identifying early focal lesions, but also evidencing diffuse or micronodular marrow infiltration patterns that are not yet detectable by conventional methods. Nevertheless, further prospective and longitudinal studies are needed to clarify the prognostic role of MRI and to establish standardized imaging-based criteria for risk stratification in SMM.

SERUM BCMA IN SMOLDERING MULTIPLE MYELOMA

B-cell maturation antigen (BCMA) ,also known as tumor necrosis factor receptor superfamily member 17 (TNFRSF17), is a transmembrane receptor of the tumor necrosis factor expressed almost exclusively on plasma cells and mature B cells. BCMA plays a critical role in plasma cell survival and proliferation [38].

In Multiple Myeloma , BCMA expression is nearly universal, making it a therapeutic target and a potential biomarker of disease burden [39].

Gamma secretase, a ubiquitous intramembranous protease, sheds membrane bound BCMA and releases it into the serum[40].

Indeed serum BCMA (sBCMA) results from proteolytic shedding of membrane BCMA by γ-secretase, reflecting tumor burden due to high plasma cell turnover. Unlike M-protein quantification, sBCMA levels are not affected by renal function or monoclonal protein catabolism, offering a direct measure of plasma cell mass. Soluble BCMA (sBCMA) has emerged as a promising prognostic marker in plasma cell dyscrasias, including Smoldering Multiple Myeloma (SMM). [41]

Ghermezi M conducted a prospective analysis of sBCMA levels in healthy donors and patients with SMM, and MM, demonstrating a stepwise increase across disease stages: levels were significantly higher in SMM compared to healthy donors, and highest in symptomatic MM.[42].

Bujarski S investigated whether baseline serum B-cell maturation antigen (sBCMA) levels could predict disease progression in 65 patients with SMM. A baseline sBCMA level ≥137.5 ng/mL was identified as the optimal cutoff distinguishing high-risk from low-risk SMM patients. Those patients with high-risk sBCMA levels experienced a shorter time to progression, although these findings require prospective validation before being incorporated into clinical practice guidelines [43].

Similarly, Visram A and colleagues, at the Mayo Clinic, examined the prognostic significance of sBCMA in MGUS and SMM. In a retrospective analysis of 99 MGUS and 184 SMM patients, baseline sBCMA levels were markedly higher in individuals who subsequently progressed to MM. Overall, elevated sBCMA levels in both MGUS and SMM were consistently associated with an increased risk of progression, underscoring the potential utility of sBCMA as a biomarker for disease monitoring and risk stratification.(Fig 4) [44].

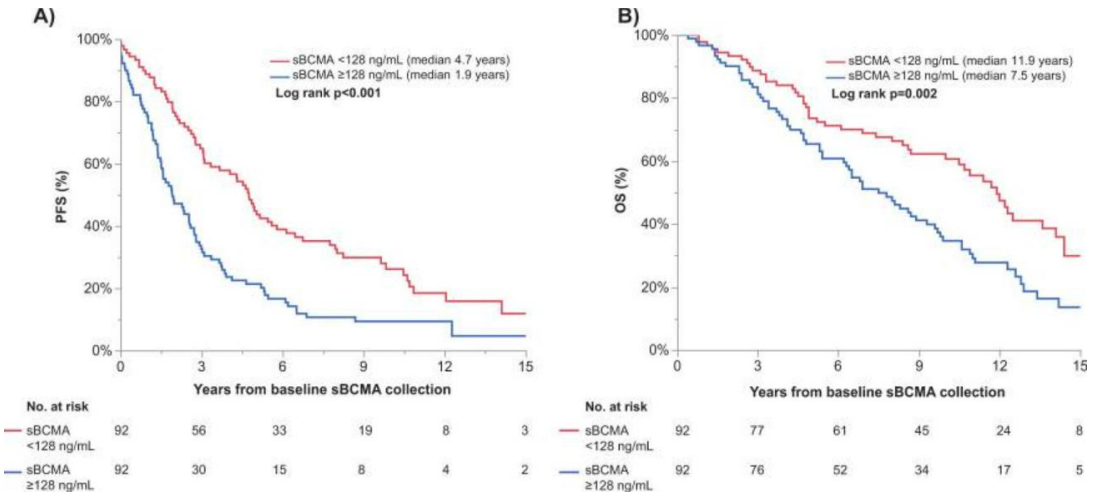


Figure 4. PFS by sBCMA in SMM [44]

In conclusion, sBCMA has emerged as a valuable biomarker in smoldering multiple myeloma, providing prognostic information beyond traditional clinical parameters and identifying patients at higher risk of progression to symptomatic MM.

MATERIALS AND METHODS

This is a retrospective and prospective study recruiting patients with smoldering multiple myeloma (SMM) within routine hematologic practice at IRST–Meldola–Forlì-Cesena. The study was approved by the local Ethics Committee. All patients were enrolled after providing written informed consent in accordance with the Guidelines for Good Clinical Practice.

Eligibility criteria included a diagnosis of SMM according to the International Myeloma Working Group (IMWG) criteria [5], no prior therapy for plasma cell disorders, and willingness to provide biological samples and assesments for research purposes.

All evaluations were performed within a maximum interval of five months. For each patient, clinical and laboratory data were collected, and the IMWG risk score for SMM was calculated [10]. Progression-free survival (PFS) was defined as the time from diagnosis until disease progression to symptomatic multiple myeloma.

Bone marrow (BM) samples were obtained for fluorescence in situ hybridization (FISH) analysis to assess the main cytogenetic abnormalities, including *t*(4;14), *t*(14;16), and del(17p), according to the revised International Staging System[45]. In addition, del(1p) and amp(1q) were evaluated as chromosomal abnormalities associated with poor prognosis[46]. Moreover, next-generation sequencing (NGS) was performed in 25 patients on bone marrow (BM) samples after isolation of $CD38^+$ cells. Gene expression profiling (GEP) was conducted using the SKY92 panel. A "virtual FISH" approach was applied to NGS data to evaluate cytogenetic alterations, a method particularly useful when standard FISH analysis could not be performed.

Peripheral blood samples were analyzed to determine serum BCMA (sBCMA) levels in 24 patients.

Skeletal assessment was conducted using whole-body diffusion-weighted MRI (WB-DWI-MRI) and ^18F-FDG PET/CT.

The primary objective of the study was to evaluate the prognostic value of WB-DWI-MRI patterns, serum BCMA (sBCMA) levels, SKY92 molecular signature, and virtual FISH findings in predicting the risk of progression from SMM to overt multiple myeloma.

SKELETAL ASSESSMENT

Skeletal assessment was performed using ^18F-FDG PET/CT and 3T whole-body magnetic resonance imaging (WB-DWI-MRI) with diffusion-weighted imaging (DWI). DWI served as the core sequence, providing excellent lesion-to-background contrast and allowing quantitative assessment of water diffusion through apparent diffusion coefficient (ADC) mapping. Additional sequences included T2-weighted imaging, Dixon-type T1 sequences, and relative fat fraction (rFF) mapping. According to the 'Guidelines for Acquisition, Interpretation, and Reporting of Whole-Body MRI in Myeloma', MRI images were categorized into distinct patterns: normal, diffuse, micronodular (focal lesions <5 mm), and focal (focal lesions ≥5 mm). Patients presenting with more than one focal lesion were excluded from the study, as this finding was considered diagnostic of symptomatic myeloma[5]. All patients also underwent ^18F-fluorodeoxyglucose positron emission tomography/computed tomography (^18F-FDG PET/CT) to confirm no progression to symptomatic multiple myeloma.

SERUM BCMA LEVEL EVALUATION

Plasma levels of circulating B cell maturation antigen /TNFRSF17 (BCMA) were measured using the Simple Plex™ automated immunoassay system (Ella™, Bio-Techne, Minneapolis, USA), a microfluidic next-generation ELISA platform. All concentrations were interpolated from the cartridge's factory-calibrated standard curve using the integrated Ella software.

GENE EXPRESSION PROFILING AND VIRTUAL FISH EVALUATION

Gene expression profiling (GEP) was performed on enriched bone marrow PCs using the SkylineDx MMprofiler™ platform (Rotterdam, The Netherlands). Data were processed

using the MMprofiler™ CE IVD assay software module (ASM), which performs normalization and quality control, and subsequently returns gene expression profiles and SKY92 risk classification.

The SKY92 gene expression signature stratifies patients into standard-risk (SR) or high-risk (HR) categories based on the expression of 92 genes involved in key oncogenic pathways, including cell cycle regulation, apoptosis inhibition, DNA repair, MYC activation, and chromosomal instability.

In addition, the platform provides information on key cytogenetic markers. Specifically, alterations evaluated by both GEP and standard FISH were t(4;14), t(14;16), del(17p), and gain(1q).

STATISTICAL ANALYSIS

Descriptive statistics were applied to summarize baseline patient characteristics. Comparisons between groups were performed using the non-parametric Wilcoxon rank-sum test and Fisher's exact test for discrete variables.

PFS(progression free surivival), that is the probability of progression to symptomatic disease, was defined as the interval from bone marrow evaluation (considered the time of smoldering myeloma diagnosis) to progression to symptomatic myeloma or last follow-up. PFS was analyzed using the Kaplan–Meier method. Mantel–Cox proportional hazards test was used to estimate *p*-values, hazard ratios (HR) for progression, and 95% confidence intervals (CI), taking into account baseline sBCMA levels, WB-DWI-MRI patterns, SKY92 gene expression profiles, standard fish and virtual fish evaluations.
In addition, we assessed the utility of virtual FISH analysis in patients for whom standard FISH could not be performed, by evaluating the increase in the proportion of patients for whom genetic data could be obtained.

Unfortunately, Standard FISH data, serum BCMA levels and gene expression profiling analyses are available only in about half of patients. These missing data therefore did not allow for a robust multivariate analysis to be performed.

In addition, about half of patients initiated treatment within clinical trials aimed at preventing progression to symptomatic disease, which may have affected the prognostic value of the data collected at diagnosis. For this reason, a separate analysis of treated and untreated patients was also conducted to explore the prognostic value, in terms of progression to symptomatic disease, of MRI findings, SKY92, standard FISH/virtual FISH, and sBCMA levels. Hhowever, due to the limited sample size—particularly for the latter three assessments—these results should be interpreted with caution.

RESULTS

A total of 52 patients diagnosed with smoldering multiple myeloma (SMM) according to IMWG criteria were enrolled between February 2021 and December 2024. Baseline characteristics are summarized in Table 1. Overall, 27% (14/52) of patients have progressed to symptomatic myeloma to date. A total of 29 patients initiated treatment within clinical trials aimed at preventing progression to symptomatic disease; however, 5 of these patients eventually progressed to symptomatic multiple myeloma. We evaluate patients characteristic in the overall cohort, as well as in patients who progressed versus those who did not.

In particular in entire cohort median age was 61 years (range, 35–83), and 42% (n = 22) of patients were at least 65 years old. Twenty-five patients (48%) were male.

The median M-protein concentration was 20.45 g/L (range, 3.1–41.1); values >20 g/L were observed in 50% (n = 26) of cases. The IgA isotype was present in 15% (n = 8) of patients. Median bone marrow plasmacell infiltration was 25% (range, 10–55), with 56% showing >20% plasmacells. The median serum free light chain ratio (FLCR) was 7.21(range,0.61-93.43), and values >20 were observed in 29% of patients. β2-microglobulin levels ≥3.5 mg/L were detected in 12% (n = 6) of patients, while Bence Jones proteinuria was present in 50% (n = 26).

According to IMWG risk stratification, 44% (n = 23) of patients were classified as high-risk (≥2 risk factors), 39% (n = 20) as intermediate-risk (1 risk factor), and 17% (n = 9) as low-risk. Notably, 71% (n = 10) of patients who progressed were classified as high-risk, compared with 34% in the non-progressed group (p=0.0191)(Fig. 5)

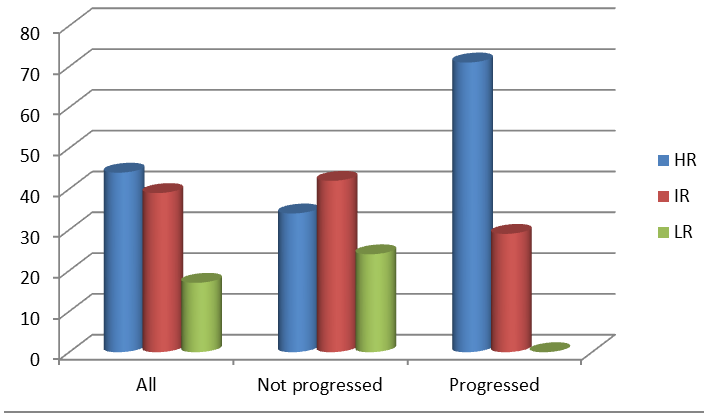


Figure 5. IMWG risk percentage in entire, not progressed and progressed cohort

FISH analysis revealed cytogenetic abnormalities—including *t*(4;14), *t*(14;16), +1q, −1p, and del(17p)—in 23% (n = 12) of patients, while 31% (n = 16) showed no abnormalities. FISH was not evaluable in 46% (n = 24) of cases. Among patients who progressed, cytogenetic abnormalities were detected in 28% (n = 4), compared with 21% (n = 8) among those who did not progress ($p = 0.5939$)(Fig. 6).

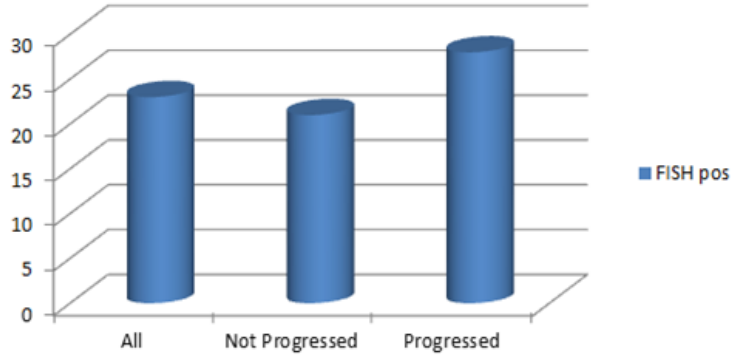


Figure 6. FISH positivity percentage in entire, not progressed and progressed cohort

Whole-body diffusion-weighted MRI (DWI-WB-MRI) demonstrated a diffuse bone marrow infiltration pattern in 25% (n = 13) and a micronodular pattern in 6% (n = 3) of patients, whereas 69% (n = 36) showed a normal MRI signal. No focal lesions or ^18F-FDG PET positivity suggestive of active disease transformation were observed. Among progressed patients, abnormal MRI signals (diffuse or micronodular) were observed in 86% (n = 12), with diffuse and micronodular patterns each present in 43% (n = 6). In

contrast, only 21% of non-progressed patients showed altered MRI signals (diffuse in 18% [n = 7] and micronodular in 3% [n = 1]) ($p = 0.0119$).(Fig 7)

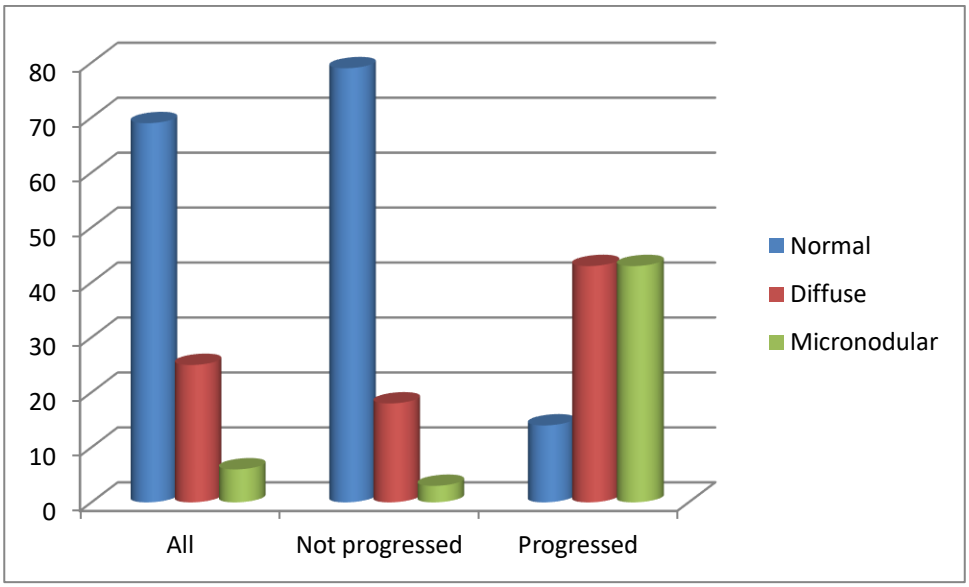


Figure 7. DWI-WB-MRI pattern percentage in entire, not progressed and progressed cohort

Characteristic	All(n=52)	Not progressed(n=38)	Progressed (n=14)
Age			
median	61(range35-83)	58(range35-79)	68(range48-83)
>o =65	22 (42%)	13(34%)	9(64%)
Sex			
Male/Female	25/27	10/18	5/9
M protein			
Median g/l	20.45 (range 3.10-41.1)	20.2 (range 3.1-41.2)	22.6 (range 7.9-38.4)
>20 g/l	26 (50%)	19 (50%)	7 (50%)
IgA isotype	8 (15%)	5 (13%)	3 (21%)
Bone marrow PC			
Median %	25 (range 10-55)	20 (range 10-55)	40 (range 25-55)
>20%	29 (56%)	15(40%)	14(100%)

FLC			
R median	7.21(range 0.61-93,43)	7.21(range 0.61-93.43)	8.65(range1.52-89.77)
R>20	15 (29%)	10 (26%)	5 (36%)
B2M			
>0=3,5 mg/l	6 (12%)	4 (10%)	2 (14%)
BJ pos	26 (50%)	19 (59%)	7 (50%)
IMWG risk			
Low	9 (17%)	9 (24%)	0
Intermediate	20 (39%)	16 (42%)	4 (29%)
High	23 (44%)	13 (34%)	10 (71%)
Standard FISH			
Positive	12 (23%)	8 (21%)	4 (28%)
Negative	16 (31%)	11 (29%)	5 (36%)
NV	24 (46%)	19 (50%)	5 (36%)
WB-DWI-MRI			
Normal	36 (69%)	30 (79%)	2 (14%)
Diffuse+micronodular	13+3 (25+6%)	7+1(18+3%)	6+6(43+43%)

Table 1 .Patients clinical, laboratoristic, cytogenetic and radiological characteristics

Table 2 summaries patient characteristics including evaluation by serum BCMA levels, gene expression profiling using the SKY92 platform and performing virtual fish . In particular sBCMA and GEP analyses have so far been performed in 24 and 25 patients, respectively

In patients evaluated for sBCMA(n=24), the median level was 106.1 ng/mL (range, 41.3–691.2). Progressed patients showed higher median sBCMA levels (152.9 ng/mL; range, 54.5–691.2) compared with non-progressed patients (104.7 ng/mL; range, 41.3–393.7; p = 0.2591) (Fig. 8).

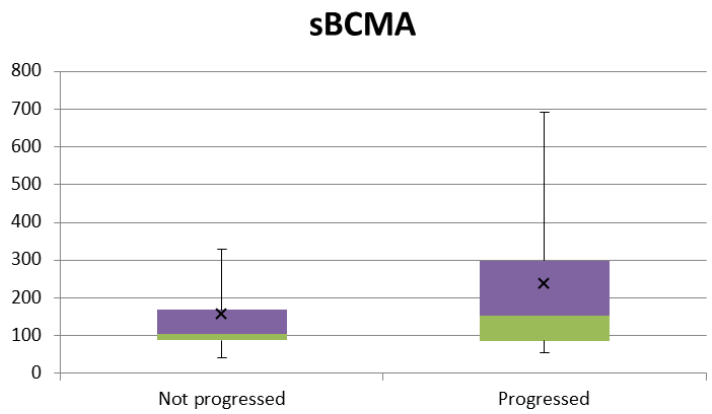


Figure 8. sBCMA levels in progressed and not progressed cohorts

Patients with sBCMA levels higher than the median value (106.1 ng/mL) represented 71% (n = 5) in progressed cohort versus 47% (n = 8) in non-progressed one (p= 0.2842)(Fig.9)

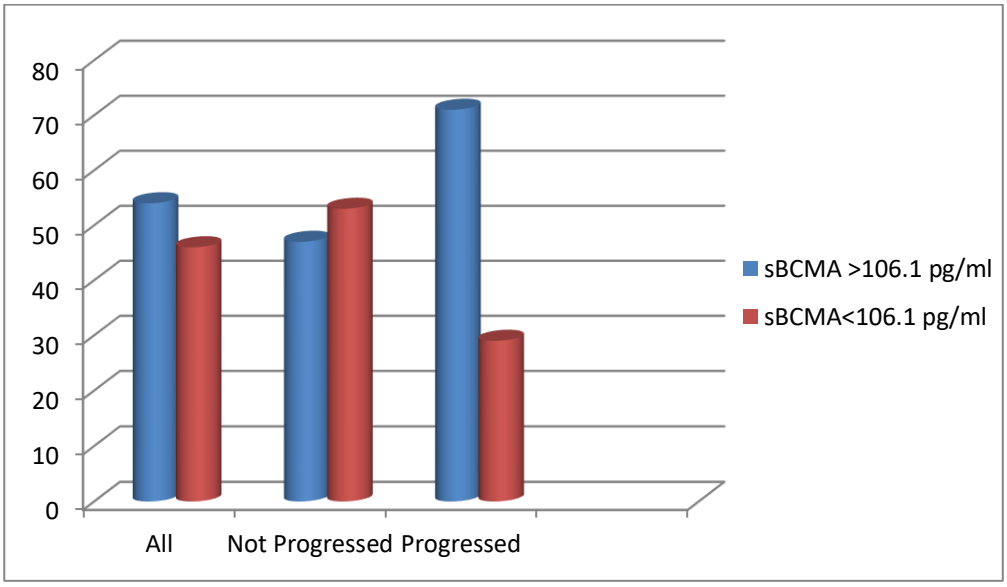


Figure 9. sBCMA levels by median level in entire, progressed and not progresse cohorts

The SKY92 gene expression profiling (GEP) assay was performed in 25 patients, identifying a high-risk signature in 24% (n = 6) and a standard-risk signature in 76% (n = 19). A high-risk SKY92 signature was detected in 43% of progressed patients compared with 17% of non-progressed patients (p = 0.2179).

Virtual FISH analysis by NGS was also conducted in 25 patients and revealed molecular abnormalities -t(4;14), t(14;16), +1q and del(17p)- in 68% (n = 17) of cases. Specifically, virtual FISH detected abnormalities in 57% (n = 4) of progressed and 72% (n = 13) of non-progressed patients (p = 0.5009).

Characteristic	All	Not progressed	Progressed
sBCMA (ng/ml)	N=24	N=18	N=7
median	106.1(range41.3-691.2)	105.9(41.3-393.7)	152.9(54.5-691.2)
>o = 106.1 ng/mL	13 (54%)	8(47%)	5(71%)
SKY 92	N=25	N=18	N=7
HR	6(24%)	3(17%)	3(43%)
SR	19(76%)	15(83%)	4(57%)
Virual fish	N=25	N=18	N=7
Positive	17 (68%)	13(72%)	4(57%)
Negative	8(32%)	5(38%)	3(43%)

Table 2 Patients characteristic by sBCMA and NGS evaluations (Sky 92 panel and virtual fish)

When considering both conventional and virtual FISH analyses, mutations were identified in 48% (n = 25) of patients, while 29% (n = 15) showed none of the evaluated alterations. Among progressed patients, alterations were observed in 57% (n=8) compared with 45% (n = 17) in non-progressed (p = 0.4459). Overall, combining both standard and virtual FISH analyses left only 23% (n = 12) of patients without any available cytogenetic evaluation(Tab.3 ; Fig 10 and 11)

Specifically, among patients in whom standard FISH could not be performed, virtual FISH revealed the presence of cytogenetic alterations in 9 patients and the absence of alterations in 3 patients. Moreover, in 4 patients with no abnormalities detected by standard FISH, virtual FISH identified a del(17p) alteration.

FISH plus VF	All(52)	Not progressed(38)	Progressed (14)
Positive	25 (48%)	17 (45%)	8 (57%)
Negative	15 (29%)	11 (29%)	4 (29%)
NV	12 (23%)	10 (26%)	2 (14%)

Table 3: Patients chatractecristic by standard and virtual fish

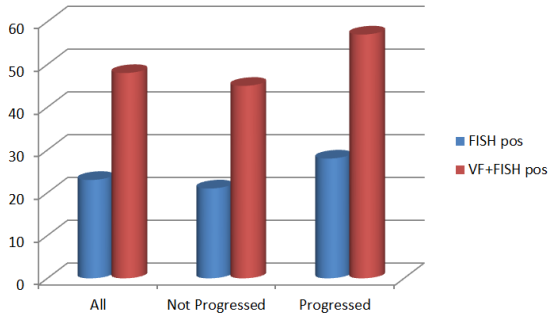


Figure 10. Standard Fish and Virtual fish positivity percentage in entire, not progressed and progressed cohort

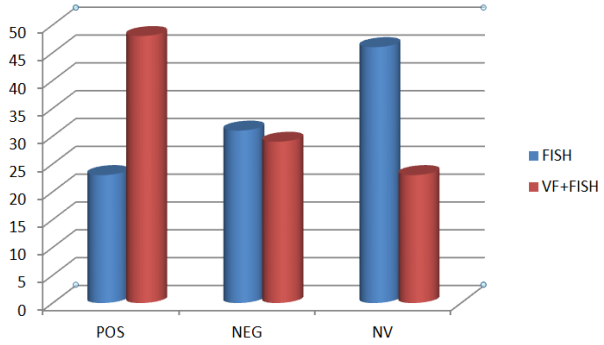


Figure 11 . Percentage of results performing standard fish alone or togheter with virtual fish (percentage) in entire cohort

Progression-free survival (PFS) was subsequently analyzed using the Kaplan–Meier method. PFS was defined as the time from bone marrow evaluation - corresponding to the diagnosis of smoldering myeloma - to progression to symptomatic disease or last follow-up.

Consistent with expectations, PFS differed significantly across IMWG risk groups (p = 0.0186)(Fig..12).

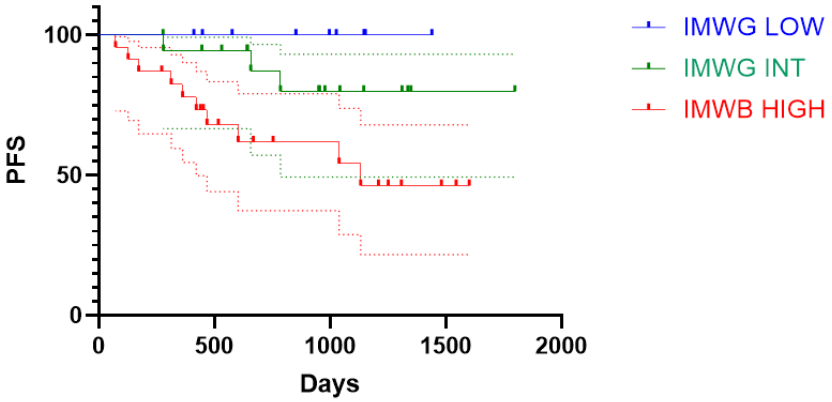


Figure 12 . Probability of progression considering IMWG risk score Low, Intermediate or High.

Progression-free survival (PFS) was also evaluated according to DWI–WB–MRI patterns , sBCMA levels, SKY92 GEP signature, and cytogenetic findings (standard and virtual FISH).

When PFS was analyzed considering DWI–WB–MRI findings, patients showing normal bone marrow signal had significantly longer PFS compared with those displaying altered signals (diffuse or micronodular patterns) (HR = 0.2753; 95% CI, 0.0844–0.8976 ;p = 0.0102). (Fig. 13)

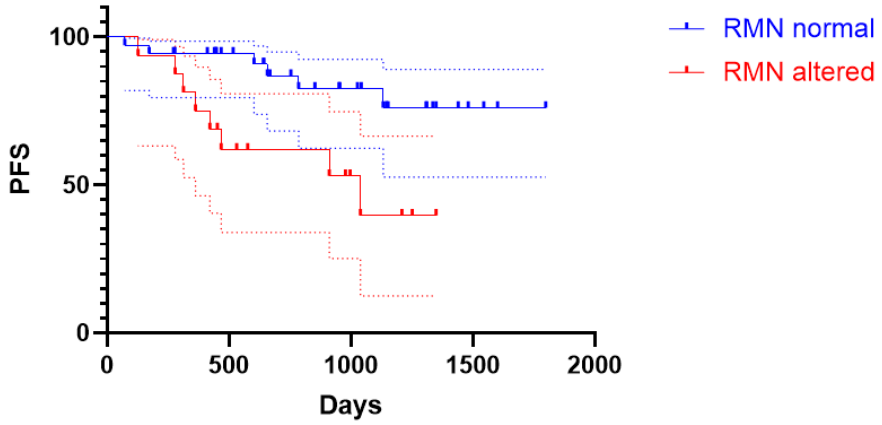


Figure 13 . Probability of progression considering DWI-WB-MRI pattern normal or altered (diffuse or micronodular) in all pazients evaluated

Evaluation of PFS according to sBCMA levels (using the median value of 106.1 ng/mL as the cut-off) showed a trend toward better PFS in patients with sBCMA levels below the median, although the difference was not statistically significant (HR = 0.4642; 95% CI, 0.1051–2.051; p = 0.3449)(Fig. 14).

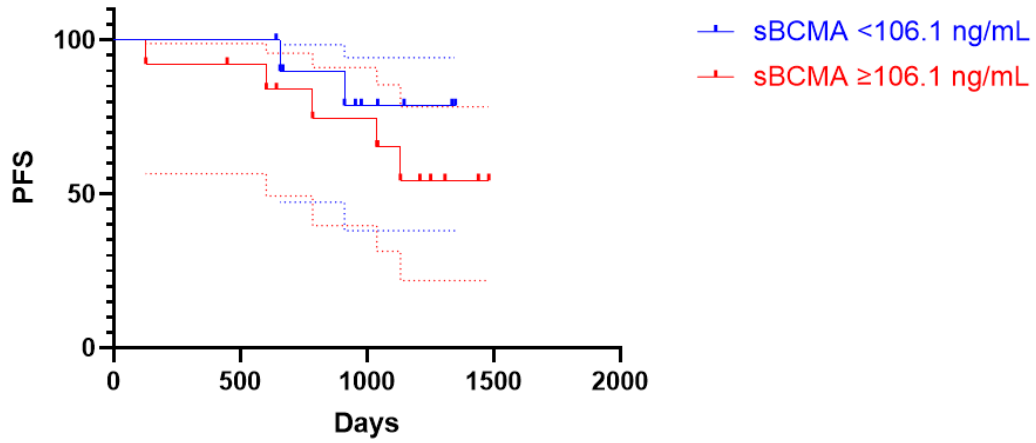


Figure 14 . Probability of progression considering sBCMA levels cut-off 106.1 ng/m in all patients evaluated

Similarly, PFS analysis based on SKY92 and standard FISH results revealed longer PFS in patients with a standard-risk SKY92 signature (HR = 0.5014; 95% CI, 0.0969–2.594 ;p = 0.3326) and in those with negative standard FISH results (HR = 0.9423; 95% CI, 0.2518–3.527; p = 0.9293), although neither comparison reached statistical significance.(Fig. 15 and Fig. 16).

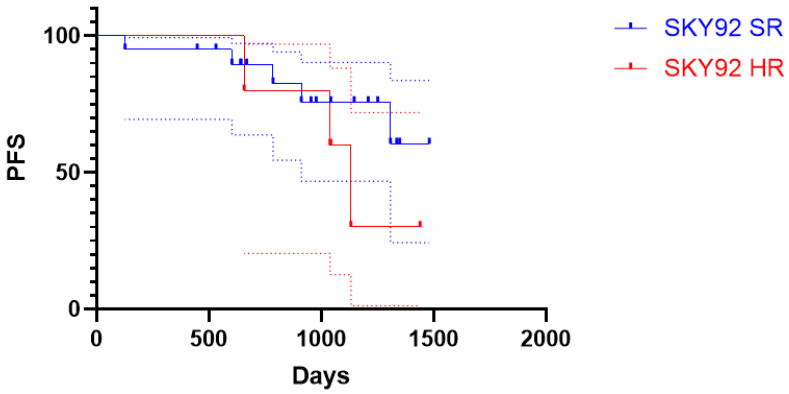


Figure15. Probability of progression considering GEP-SKY92 signature Standard Risk (SR) or High Risk (HR) in all patients evaluated

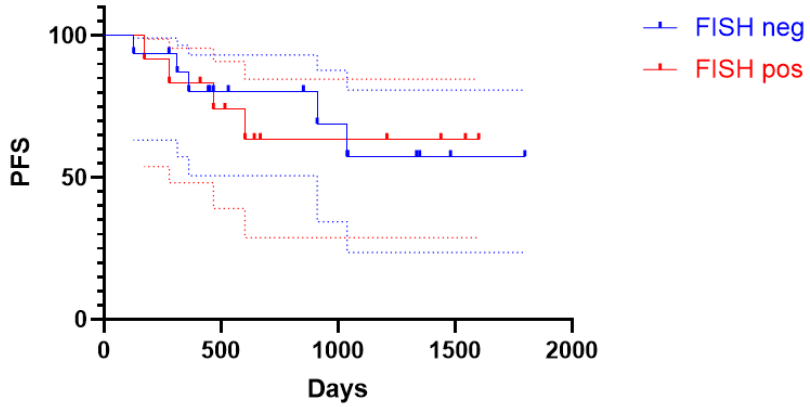


Figure 16. Probability of progression considering Standard Fish results positive or negative in all patients evaluated

Interestingly, when evaluated by virtual FISH, PFS was paradoxically longer in patients with detected cytogenetic alterations (HR = 2.019; 95% CI, 0.3812–10.69 ;p = 0.3461), though this finding was not statistically significant.(Fig. 17)

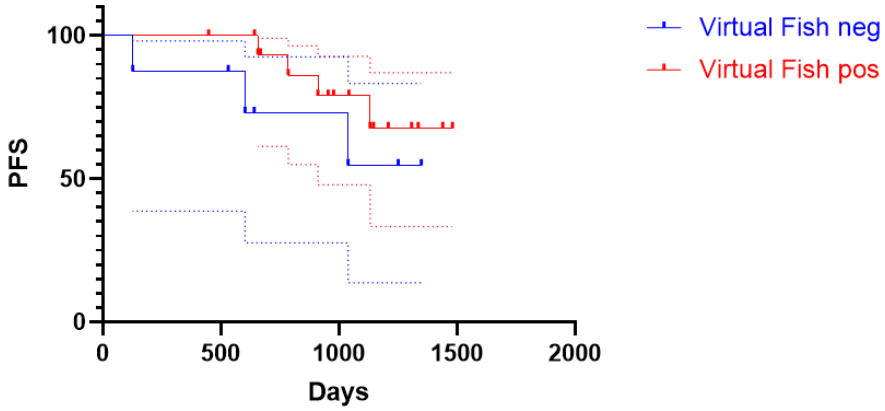


Figure 17. Probability of progression considering Virtual Fish results positive or negative in all patients evaluated

When considering both standard and/or virtual FISH together, patients without any detected alterations showed a trend toward longer PFS, but again without statistical significance (HR = 1.084; 95% CI, 0.3208–3.661 ;p = 0.8951). (Fig.18)

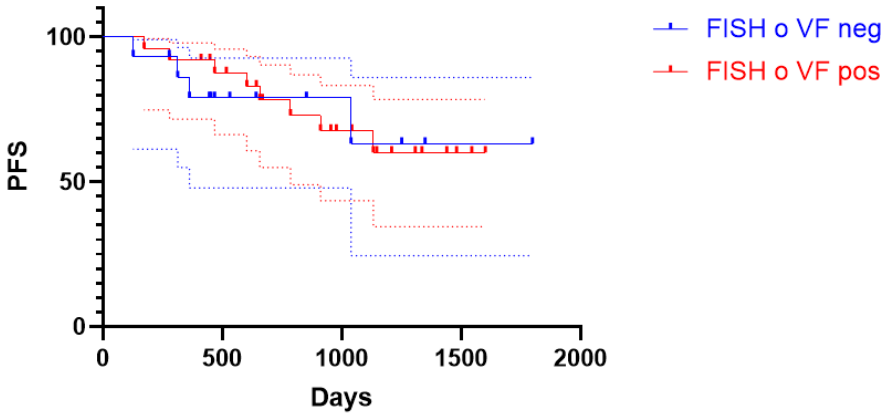


Figure 18: Probality of progression considering both standard FISH and/or Virtual fish results in all patients evaluated

Finally, given that 29 patients after enrollement initiated treatment within clinical trials to prevent progression to symptomatic disease, we performed a survival analysis separately in treated and untreated patients, according to DWI–WB–MRI patterns , sBCMA levels, the SKY92 GEP signature, and cytogenetic findings (standard and virtual FISH).

PFS was analyzed according to DWI–WB–MRI findings separately in treated and untreated patients. Patients with normal bone marrow signal exhibited longer PFS compared with those showing altered signals; however, this difference was statistically significant only in untreated patients (HR = 0.1535.084; 95% CI, 0.03288- 0.7169 ; p = 0.0014) (fig 19) , and not in those who received treatment (HR = 0.07318; 95% CI 0.1020 to 4.499;p = 0.6671)(fig 20).

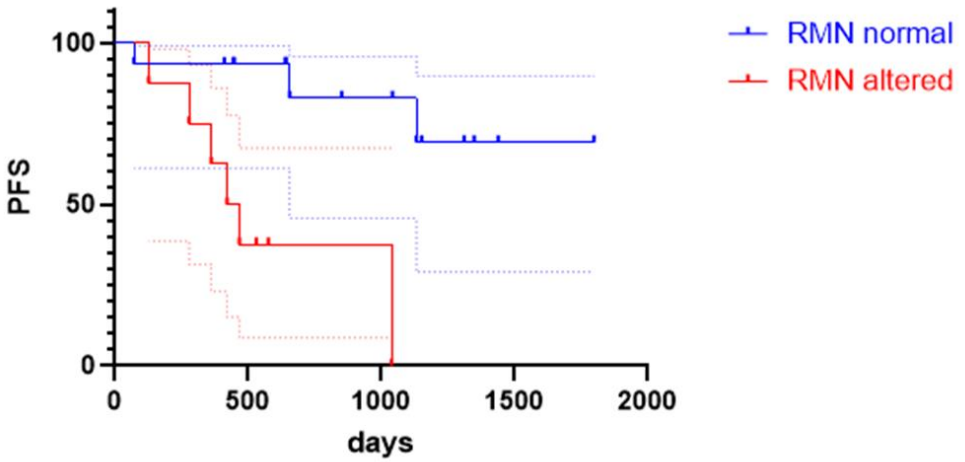


Figure 19: Probality of progression considering DWI-WB-MRI pattern normal or altered (diffuse or micronodular) in untreated patients

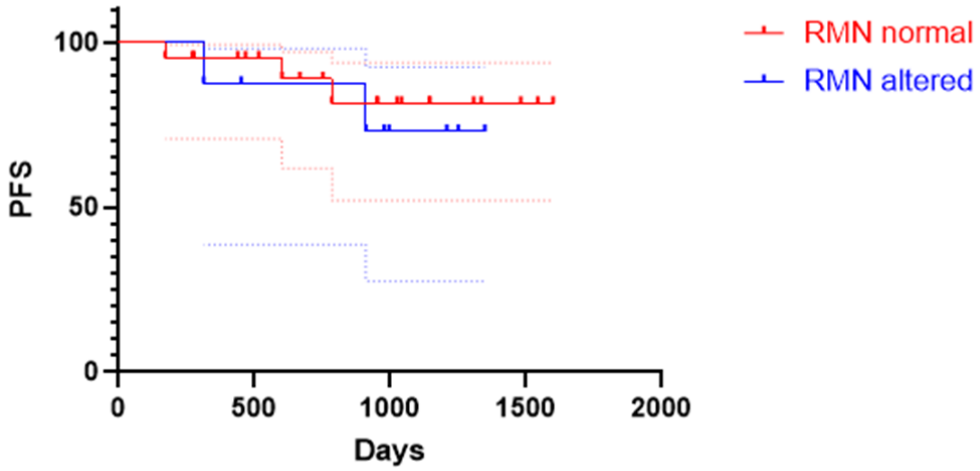


Figure 20: Probality of progression considering DWI-WB-MRI pattern normal or altered (diffuse or micronodular) in treated patients

Evaluation of PFS according to sBCMA levels (using the median value of 106.1 ng/mL as the cut-off) showed a trend toward better PFS in those with sBCMA levels below the median, both in untreated (HR = 0.4830 ; 95%CI, 0.06577-3,547 ;p = 0.5163) (fig 21) and treated groups (HR = 0.4706; 95% CI, 0.04890- 4.529 ;p = 0.5282) (fig 22), although the difference was not statistically significant.

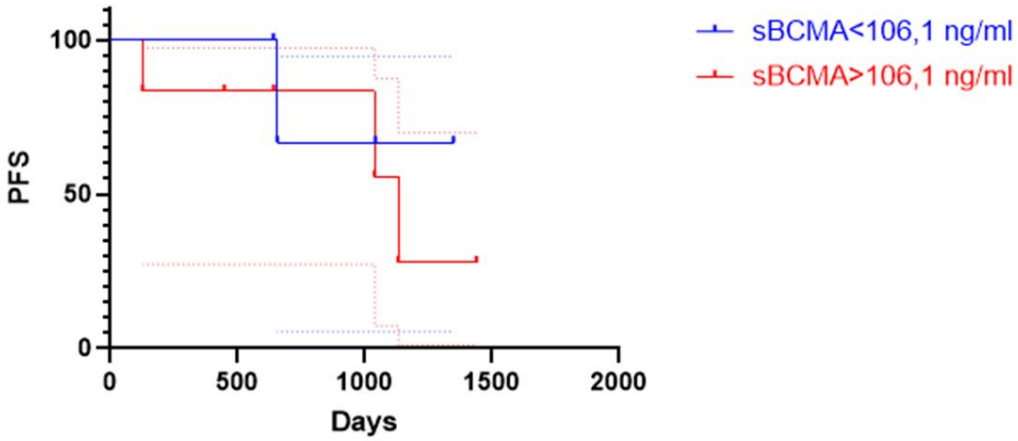


Figure 21 : Probality of progression considering sBCMA levels cut-off 106.1 ng/ml in untreated patients

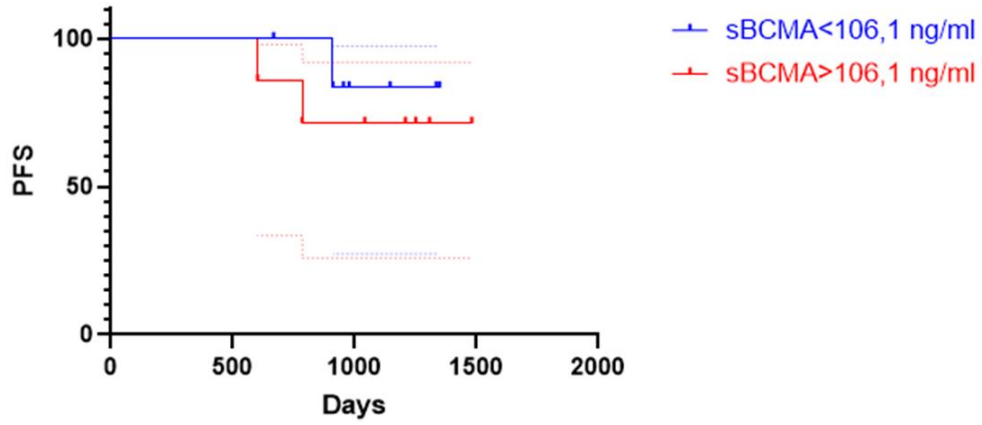


Figure 22 : Probality of progression considering sBCMA levels cut-off 106.1 ng/ml in treated patients

Similarly, PFS analysis considering both standard FISH and/or virtual FISH results revealed a trend toward longer PFS in patients without cytogenetic alterations, both in untreated (HR = 0.9590; 95% CI, 0.1935-4.753; p = 0.9591) (fig 23) and treated groups untreated (HR = 0.9126; 95% CI, 0.1136- 7.333; p = 0.9328) (fig 24), but the difference was not statistically significant.

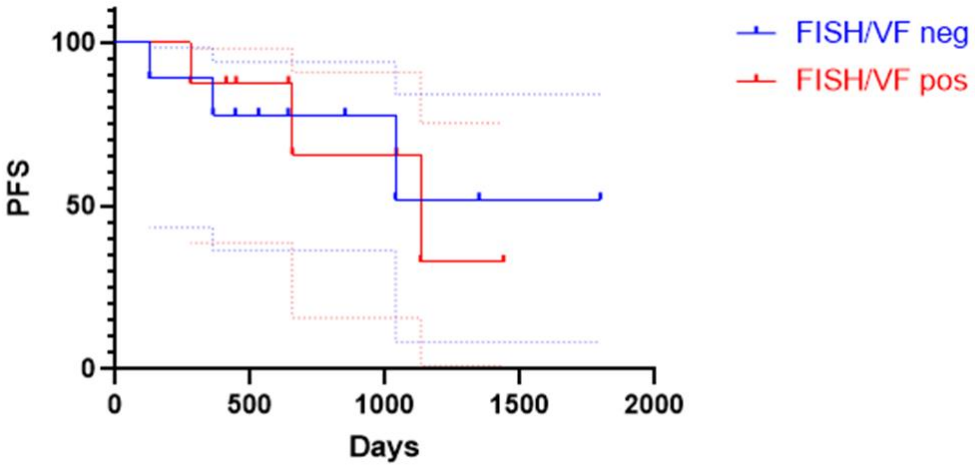


Figure 23 : Probality of progression considering considering both standard FISH and/or Virtual fish results in untreated patients

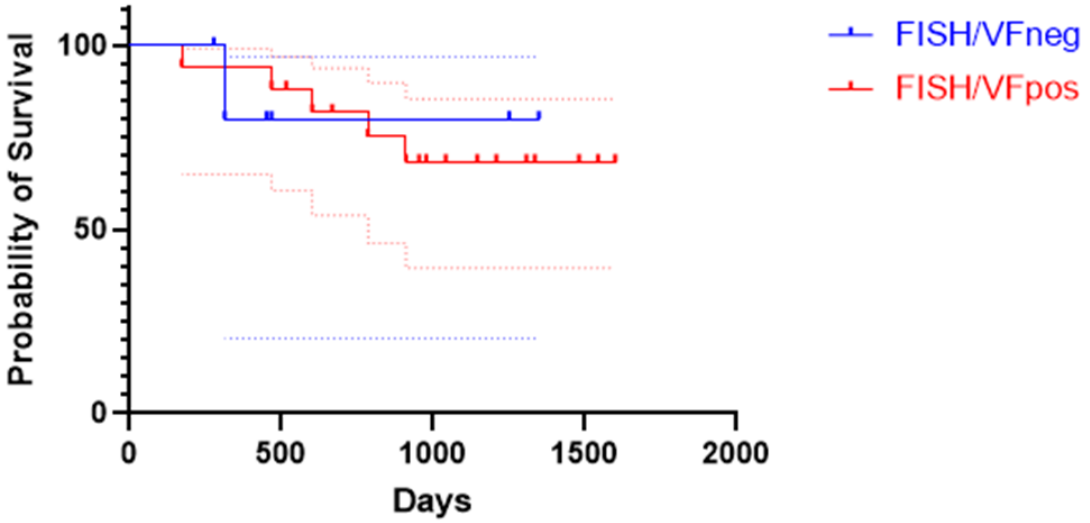


Figure 24 : Probality of progression considering considering both standard FISH and/or Virtual fish results in untreated patients

Finally, a separate survival analysis was performed according to the SKY92 signature; however, this evaluation could only be conducted in untreated patients, as none of the treated patients were classified as high-risk. Nonetheless, analysis of survival data according to SKY9 2 signature in the untreated cohort revealed a trend toward better PFS

in patients with a standard risk signature respect to hish risk , although this difference was not statistically significant (HR = 0.7372; 95% CI, 0.08881-6.120;p = 0.7796) (fig 25)

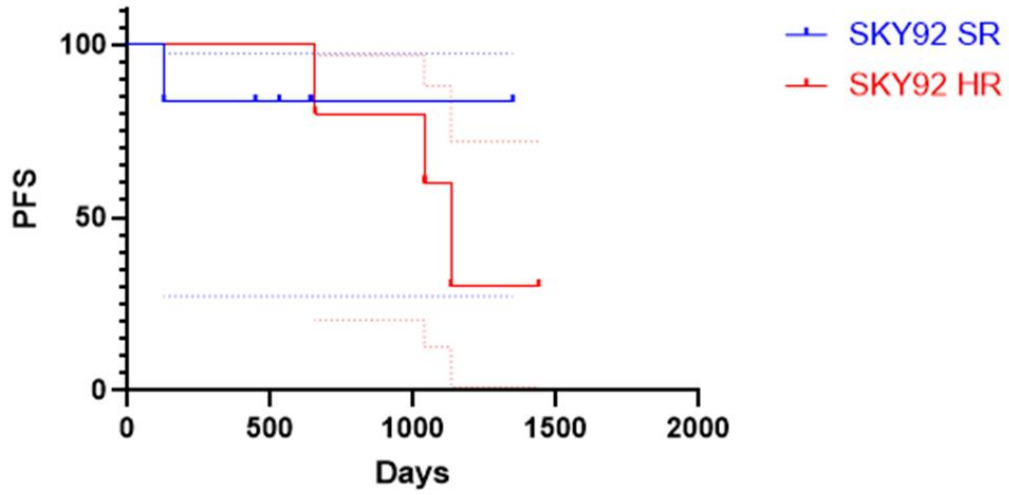


Figure 25: Probability of progression considering GEP-SKY92 signature SR or HR in untreated patients

DISCUSSION

Smoldering multiple myeloma (SMM) is a plasma cell dyscrasia characterized by an increased clonal plasma cell burden compared with monoclonal gammopathy of undetermined significance (MGUS), but without evidence of end-organ damage or active myeloma-defining biomarkers, according to the updated International Myeloma Working Group (IMWG) criteria[5]. The estimated incidence of SMM is approximately 0.9 cases per 100,000 persons per year amd the median age at diagnosis is around 67 years, with a slight male predominance[6].

SMM represents an asymptomatic form of myeloma, yet it is not merely a biological intermediate stage between MGUS and multiple myeloma (MM). It is a heterogeneous, clinically defined condition, in which approximately two-thirds of patients demonstrate a clinical course similar to MGUS (premalignant behavior), whereas about one-third exhibit biological features more akin to MM (malignant behavior)[11]. The risk of progression to symptomatic MM is estimated at approximately 10% per year during the first five years, 3% per year over the following five years, and 1% per year thereafter. However, a subset of patients shows significantly higher progression risks, defining the category of high-risk SMM[7], [8].

Several risk stratification models have been proposed for SMM. The IMWG "2/20/20" model is currently the most widely adopted and is based on bone marrow plasma cell percentage, serum M-protein concentration, and free light chain (FLC) ratio. High-risk smoldering multiple myeloma, as defined by the IMWG risk stratification model, is associated with a substantial risk of progression, with approximately 45–60% of patients developing symptomatic multiple myeloma within two years of diagnosis.[10].

In Italy, the current standard of care for SMM remains observation, with treatment reserved for the onset of symptomatic disease. Nevertheless, recent randomized clinical trials have suggested that early intervention in patients with high-risk SMM may improve

progression-free survival, fueling an ongoing debate regarding early therapeutic strategies in selected subgroups[12], [13].

Moreover, emerging scientific evidence has identified additional risk factors potentially associated with SMM progression that are not included in the IMWG risk score. Among these are findings from whole-body MRI (WB-MRI), gene expression analyses, and serum B-cell maturation antigen (s BCMA) levels.

In this study Whole-body DWI–MRI revealed abnormal marrow infiltration patterns (diffuse or micronodular) in 31% of patients. Among those who progressed, abnormal MRI findings were significantly more frequent compared with non-progressed patients (86% vs. 21%; p = 0.0119). Moreover, patients with normal DWI–WB–MRI signals had a significantly longer PFS than those with diffuse or micronodular patterns (HR = 0.2753; 95% CI, 0.0844–0.8976; p = 0.0102).

These findings highlight the strong prognostic value of DWI–WB–MRI in SMM[5], [31], [37] likely related to the presence of abnormal marrow infiltration patterns, which are clearly associated with a higher risk of progression to symptomatic myeloma. Conversely, the high prevalence of normal MRI findings among non-progressed patients (79%) further supports DWI–WB–MRI as a sensitive and non-invasive tool for early risk stratification. Its ability to detect subtle diffuse or micronodular marrow changes may help guide closer monitoring or earlier therapeutic interventions in patients identified as high-risk.

In the present study, among the 24 patients evaluated for serum BCMA (sBCMA), the median sBCMA level was 106.1 ng/mL. Elevated sBCMA levels (above the median) were more frequent among patients who progressed compared with those who did not (71% vs. 47%, respectively), and the median sBCMA concentration was higher in progressed patients (152.9 ng/mL vs. 105.9 ng/mL). Trends toward longer PFS were also observed in patients with sBCMA levels below the median, although not statistically significant.

These findings support the concept that sBCMA levels, which reflect tumor burden, may serve as a dynamic biomarker of myeloma activity[41]. Elevated sBCMA levels have previously been associated with inferior progression-free and overall survival in active multiple myeloma, and may also predict progression from smoldering to symptomatic disease as reported in literature[42], [43], [44]. In the present study , however, the relatively small cohort size likely limited the statistical power to detect significant differences. Overall, sBCMA remains a promising, minimally invasive biomarker for monitoring disease kinetics, and its role warrants validation in larger prospective studies.

The SKY92 gene expression profiling (GEP) assay was performed in 25 patients, identifying a high-risk signature more frequently among those who progressed compared with non-progressed patients (43% vs. 17%, respectively). Although not statistically significant, trends toward longer PFS were also observed in patients with low-risk SKY92 profiles.

These results are consistent with previous findings in symptomatic multiple myeloma, where the SKY92 signature effectively stratifies patients according to prognosis, identifying those with significantly shorter progression-free survival (PFS)[23], [24]. The observed trend in this study further supports the integration of gene expression profiling into risk stratification frameworks, even within SMM populations, to improve early identification of high-risk disease.

Virtual fish , performed via next-generation sequencing (NGS) to detect cytogenetic abnormalities such as *t*(4;14), *t*(14;16), +1q, and del(17p), can provide valuable complementary information in cases where conventional FISH cannot be performed.

In our cohort, trends toward longer progression-free survival (PFS) were observed in patients who were negative by standard FISH, but not in those who were negative by virtual FISH. Interestingly, PFS appeared paradoxically longer among patients with alterations detected by virtual FISH, likely reflecting small-sample variability. When considering both standard and virtual FISH together, patients with no detectable

cytogenetic abnormalities demonstrated slightly better PFS, although the difference was not statistically significant.

When considering both standard and virtual FISH together, patients with no detectable cytogenetic abnormalities demonstrated slightly better PFS, although the difference was not statistically significant.

In our study, virtual FISH successfully provided additional data in cases where standard FISH was not feasible, detecting cytogenetic alterations in 9 patients and the absence of abnormalities in 3. This approach reduced the proportion of patients without evaluable cytogenetic data and increased the percentage of patients identified with relevant abnormalities. Notably, in 4 patients with normal standard FISH results, virtual FISH identified a del(17p) alteration, highlighting its enhanced sensitivity and potential value as a complementary tool for comprehensive genomic evaluation.

Overall, these findings emphasize the potential of integrating virtual FISH with conventional cytogenetics to improve the completeness and accuracy of molecular characterization in smoldering multiple myeloma.

The principal limitation of this study is related to the fact that 29 patients, after enrollement, were treated within clinical trials aimed at preventing progression to symptomatic myeloma. This may have affected the prognostic value of the data collected at diagnosis. For this reason, a separate analysis of treated and untreated patients was also conducted to explore the prognostic value— in terms of progression to symptomatic disease—of DWI–WB–MRI findings, sBCMA levels, standard FISH/virtual FISH and Sky92 signature.

Due to the limited sample size, analyses involving sBCMA levels and standard/virtual FISH results did not reach statistical significance. Nevertheless, a trend toward improved PFS was observed in patients with low sBCMA levels and negative standard/virtual FISH findings in both treated and untreated subgroups.

The analysis of the probability of progression to symptomatic disease according to the SKY92 signature was not feasible in treated patients owing to the absence of high-risk profiles in this subgroup. In contrast, among untreated patients, the SKY92 standard –risk signature was associated with a trend toward longer progression-free survival, although this association did not reach statistical significance.

Notably, among all analyses, only the evaluation based on DWI–WB–MRI findings showed a statistically significant improvement in progression-free survival in untreated patients, while in treated patients it revealed a non-significant trend. In particular, the differences in MRI-based outcomes between treated and untreated patients may highlight the potential impact of early intervention in SMM. Effective treatment of the medullary neoplastic infiltrate, which is likely reflected in the MRI pattern, could negate its prognostic value in treated group.

In conclusion, results of this study, suggest that imaging, serum, and molecular markers provide complementary prognostic information. DWI–WB–MRI emerged as the strongest predictor of early progression, while sBCMA, SKY92, and FISH-based parameters demonstrated consistent but non-significant trends. The main limitations of this study are the small sample size, the presence of treated and unttreated patients and limited follow-up duration, which may have influenced statistical power. Moreover Standard FISH data, serum BCMA levels and gene expression profiling analyses are available only in about half of patients. These missing data therefore did not allow for a robust multivariate analysis to be performed.

Despite these constraints, observed trends align with existing literature and highlight the value of integrating imaging, serumBCMA, and molecular markers for more precise risk stratification.

In particular our findings support the combined use of sBCMA, SKY92, cytogenetic assessment (including virtual FISH), and DWI–WB–MRI for risk stratification in smoldering myeloma. DWI–WB–MRI emerged as the most robust predictor of

progression, while serum and molecular markers provided complementary trends consistent with known biology. Virtual FISH enhanced evaluability and identified additional high-risk lesions, such as del(17p). These results underscore the potential of a multimodal approach for early identification of high-risk patients and warrant validation in larger, prospective studies.

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