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MEDICINA E ONCOLOGIA MOLECULAR

**SEARCHING FOR BIOMARKERS OF MEASURABLE RESIDUAL DISEASE
AND OF DRUG RESISTANCE IN LIQUID BIOPSIES FROM MULTIPLE
MYELOMA PATIENTS**

Rui Filipe Cordeiro Bergantim

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SEARCHING FOR BIOMARKERS OF MEASURABLE RESIDUAL DISEASE AND OF DRUG RESISTANCE IN LIQUID BIOPSIES FROM MULTIPLE MYELOMA PATIENTS

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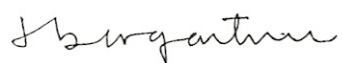
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STATEMENT OF INTEGRITY

Under the terms of the “Decreto-lei nº 216/92, de 13 de Outubro”, is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published manuscripts included in this dissertation.

The Ethical Committee for Health at Unidade Local de Saúde São João, Porto - Portugal, approved all experimental research in this thesis dissertation.

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ABSTRACT

Multiple myeloma (MM) is a highly heterogeneous hematological neoplasia that experienced several innovative treatments over the last years. Current strategies involve the sequential and integrated use of various drug classes, improving patient outcomes, including better responses, prolonged progression-free periods, enhanced survival, and better quality of life. The treatment response spectrum is broad, reflecting diverse survival outcomes, from primary refractory to negative measurable residual disease.

Effective biomarkers that predict drug response and guide treatment decisions are essential to improve therapeutic efficacy and the result of MM treatment. Current biomarkers are insufficient, and the most sensitive tools rely on bone marrow evaluation, which requires a painful and invasive procedure, limiting sequential MM evaluation over the treatment course and failing to address the spatial heterogeneity of MM. Liquid biopsies using biomarkers in the peripheral blood, such as Extracellular Vesicles (EVs) cargo and free microRNAs (miRNAs), offer a promising, less invasive approach for monitoring disease and guiding treatment decisions.

Therefore, this thesis aimed to identify biomarkers of measurable residual disease and drug response/resistance using liquid biopsies from patients with Multiple Myeloma and contribute to guiding personalized therapeutic decisions.

Using samples from ten MM patients treated with the same chemotherapy protocol, it was possible to isolate EVs from peripheral blood (PB) and bone marrow (BM), both at diagnosis and complete remission, carrying MM markers such as CD38 and CD138. Diagnostic EV samples had different levels of MM markers between PB and BM paired samples, but no differences between PB and BM were found at complete remission. Thus, this proof of concept study suggested that PB EVs from MM remission could be used as a source of MM biomarkers and as a non-invasive approach for monitoring response to treatment, including measurable residual disease. EVs concentration was lower in the PB of healthy controls than patients, and MM markers were mostly not detected in EVs from the healthy controls.

In another approach, also using samples from MM patients treated with the same chemotherapy protocol, Next-generation sequencing (NGS) profiling revealed several miRNAs expressed differently between treatment primary refractory and sensitive patients and between PB and BM. Preliminary results (with twenty patients) showed that among the differentially expressed miRNAs, miR-665, miR-483-5p, miR-143-3p, and miR-145-5p were selected for further validation using qPCR, and it was observed that miR-665 retained statistical significance being elevated in treatment-primary refractory patients compared to those sensitive to treatment. This miRNA was also more abundant in PB than in BM. High miR-665 levels were associated with aggressive disease features, such as higher lactate dehydrogenase, higher b2-microglobulin, higher creatinine, and more frequent extramedullary disease. Likewise, it was associated with poorer survival outcomes, including reduced overall survival.

Finally, it was demonstrated that it was possible to successfully isolate and quantify miRNAs from the cargo of EVs isolated from the PB and BM of MM patients at the diagnosis and remission stages. Notably, at diagnosis, miRNA levels were higher in the BM than in the PB, but this difference was not observed at remission.

In conclusion, this thesis underscores preliminary results showing the promising potential of liquid biopsies in MM, using EVs cargo and miRNAs as analytes. Their application as a minimally invasive approach could lead to improved and more frequent longitudinal monitoring of patients, detection of early signs of relapse/progression, and identification of more profound and persistent responses to treatment. Additionally, they could be essential to the early detection of drug resistance and more personalized treatment strategies.

KEYWORDS

Multiple Myeloma; Liquid Biopsy; Biomarkers; Extracellular Vesicles; MicroRNAs; Drug Resistance; Measurable Residual Disease.

RESUMO

O Mieloma Múltiplo (MM) é uma neoplasia hematológica heterogénea cujo tratamento evoluiu de forma substancial ao longo dos últimos anos. A abordagem actual envolve a utilização sequencial e integrada de várias classes de fármacos, com melhoria das respostas ao tratamento, maior sobrevivência livre de progressão e sobrevivência global, assim como de maior qualidade de vida dos doentes. O espectro de respostas possíveis ao tratamento é amplo, reflectindo o impacto dos diferentes tratamentos na sobrevivência destes doentes, indo desde doença primariamente refractária a doença residual mensurável negativa.

A existência de biomarcadores eficazes que antecipem a resposta ao tratamento e orientem as decisões do mesmo é essencial para melhorar a eficácia terapêutica no MM. Os biomarcadores actuais são insuficientes e as abordagens mais sensíveis baseiam-se na avaliação da medula óssea, o que exige um procedimento doloroso e invasivo, limitando a avaliação seriada do MM ao longo do tratamento e não abordando a heterogeneidade espacial do MM. As biópsias líquidas que utilizam biomarcadores no sangue periférico, como o conteúdo das vesículas extracelulares (EVs) e os microRNAs (miRNAs) circulantes, oferecem uma abordagem promissora e menos invasiva para monitorizar a doença e orientar as decisões de tratamento.

Assim, esta tese teve como objetivo identificar biomarcadores de doença residual mensurável e de resposta/resistência a fármacos, utilizando biópsias líquidas de doentes com MM, e contribuir para orientar decisões terapêuticas, promovendo uma medicina de precisão.

Utilizando amostras de dez doentes com MM tratados com o mesmo protocolo de quimioterapia, foi possível isolar EVs no sangue periférico (SP) e medula óssea (MO), tanto ao diagnóstico como em remissão completa, contendo marcadores de MM, entre os quais CD38 e CD138. As amostras de diagnóstico apresentaram níveis diferentes dos marcadores de MM nas várias amostras emparelhadas de SP e MO, não tendo sido encontradas diferenças entre SP e MO nas amostras de remissão completa, sugerindo que estes marcadores poderão ser utilizados como biomarcadores para monitorizar de forma não invasiva a resposta ao tratamento, incluindo doença residual mensurável. A quantidade de EVs foi menor no SP dos controlos saudáveis comparativamente aos doentes, e os marcadores de MM não foram, na sua maioria, detectados nas EVs dos controlos saudáveis.

Noutra abordagem, utilizando amostras de doentes com MM tratados com o mesmo protocolo de quimioterapia, a análise por NGS revelou vários miRNAs expressos de forma diferente entre doentes sensíveis e resistentes ao tratamento, e entre SP e MO. Resultados preliminares de vinte doentes, mostraram que dos miRNAs diferencialmente expressos, os miR-665, miR-483-5p, miR-143-3p e miR-145-5p foram posteriormente validados por qPCR, tendo-se observado que o miR-665 manteve significado estatístico, estando elevado nos doentes resistentes em comparação com os sensíveis ao tratamento. Verificou-se também que este miRNA foi mais abundante no SP do que na MO, sendo que níveis elevados de miR-665 estavam associados a características

agressivas da doença, tais como LDH elevada, b2-microglobulina elevada, creatinina elevada e doença extramedular mais frequente. Do mesmo modo, este miRNA foi associado sobrevivência global mais curta.

Finalmente, foi demonstrado que é possível isolar e quantificar os miRNAs contidos na carga das EVs isolados do SP e MO dos doentes com MM ao diagnóstico e no momento de remissão completa. De notar que os níveis de miRNA foram mais elevados na MO do que no SP aquando do diagnóstico, sendo que esta diferença não foi observada no momento da avaliação da remissão.

Em conclusão, esta tese destaca resultados preliminares evidenciando o potencial das biópsias líquidas no MM, principalmente utilizando o conteúdo das EVs e os miRNAs circulantes. A sua aplicação como abordagem não invasiva poderá levar a uma melhor monitorização seriada, detectando sinais precoces de recaída/progressão e identificando respostas mais profundas e persistentes ao tratamento. Além disso, poderá ser fundamental para a deteção precoce da resistência a fármacos e para estratégias de tratamento personalizadas.

PALAVRAS-CHAVE

Mieloma Múltiplo; Biópsia Líquida; Biomarcadores; Vesículas Extracelulares; MicroRNAs; Resistência a fármacos; Doença Residual Mensurável

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ABBREVIATIONS

A-B-C

ABC: ATP-binding cassette
ABCB1: ATP-binding cassette sub-family B member 1
ABCC1: ATP-binding cassette sub-family C member 1
ABCG2: ATP-binding cassette sub-family G member 2
ADC: Antibody-drug conjugates
ADCC: Antibody-dependent cellular cytotoxicity
ADCP: Antibody-dependent cellular phagocytosis
AID: Activation-induced cytidine deaminase
AKT: Protein kinase B
ALDH: Aldehyde dehydrogenase
ANXA2: Annexin A2
APOBEC: Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
APE1: Apurinic/Apyrimidinic Endonuclease 1
APRIL: A Proliferation-Inducing ligand
ASM: Acid sphingomyelinase
ASO-qPCR: allele-specific oligonucleotide qPCR
ASCT: Autologous stem cell transplantation
ATP: Adenosine triphosphate
ATF4: Activating transcription factor 4
ATF6: Activating transcription factor 6
ATM: Ataxia Telangiectasia Mutated
ATR: Ataxia Telangiectasia and Rad3-related
AREG: Amphiregulin
ARF: Alternate Reading Frame
AURKA: Aurora kinase A
BAFF: B-cell activating factor
Bad: Bcl-2-associated death promoter
BAX: Bcl-2-associated X protein
BCL2: B-cell lymphoma 2
BCL9: B-cell CLL/lymphoma 9
BCMA: B-cell maturation antigen
BelaMaf: Belantamab Mafodotin
BsAbs: Bispecific antibodies
BiTE: Bispecific T-cell engager
BIRC2: Baculoviral IAP repeat-containing protein 2
BIRC3: Baculoviral IAP repeat-containing protein 3
BLIMP1: B lymphocyte-induced maturation protein 1
BM: Bone marrow
BMF: Bcl-2 Modifying Factor
BMSCs: Bone marrow stromal cells
Bmi1: Moloney murine leukemia virus integration site 1
BLIMP-1: B Lymphocyte-Induced Maturation Protein-1
Bregs: B regulatory cells
CAFs: Cancer-associated fibroblasts
CAM-DR: Cell adhesion mediated drug resistance
CAR-T: Chimeric antigen receptor T-cell therapy
CCND1-3: Cyclin D genes
CDH1: Cadherin 1
CDK: Cyclin-dependent kinase
CDKN: Cyclin-dependent kinase inhibitor
CELMoDs: Cereblon E3 Ligase MoDulators
circMyc: Circular RNAs
CfDNA: Cell-free DNA
CHK1/2: Checkpoint Kinase 1/2
CNA: Copy number abnormalities
CR: Complete response

CRBN: Cereblon
CSCs: Cancer stem cells
CTLA-4: Cytotoxic T-lymphocyte associated antigen 4
CTSK: Cathepsin K
CTCs: Circulating tumor cells
CTX: C-terminal cross-linking telopeptide of type-1 collagen
CXCL12: C-X-C motif chemokine ligand 12
CXCR4: C-X-C chemokine receptor type 4

D-E-F

DAPK1: Death-associated protein kinase 1
DCs: Dendritic cells
ddPCR: Droplet digital Polymerase Chain Reaction
DIME1: Dimethyladenosine Transferase 1
DLK1-DIO3: Delta-Like 1 Homolog - Type III Iodothyronine Deiodinase
DNA: Deoxyribonucleic acid
DNase: Deoxyribonuclease
DNMT3A/B: DNA methyltransferase
DSBs: Double-strand DNA breaks
ECM: Extracellular matrix
ECs: Endothelial cells
ERK: Extracellular signal-regulated kinase
eIF4E: Eukaryotic translation initiation factor 4E
EGFR: Epidermal growth factor receptor
EMD: Extramedullary disease
ER: Endoplasmic reticulum
ERG: erythroblast transformation-specific related gene
EVs: Extracellular Vesicles
FAP: Fibroblast activation protein
FGF: Fibroblast growth factor
FGFR3: Fibroblast growth factor receptor 3
FISH: Fluorescence in situ hybridization
FOXP1: Forkhead box protein P1
FSP1: Fibroblast-specific protein 1

G-H-I

GEP: Global gene expression profiling
GPCR5D: G-protein-coupled receptor family C group 5 member D
GR: Glucocorticoid receptor
GSK3: Glycogen synthase kinase 3
HDAC: histone deacetylase
HIF-1: Hypoxia-inducible factor 1
HLA-DR1: Human leukocyte antigen – DR isotype
IAP: Inhibitor of apoptosis protein
ICAM1: Intercellular adhesion molecule 1
ICD: Immunogenetic cell death
IFN γ : interferon γ
IGF-1: Insulin-like growth factor 1
IGH: Immunoglobulin-heavy chain
HSP: Heat shock protein
IKK α/β : Inhibitory Kappa B Kinase α/β
IL: Interleukin
IKZF1: Ikaros
IKZF3: Aiolos
IMiDs: Immunomodulatory drugs
IMWG: International Myeloma Working Group
IRE1 α : Inositol-requiring enzyme 1 α

IRF4: Interferon regulatory factor 4
ISS: International Staging System
ITGA9: Integrin subunit alpha 9

J-K-L

JAK-STAT: Janus kinase-signal transducer and activator of transcription pathway
KPNA2: Karyopherin subunit alpha 2
LAT1: L-type Amino Acid Transporter 1
LAG3: Lymphocyte-activation gene 3
LDH: Lactate dehydrogenase
LDHA: Lactate dehydrogenase A
LNA: Locked nucleic acid
lncRNAs: long-non-coding RNAs

M-N-O

MALDI-TOF-MS: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MAF: Musculoaponeurotic fibrosarcoma
MAPK: Mitogen-activated protein kinase
MCL1: Myeloid cell leukemia 1
MDM2: Murine double minute 2
MDR: Multidrug resistance
MGUS: Monoclonal gammopathy of undetermined significance
MHC: Major histocompatibility complex
miRNA: MicroRNA
miRISC: miRNA-induced silencing complex
MMP: Matrix metalloproteinase
MM: Multiple myeloma
MMSET: Multiple Myeloma SET Domain Protein
MoAbs: Monoclonal antibodies
MRD: Measurable residual disease
MRI: Magnetic resonance imaging
MRP1: Multidrug resistance-associated protein 1
MSC: Mesenchymal stem cells
MS: Mass spectrometry
MUC1: Mucin 1
MYC: V-Myc avian myelocytomatosis viral oncogene homolog
NAD: Nicotinamide adenine dinucleotide
NEDD4: Neural Expressed, Developmentally Down-regulated 4
NFATc1: Nuclear factor of activated T cell c1
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS: Next-generation sequencing
NK: Natural killer
NO: Nitric oxide
NSD2: Nuclear receptor binding SET domain protein 2
NTA: Nanoparticle Tracking Analysis
NUPR1: Nuclear protein 1
OPG: Osteoprotegerin

P-Q-R

PCL: Plasma cell leukemia
PD-1: Programmed death-1
pDCs: Plasmacytoid dendritic cells
PDGF-BB: Platelet-derived growth factor-BB
PD-L1: Programmed death-ligand 1
PET-CT: Positron emission tomography-computed tomography

PFS: Progression-free survival
PIAS3: Protein Inhibitor of Activated STAT3
PI3K: Phosphoinositide 3-kinase
PIM-1: Proto-oncogene serine/threonine-protein kinase Pim-1
PIs: Proteasome inhibitors
PSMA3-AS1: Proteasome subunit alpha 3 antisense RNA 1
PTEN: Phosphatase and tensin homolog
PUMA: p53 up-regulated modulator of apoptosis
qPCR: quantitative Polymerase Chain Reaction
RANKL: Receptor activator of nuclear factor kappa-B ligand
RB1: Retinoblastoma protein
Rho-B: Ras Homolog Family Member BAKy
R-ISS: Revised International Staging System
RNA: Ribonucleic acid
RNase: Ribonuclease
RMRP: Mitochondrial RNA processing endoribonuclease
ROS: Reactive oxygen species
RUNX2: Runt-related transcription factor 2

S-T-U

SAA: Serum amyloid A
SDF-1: Stromal cell-derived factor 1
sFLC: Serum-free light chain
SHM: Somatic hypermutation
SINE: Selective inhibitor of nuclear export
SKP2: S-phase kinase-associated protein 2
SLAMF7: Signaling lymphocytic activation molecule 7
SLC7A5/LAT: Solute Carrier Family 7 Member 5
SMA: Smooth muscle actin
SMM: Smoldering Multiple Myeloma
SOCS1: Suppressor of cytokine signaling 1
Sp1: Specificity Protein 1
SPRY2: Sprouty homolog 2 SHIP-1
STAT3: Signal transducer and activator of transcription 3
STING: Stimulator of interferon genes
TAMs: Tumor-associated macrophages
TEM: Transition Electron Microscopy
TCE: T-cell engagers
TGF-β: Transforming growth factor beta
TH: T helper
TIGIT: T cell immunoreceptor with Ig and ITIM domains
TLR2: Toll-like receptor 2
TNF: Tumor necrosis factor
TNFRSF17: TNF receptor superfamily member 17
TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand
Tregs: Regulatory T cells
UPR: Unfolded protein response
UbcH10: Ubiquitin-Conjugating Enzyme E2 C
USP5: Ubiquitin-Specific Peptidase 5

V-W-X-Y-Z

VCAM1: Vascular cell adhesion molecule 1
VEGF: Vascular endothelial growth factor
WES: Whole exome sequencing
XBP1: X-box binding protein 1
XPO1: Exportin-1

CHAPTER 1

INTRODUCTION

The content of this chapter includes sections that were either extracted or adapted from the following papers/book chapters developed as part of this dissertation, as stated in the “list of publications” (pages VII-IX). Authorizations from the publishers were granted.

Pinto, Vanessa; **Bergantim, Rui**; Caires, Hugo R.; Seca, Hugo; Guimarães, José E.; Vasconcelos, M.Helena. **“Multiple Myeloma: Available Therapies and Causes of Drug Resistance”**. Cancers (Basel). 2020;12(2):407. DOI: <https://doi.org/10.3390/cancers12020407>

- In section 2.1.1 to 2.1.3

Bergantim, Rui; Alves, Raquel; Peixoto da Silva, Sara; Jorge, Joana Verdasca; Xavier, Cristina P.R.; Gonçalves, Ana Cristina; Guimarães, José E.; Sarmiento-Ribeiro, Ana Bela; Vasconcelos, M. Helena. **“Drug Resistance in hematological malignancies: mechanisms and counteracting strategies”**. In preparation.

- In section 2.2.1 to 2.2.8

Peixoto da Silva, Sara; Caires, Hugo R.; **Bergantim, Rui**; Guimarães, José E.; Vasconcelos, M. Helena (2022). **“miRNAs mediated drug resistance in hematological malignancies”**. Seminars in Cancer Biology. 2022; 83: 283–302. DOI: <https://doi.org/10.1016/j.semcancer.2021.03.014>

- In section 2.2.9

Bergantim, Rui; Jorge, Joana; Peixoto da Silva, Sara; Alves, Raquel; Gonçalves, Ana Cristina; Sarmiento-Ribeiro, Ana Bela; Guimarães, José Eduardo; Vasconcelos, M.Helena; 2025. **“The Role of MicroRNAs in Mature B-Cell Neoplasias Drug Resistance”**. In: Rezaei, N. (Ed.), Comprehensive Hematology and Stem Cell Research, vol. 2, pp. 484–505. US: Elsevier. <https://dx.doi.org/10.1016/B978-0-443-15717-2.00013-5>. ISBN: 9780443157172

- In section 2.2.9
- In section 3.2.4

1. MULTIPLE MYELOMA OVERVIEW

1.1. Definitions

Multiple Myeloma (MM) is a hematological neoplasia characterized by the clonal proliferation and accumulation of terminally differentiated plasma cells derived from post-germinal-center B cells.[1] Monoclonal plasma cells are primarily found in the bone marrow either focally and/or diffusely, but they can be seen in peripheral blood and other extramedullary locations, mainly in the later stages of the disease history[2,3]. These plasma cells will produce non-functional intact immunoglobulin or parts of thereof, known as monoclonal protein or M protein, that can be found both in peripheral blood and urine[3,4]. Clinically, MM is typified by the clonal proliferation of malignant plasma cells in the bone marrow, identification of monoclonal protein in the blood and/or urine, and associated organ dysfunction[1,3,4]. The clinical manifestations are driven by the monoclonal plasma cell, cytokines secreted by those, and excessive production of the M protein leading to organ dysfunction, characterized by hypercalcemia, renal insufficiency, anemia, and/or bone disease, collectively known by the CRAB acronym[3,4].

1.2. Epidemiology

Multiple Myeloma is estimated to represent 1.7% of all malignancies, yet it is the second most common hematological malignancy, constituting 10% of all hematological malignancy diagnoses[5,6]. According to the Global Cancer Observatory, the global incidence of MM in 2022, is 1.8 per 100 000 people, occupying the 21st place of all cancers, representing 187 952 new cases. Of this, 103 805 were male and 84 147 were female, representing an age-standardized incidence of 2.2/100 000 and 1.5/100 000, respectively[7]. From 1990 to 2016, the global incidence of MM increased by 126%[8]. The incidence varies and is highest in more developed countries such as Northern America, Australia-New Zealand, and Northern Europe[5,6]. This higher incidence in these countries reflects the availability of better diagnostic techniques and greater clinical awareness. The lifetime risk of being diagnosed with MM from birth up to the age of 74 is 0.24% for men and 0.17% for women, indicating that men are about 1.5 times more likely to develop MM[8]. Racial disparities are also evident in MM, with a 2 to 3 times higher incidence in black individuals than in white ones and lower in Asian and Hispanic populations[9-12].

In 2022, an estimated 121 388 people died with MM, accounting for 1.1% of all cancer deaths, being the 17th cancer responsible for death related to cancer. Of this, 66 966 were male and 54 422 were female, representing an age-standardized mortality of 1.3/100 000 and 0.9/100 000, respectively[7]. Resembling sex discrepancy in MM risk, the risk of death from MM was 0.15% among males and 0.10% among females[8].

Using the Surveillance, Epidemiology, and End Results registries in the United States, it was observed that from 1993 to 2012, the incidence of MM increased in younger patients, probably reflecting a greater utilization of healthcare services, allowing for earlier identification of cases[10]. While incidence has risen over the last decades, mortality has fallen due to the drastically increased survival rate, reflecting the widespread use of autologous stem

cell transplantation and the development of novel therapeutic agents[8]. The overall mortality rate dropped for all ages from 3.3/100 000 in 2013 to 3.2/100 000 in 2017, being more expressive among patients older than 65, reducing from 21.7/100 000 to 20.5/100 000[8]. Nonetheless, above 75 years of survival improvement is limited because of constraints to access to differentiated care and cultural patterns of interaction rather than biological aspects[10,13]. It was also observed that the survival of MM is improving among racial/ethnic minorities. However, the difficulty in accessing novel treatments and the poor accrual of these patients in epidemiological studies can limit our understanding of this disparity [10,14].

In Portugal, MM has an incidence of 7.8/100 000 individuals, representing 780 new cases per year, with a median age of diagnosis at 69 years old, 58% of the diagnosed patients being 70 years old or older, and a smaller portion, 9%, being younger than 55 years. A detailed analysis of MM diagnosis in public hospitals revealed that in 2018, there were 1941 identified patients with MM, leading to a prevalence rate of 18.9/100 000. The mortality rate for MM in Portugal was reported at 6.9/100 000, with the median age at death for MM patients of 75 years[15].

1.3. Risk Factors

Risk factors directly responsible for MM are unknown, even though several studies have evaluated potential causes[3,4]. Established risk factors for MM include age, male sex, African ancestry, obesity, and chronic inflammation[16,17]. Antigens triggering a MM clone may also be endogenous. Notably, Monoclonal Gammopathy of Unknown Significance (MGUS) prevalence is elevated in Gaucher patients, which is characterized by glucocerebrosidase deficiency resulting in the accumulation of glucosylceramide and high levels of specific immunoglobulin for deacylated lysolipid glucosyl sphingosine, compared to the control population[18]. Environmental and occupational exposures include radiation, pesticides, solvents, hair dyes, benzene, and petroleum. Nonetheless, some of the reports offer little evidence to support a causal relation with the development of MM[3,19]. Familial aggregation of MM and its precursor MGUS suggests that genetic factors may play a significant role in developing MM[20,21]. In addition, the risk of MGUS in first-degree relatives of patients with MM is increased by twofold[22]. Genome-wide association studies identified more than 20 genetic loci associated with an increased risk for MM and increased death in those already diagnosed[23]. For instance, several single-nucleotide polymorphisms can lead to MYC activation, associated with MM progression, and increased levels of interleukin-6 (IL-6), a cytokine with a defined role as a growth and survival factor in MM[23,24].

1.4. Mechanisms of disease/pathophysiology

1.4.1. Ontogenesis

Gaining a deep understanding of B cell development and plasma cell biology is crucial for unraveling the complexities of MM. It represents the neoplastic counterpart of terminally differentiated, immunoglobulin-producing, long-live plasma cells (PCs)[25]. Normal PCs develop from hematopoietic stem cells, which undergo a complex differentiation process in the bone marrow and secondary lymphoid organs, to B cells and PCs[25-27]. In

the bone marrow, during early B-cell development to PCs, immature B cells undergo V(D)J rearrangements, first in the heavy chain genes and then the genes coding for the light chain, generating their diverse primary immunoglobulin repertoire[28,29]. B cells then leave the BM and migrate to secondary lymphoid organs, such as the lymph nodes or the spleen. Here, during the germinal center reaction that results in the production of antibodies with high specific antigens and different functional properties, B cells undergo two types of somatic alterations, namely somatic hypermutation (SHM) and class-switch recombination, both mediated by the activation-induced cytidine deaminase (AID) and double-strand DNA breaks(DSBs)[29,30]. Also, AID deaminates cytosines on single-stranded DNA, resulting in immunoglobulin heavy and light chains or SHM mutations[26,28]. Although AID-induced DSBs are mostly repaired locally, they can be joined to DSBs occurring elsewhere in the genome, leading to aberrant fusions of DNA and aberrant chromosomal translocations. If involved, specific oncogenes can give cells a growth advantage, leading to pathological conditions such as plasma cell dyscrasias[3,31]. Indeed, the MM precursor stages, MGUS and Smoldering Multiple Myeloma (SMM), may carry some of these initial abnormalities, suggesting that they are necessary but insufficient for the MM pathogenesis. It is believed that late-stage oncogenic events occur in the bone marrow, following the complete differentiation of the initial MM clone into a long-lived plasma cell[31].

1.4.2. Genetic alterations

MM is clinically and biologically heterogeneous, harboring several genetic alterations proposed as driving events in myelomagenesis. These genetic events can be broadly categorized into two distinct groups: primary and secondary[26,31].

Regarding the **primary genetic alterations**, MM is typically divided into two subtypes: hyperdiploid and non-hyperdiploid). The initiating event driving malignant development is either the acquisition of hyperdiploidy or the translocation involving the immunoglobulin heavy chain (IGH) genes locus on chromosome 14[26,31,32]. These clonal alterations are present in almost all cells and have been seen in the precursor conditions MGUS and SMM[33]. **Hyperdiploidy** is the most common form of aneuploidy in MM, being present in half of patients, and is characterized by the co-occurring trisomies of some or all of chromosomes 3, 5, 7, 9, 11, 15, 19 and 21. These patients are less likely to have primary IGH translocation. Although these trisomies often occur together, trisomies 3 and 5 are associated with favorable outcomes, unlike trisomy 21, which is associated with poor prognosis. Interestingly, the poor prognosis conferred by t(4;14) appears to be mitigated by the trisomies 3 and 5 co-occurrence[34,35]. **Translocations** involving the IGH and a limited set of recurrent partner genes, such as Cyclin D genes (CCND1-3), WHSC1 (also known as NSD2 or MMSET), MAF, or MAFB, represent an important class of primary alterations[36]. Due to these translocations, the resulting fusion products with enhanced expression of the partner genes end under the control of the IGH enhancer, one of the most active ones in PCs. As referred previously, the underlying mechanism of translocations is mostly abnormal class-switch recombination during plasma cell development and abnormal V(D)J rearrangements[37]. Translocations t(11;14), observed in 15-20% of MM patients, and t(6;14), observed in 1-4% of MM patients, juxtapose the IGH enhancer with cyclin D genes (CCND1 and CCND3, respectively), inactivating the RB1 gene and allowing for E2 factor activation and cell cycle progression[38,39].

Translocation t(4;14) is observed in 15% of MM and leads to the overexpression of 2 genes, FGFR3 (Fibroblast Growth Factor Receptor 3) and NSD2 (Nuclear Receptor Binding SET Domain Protein 2), commonly known as MMSET (Multiple Myeloma SET Domain Protein). The overexpression of these genes contributes to increased tumor proliferation and high tumorigenicity. This translocation is associated with a dismal prognosis, and patients are classified as high-risk[26,40]. Translocations t(14;16) and t(14;20), observed in 3% and 1.5% of MM patients, respectively, deregulate the MAF (Avian Musculoaponeurotic Fibrosarcoma) and MAFB (Avian Musculoaponeurotic Fibrosarcoma Homolog B) genes, respectively. The resulting overexpression induces proliferation and protects cells from drug-induced apoptosis, conferring a bad prognosis [26,41,42].

Secondary genetic alterations with a crucial role in MM progression include a wide range of alterations, including copy number alterations, translocations, and recurrent mutated genes grouped in specific signaling pathways[31,32,43]. **Copy number abnormalities (CNA)** result in chromosomal regions of loss or gain[44]. The loss of tumor suppressor genes is typically due to deletions on either the short arm (p) or long arm (q) of a chromosome – del(1p), resulting in loss of CDKN2C, FAF1, and FAM46C (also known as TENT5C), del(11q) affect BIRC2 and BIRC3, del(13q) impact RB1 and DIS3, and del(17p) resulted in the loss of TP53[26,31,32]. This last one has a major impact on cell cycle control and DNA damage response and has been described as a secondary event since, although only 10% of newly diagnosed MM (ndMM) patients harbour this deletion, it reaches up to 80% in later stages of the disease, associated with poor prognosis [45]. Gain in the long arm of chromosome 1 is found in around 40% of patients, often in association with t(4;14), carrying a bad prognosis, even with the gene or genes responsible remain unknown[46]. In addition to CNA, **translocations involving MYC** (V-Myc Avian Myelocytomatosis Viral Oncogene Homolog), either via t(8;14) or without involving the IGH gene, are also considered secondary genetic events since they are found at low frequencies in MGUS or SMM patients (3-4%). In contrast, in ndMM, the frequency rises to 15-20%, and in relapse or refractory MM (rrMM), it rises to 50%[39,47]. MYC regulates up to 15% of all genes, including upregulation of CCND2, which is involved in cell cycle regulation, and upregulation of Enolase 1, which is involved in glycolysis. Importantly, MYC rearrangement has become central in transitioning from premalignant conditions to symptomatic MM[48,49]. **Somatic mutations** are highly variable among MM patients and occur most often in genes of the mitogen-activated protein kinase (MAPK) pathway (mutations in KRAS, NRAS, BRAF, EGR1, and FGFR3 genes). Around 50% of patients have those mutations (KRAS 22%, NRAS 17%, BRAF 8%), mostly subclonal and detected at diagnosis and relapse, consistent with their role in tumor progression rather than initiation[44]. Moreover, the DNA repair pathway is mutated in 15% of patients, with several gene mutations such as TP53, ATR, ATM, and ZFHX4. Other commonly mutated genes include BRAF, TRAF3, PRDM1, CYLD, RB1, IRF4, EGR1, MAX, HIST1H1E, ACTG1, TENT5C and DIS3, found in around 10% of patients[31,39]. Interestingly, an APOBEC mutation signature is seen in around 4% of cases and is linked to the translocation-mediated deregulation of MAF and MAFB, explaining the rapid progression and drug resistance of these MM cases carrying t(14;16) and t(14;20)[50]. Overall, the frequency of mutations increases during disease progression, and high-risk stages also have a higher mutation burden. Other affected signalling pathways include the NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, which is impacted by copy number losses, mutations, and translocations, and the PI3K (Phosphoinositide 3-kinase) pathway, which becomes dysregulated even without genetic changes[51,52]. In fact, in 20% of MM patients, genetic abnormalities activate the non-

canonical NF- κ B pathway, leading to elevated expression of several anti-apoptotic proteins. Also, the apoptotic pathway may be disrupted, evidenced by the BCL2 (protein B-cell lymphoma 2) dependency in patients with the t(11;14) and MCL1 (myeloid cell leukemia 1) dependency in others[53,54].

1.4.3. Epigenetic alterations

Beyond the previously outlined established genetic changes, recent evidence indicates that epigenetic modifications may also significantly contribute to developing MM. Fundamental epigenetic changes observed in PCs include irregular DNA methylation, alterations in histone modification, and atypical expression of miRNAs.

Despite the general and extensive **hypomethylation**, MM cells frequently present regional DNA hypermethylation[55,56]. In this context, the promoter of the cyclin-dependent kinase inhibitor genes CDKN2B and CDKN2A is hypermethylated, leading to transcriptional silencing of these genes and, therefore, activation of the cell cycle, enhancing cell proliferation and disease progression[57]. Numerous other genes that inhibit tumor growth, such as CDH1, DAPK1, SOCS1, and SHP1, were rendered inactive due to DNA hypermethylation at the CpG islands associated with their promoters[55]. The patterns of DNA methylation separate normal PCs from MGUS and MM cells, being observed that the extent of hypermethylation is comparable between MGUS and MM. In contrast, hypomethylation levels are elevated in MM compared to MGUS[55,56]. In patients harboring the t(4;14), it is possible to verify high levels of gene-specific DNA hypermethylation compared to other cytogenetic subgroups, exhibiting overexpression of MMSET, a gene encoding a histone methyltransferase that acts as a transcriptional repressor. MMSET is responsible for the methylation of histone H3 lysine 36 (H3K36), and its misregulation leads to widespread alterations in histone modifications, enhancing cell survival, proliferation, and DNA repair mechanisms[58]. Additionally, histone can also undergo **acetylation**. In MM, histone deacetylases (HDACs) are overexpressed, contributing to an aberrant acetylome in the malignant MM phenotype[55,56]. HDAC1 overexpression can lead to proliferation through repression of tumor suppressor cyclin-dependent kinase inhibitors and angiogenesis by repression of p53 and induction of HIF-1 (Hypoxia Inducible Factor) and hypoxia-responsive genes such as VEGF (Vascular Endothelial Growth Factor)[59]. HDAC4 expression is also significantly upregulated in MM to the extent that its knockdown increases the ATF4 (activating transcription factor 4) expression and suppresses the MM cell growth, triggering apoptosis and autophagy[60].

The recent development of RNA-sequencing technology facilitated the discovery of several **miRNAs** with different expression profiles associated with the pathogenesis of MM (Table 1). This is particularly interesting because miRNAs are found within genome regions that encode for either oncogenes or tumor suppressor genes[56]. In addition, miRNAs have been demonstrated to play essential roles in various biological processes in MM cells, including proliferation, differentiation, migration, apoptosis, angiogenesis, and the modulation of the bone marrow microenvironment[61-65].

Table 1. miRNAs influencing Multiple Myeloma pathogenesis and their potential targets.

microRNA promoting MM				
Expression	miRNA	Targets	Cell Processes	Ref.
Downregulated	miR-23b	Sp1	↑ Cell growth ↑ Survival	[66]
	miR-126	VEGF/PI3K/Akt	↑ Survival ↑ Angiogenesis	[67]
	miR-34a-5p	RMRP	↑ Proliferation ↓ Apoptosis	[68]
	miR-342 miR-363	Runx2	↓ Antitumor immunity ↑ Osteolysis	[69]
	miR-637	NUPR1	↑ Proliferation ↓ Apoptosis	[70]
Upregulated	miR-19a/miR-19b	SOCS1	↑ Cell growth ↑ Proliferation ↑ Survival	[71]
	miR-27b-3p miR-214-3p	FBXW7, PTEN/AKT/GSK3	↑ Cell growth ↓ Apoptosis ↑ Fibroblasts proliferation	[72]
	miR-19b/miR-20a	PTEN	↑ Proliferation ↑ Migration ↓ Apoptosis	[73]
	miR-221/miR-222	ATG12, p27/mTOR, p27Kip1, p57Kip2, PTEN, PUMA	↑ Cell growth ↓ Autophagia	[74,75]
	miR-21	Rho-B, PTEN, AKT, ERK, PIAS3 STAT3	↑ Cell growth ↑ Proliferation ↑ Survival ↑ Clonogenic development of MM in stromal free circumstances ↑ Osteoclast differentiation ↑ Fibroblasts proliferation	[76]
	miR-25-3p	PI3k/AKT	↑ Proliferation	[77]
	miR-27	SPRY2	↑ Proliferation ↑ Migration ↑ Cell invasion	[78]
	miR-181	CCND1	↑ Proliferation ↑ Migration	[79]
	miR-32 miR-17/92 cluster	SOCS1, BCL2	↑ Proliferation ↑ Apoptosis resistance	[80]
	miR-129-5p	Sp1	↓ Osteoblast differentiation	[81]
	miR-135	HIF-1	↑ Angiogenesis	[82]
	miR-21/miR-155	PTEN, SOCS1, BLC6, IL13RA	↑ MDSCs ↑ Macrophage activation ↑ Pro-inflammatory response	[83]
	miR-155/miR-221	IL-6, TNF	↑ IL-12 activity in DC ↑ Pro-inflammatory response	[84]
	miR-125a-5p	USP5, p53	↑ Cell growth ↑ Cell viability ↓ Apoptosis ↑ Migration	[85,86]
microRNAs suppressing MM				
Downregulated	miR-26a	CD38	↓ Proliferation ↓ Migration ↑ Apoptosis	[87]
	miR-29b	FOXP1, STAT3, NF-KB, MCL-1, CDK6 and SP1, DNMT3A/B and HDAC4, IFN	↓ Proliferation ↑ Apoptosis ↑ Cell Cycle Arrest ↓ Osteoclast differentiation/activation	[88]
	miR-489	LDHA	↓ Proliferation ↓ Cell viability ↓ Glucose uptake and ATP production	[89]
	miR-15a miR-16-1	MAP-kinases, AKT serine/threonine-protein-kinase, NF-kB-activator MAP3KIP3 and ribosomal-protein-S6, VEGF	↓ Cell growth ↑ Apoptosis ↓ Capillary formation	[90]
	miR-338-3p	CDK4	↓ Proliferation ↑ Apoptosis	[91]
	miR-19a-3p	Wnt1	↓ Proliferation ↓ Migration ↓ Cell invasion	[92]
	miR-194-2/192 miR-251/194-1	p53, MDM2	↑ Augmented the therapeutic efficacy of MDM2 inhibitors	[93]
	miR-192	TGIF2	↓ Proliferation	[93]
	miR-106b/-25	p38/MAPK	↑ Apoptosis ↓ Cell viability	[94]
	miR-125b	IRF4, BLIMP-1	↑ Apoptosis ↑ Autophagia	[95]
Upregulated	miR-33b	PIM-1	↓ Cell growth ↑ Apoptosis ↓ Linkage between Bad and Bcl2/I-xl	[96]
	miR-429	Bmi1	↓ Proliferation ↓ Migration ↓ Cell invasion	[97]
	miR-34a/b/c	MYC, CDK6	↓ Proliferation	[86,98,99]
	miR-340	c-Met	↓ Angiogenesis	[100]
	miR-485-5p miR-22	DLK1-DIO3 p38	↓ Cell cycle progression ↓ IL-6, IL-23, Th-17 response	[101] [102]

↑—increased, ↓—decreased

Several miRs contribute to the pathogenesis of MM through diverse signaling pathways (Table 1).

Epigenetic regulation of the tumor suppressor miRNA-23b has been observed in MM, in which c-Myc-induced miRNA-23b silencing may favor the oncogenic Sp1-activity, enhancing MM cell growth and survival[66]. Moreover, MMSET overexpression observed in t(4;14) reduces levels of miR-126, ultimately favoring c-Myc upregulation, which could increase cell survival[67]. Also, miR-19a and miR-19b can contribute to the JAK-STAT pathway activation by targeting the JAK-STAT inhibitor SOCS1 (Suppressor of cytokine signaling 1), regulating sensitivity to cytokines, namely IL-6, and consequentially cell growth, proliferation, and survival in PCs[71]. The overexpression of miR-27b-3p and miR-214-3p induced increased cell growth and resistance to apoptosis in MM fibroblasts. This was achieved through the activation of the FBXW7 (F-box and WD repeat domain containing 7) and the PTEN (Phosphatase and tensin homolog)/AKT (Protein kinase B)/GSK3 (Glycogen synthase kinase 3) pathways. When inhibitors for miR-27b-3p and miR-214-3p were introduced, the anti-apoptotic factors were suppressed, resulting in the induction of MCL1 (myeloid leukemia cell differentiation protein)[72]. miR-19b and miR-20a are upregulated in MM PC cells, enhancing cell proliferation and migration and inhibiting cell apoptosis in MM. Transfection of miR-19b/20a resulted in the downregulation of PTEN protein (a tumor suppressor protein with anti-proliferation and pro-apoptosis effects). In turn, lentivirus-mediated delivery of miR-20a boosted tumor growth[73]. miR-221/222 down-regulated other tumor suppressor genes, leading to the inhibition of MM growth in vitro and in vivo. Moreover, miR-221/222 activates several pathways, including p27Kip1, p57Kip2, PTEN, and PUMA (p53 up-regulated modulator of apoptosis)[74,75]. miR-21 is crucial for the growth and development of MM PCs by activating pro-survival signaling and targeting tumor suppressor genes such as Ras homolog (Rho)-B and PTEN, together with functional impairment of AKT and ERK (Extracellular signal-regulated kinase) signaling. Furthermore, it was also shown that using miR-21 inhibitors upregulates PTEN and Rho-B while decreasing AKT and ERK phosphorylation in MM cells[76]. Moreover, by targeting the tumor suppressor PTEN, miR-25-3p overexpression accelerates the advancement of MM by activating the oncogenic PI3k/AKT (phosphoinositide-3-kinase/protein kinase B) pathway[77]. miR-27 was overexpressed in MM PCs compared to controls, which was associated with shorter survival. Functional studies demonstrated that miR-27 overexpression by targeting the SPRY2 (Sprouty homolog 2) promoted MM cell proliferation, facilitated cell cycle progression, and expedited cell migration and invasion, whereas miR-27 knockdown inhibited cell proliferation, induced cell cycle arrest, and slowed down cell motility[78]. The miR-181a overexpression enhanced the migration and proliferation of the MM. It was also found that miR-181a down-regulation causes cell cycle arrest via the down-regulation of cell cycle regulatory protein CCND1 (Cyclin D1)[79]. In MM cell lines, mitochondrial RNA processing endoribonuclease (RMRP) was up-regulated, whereas miR-34a-5p was down-regulated. Over-expression of RMRP enhanced cell proliferation of MM cells, and its knockdown led to a marked reduction in c-Myc expression and induced apoptosis in the same cells. Likewise, the silencing of miR-34a-5p was associated with high RMRP levels. High expression of miR-34a-5p inhibited proliferation and fostered apoptosis, which indicates that RMRP acts as a miR-34a-5p sponge to boost cell proliferation and suppress cell apoptosis through upregulation of c-Myc in MM[68].

Various other miRs are crucial in suppressing the pathogenesis of MM by targeting diverse signaling pathways (Table 1). Overexpression of miR-26a, which is found to be down-regulated in MM PCs, reduced proliferation and migration and prompted apoptosis in MM cell lines. Also, miR-26a targets and downregulates the

CD38 protein, which is indicated as responsible for activating cell proliferation and restricting cell apoptosis[87]. miR-29b expression was markedly decreased in MM cell lines and tissues. Exogenous overexpression of miR-29b inhibited cell proliferation and prompted cycle arrest and apoptosis in MM PCs. Studies identified the Forkhead box protein P1 (FOXP1) as a direct target gene for miR-29b and that restoring FOXP1 reduced the pro-apoptosis and anti-proliferation effects induced by miR-29b in MM cell lines. In fact, miR-29b fades the progression of MM via downregulating FOXP1, providing a potential biological target for MM treatment[88]. miR-489 was significantly reduced in MM, serving as a tumor suppressor gene, reducing cell proliferation and viability of MM cells via lactate dehydrogenase A (LDHA)-mediated glycolytic metabolism. miR-489 inhibited aerobic glycolysis via decreased glucose uptake, lactate, and adenosine triphosphate (ATP) production. LDHA was identified as a target of miR-489, suggesting a negative correlation between miR-489 and LDHA in MM cells[89]. miR-15a and miR-16-1 were downregulated in MM cases and were found to inhibit tumor survival pathways, including MAPKs, AKT serine/threonine-protein-kinase, NF- κ B-activator MAP3KIP3, and ribosomal-protein-S6. Malignant plasma cells transfected with miR-15a/16-1 showed cellular apoptosis and repressed tumor growth[90]. miR-125b was downregulated in MM patients by cancer-secreted growth factors such as tumor necrosis factor (TNF) and insulin growth factor (IGF)-1. It was observed that apoptotic and autophagy-associated cell death were triggered in MM cells on miR-125b-5p ectopic expression and that the anti-MM activity of miR-125b-5p was mediated via direct downregulation of IRF4 and its downstream effector BLIMP-1[85]. Remarkably, miR-125b up-regulates miR-34a, inhibiting the IL-6 receptor/STAT3/miR-34a feedback loop and sequentially activating cell death mechanisms in MM[86]. miR-342 and miR-363 are downregulated in MM, and their overexpression inhibits MM progression, decreases osteolysis, and enhances antitumor immunity via targeting Runx2 (runt-related transcription factor 2) and its related target downstream[69]. Similarly, miR-637 was downregulated in MM, and its overexpression suppressed the MM progression by promoting apoptosis and suppressing proliferation via targeting of nuclear protein 1 (NUPR1)[70]. Also, miR-429 overexpression suppresses the proliferation, migration, and invasion capabilities of MM PCs and inactivates the AKT pathway by targeting Bmi1 (B cell-specific Moloney murine leukemia virus integration site 1)[97]. miR-33b targets a serine/threonine kinase known as PIM-1, which blocks the linkage between BCL2-associated agonist of cell death (Bad) and Bcl2/l-xl to inhibit apoptosis. This results in significant apoptosis of MM plasma cells, thus suppressing cancer growth and enhancing survival rates[96]. In MM tissues and cell lines, miR-338-3p levels were considerably reduced. By targeting the expression of cyclin-dependent kinase 4 (CDK4), miR-338-3p overexpression in MM cells significantly reduced proliferation and aided in promoting apoptosis, caspase 3 and caspase 8 activity[91]. Also downregulated in MM cell lines and patient samples, miR-19a-3p targets Wnt1 through the Wnt/ β -catenin signaling pathway, preventing MM cells from proliferating, migrating, and invading their surrounding tissue. In this study, β -catenin, cyclin D1, and c-Myc expression were found to be reduced by miR-19a-3p expression[92].

The TP53 gene, a potent tumor suppressor gene, has been identified as critical in preventing MM development. Several miRNAs, including miR-194-2/192 and miR-251/194-1 clusters, have been reported to regulate p53 during the progression of MM[93]. The normal function of p53 is suppressed by MDM2 (murine double minute 2), which is highly expressed in MM cells. Reduced miR-194-2/192 and miR-251/194-1 expression can inhibit MDM2 and amplify the regular p53 activity, highlighting how these miRs can be involved in MM pathogenesis[93]. The ectopic

miR-125-5p reduced the expression of p53 pathway-related genes, including the expression of miR-192 and miR-194. It was observed that inhibition of miR-125a-5p expression in wild-type p53 MM cells decreased cell growth, enhanced apoptosis, and reduced cell migration, further amplified by the co-expression of miR-192 or miR-194[95]. The expression of miR-34a and miR-34-b/c is augmented by the binding of p53 to their promoter regions, inhibiting the expression of MYC and the phosphorylation of cyclin-dependent kinase 6 (CDK6), which promotes cell proliferation. In this context, miR-34a and miR-34b/c act as tumor suppressor genes that mediate the function of p53[99,103]. It was also observed that the expression of these miRNAs was decreased in MM cells harboring the 17p deletion/TP53 mutation, and its promoter region was frequently methylated[98].

As mentioned above, MM is characterized by multistep stages and a diverse altered expression of several miRNAs. In BM and PB samples from MGUS and MM patients, different miRNA profiles were found to be involved in the progression from earlier stages to overt symptomatic MM[56,71]. For instance, it was reported that the miR-106b/-25 cluster, miR-181a/b, miR-106b, and miR-93 are overexpressed in samples from patients with MM and MGUS patients compared to healthy individuals. Anti-miR-106 b/25 reduced cell viability and induced apoptosis in MM plasma cells by inhibiting the p38/MAPK-dependent signaling pathway, highlighting its role in MM pathophysiology[94]. Additionally, the upregulation of miR-32 and the miR-17/92 cluster, targeting SOCS1 and BCL2, related to apoptosis resistance and cell proliferation, was observed exclusively in MM, suggesting their role in disease progression[80].

1.4.4. Interaction of PCs with BM microenvironment'

The interaction between PCs and the bone marrow microenvironment plays a central role in the pathogenesis of MM (Figure 1). The BM microenvironment comprises different cellular compartments (endothelial cells, stromal cells, osteoclasts, osteoblasts, and immune cells) and non-cellular fractions, including the liquid milieu (growth factors, chemokines, and cytokines) or the extracellular matrix. The interplay between PCs and BM microenvironment occurs mainly through direct cell-to-cell contact, release of soluble factors, and, as recently identified, via extracellular vesicles (EVs). These vesicles facilitate cell communication by transferring proteins, small non-coding RNAs, including miRNAs, and lipids, among other cargo. Overall, cellular and non-cellular components of the MM BM microenvironment contribute to generating an immunosuppressive tumor microenvironment, supporting MM cell growth and survival[3,25,104].

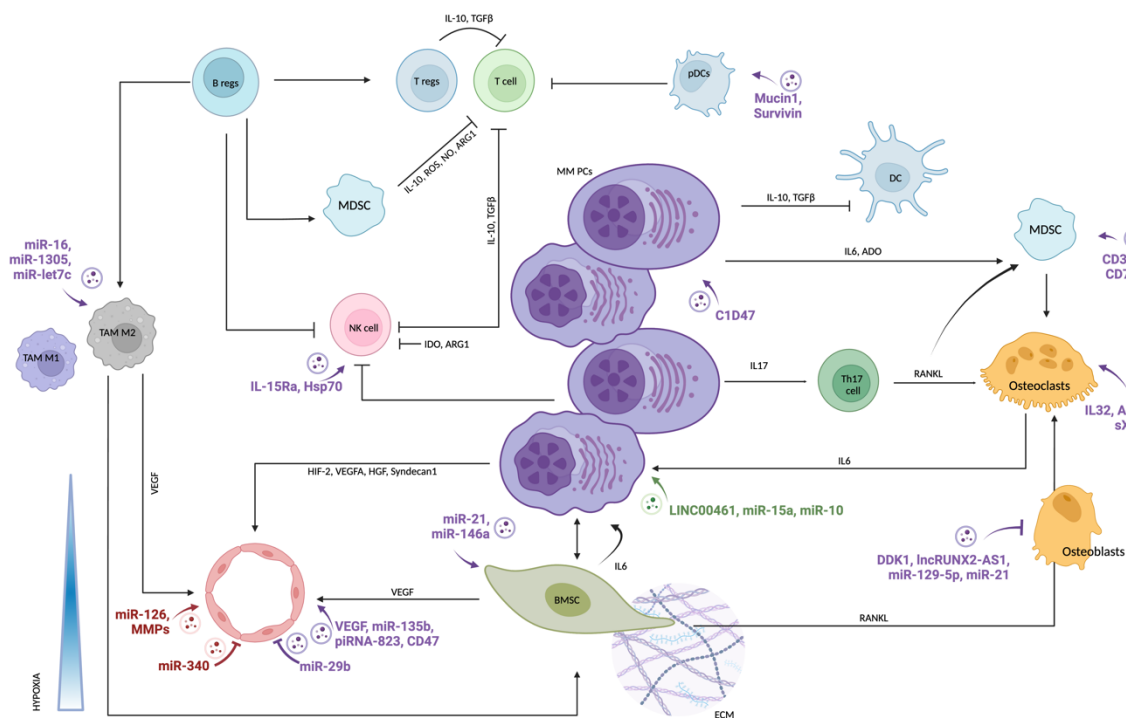


Figure 1. Interaction of MM plasma cell with the bone marrow microenvironment. The complex network of interactions between MM PCs and the different components of the BM microenvironment plays an essential role in the pathogenesis of MM, working together to sustain the malignant behavior of MM PCs and to facilitate immune evasion. Reciprocal interactions between BMSCs and MM cells involve cell-to-cell contacts and soluble factors. BMSCs are the primary source of IL-6, also produced by osteoclasts, TAMs, or MM cells. IL-6 promotes myeloma cell proliferation, survival, and drug resistance. In addition, BMSCs and TAMs secrete vascular VEGF, thereby favoring angiogenesis. External stimuli, such as hypoxia, and contributions from endothelial cells further enhance angiogenesis, supporting tumor growth and survival. Interactions involving MM cells, osteoblasts, and osteoclasts imbalance the bone remodeling process and result in bone lysis, mainly by activating Th17 cells, which produce IL-17 to promote osteoclast activation through RANKL signaling. Bregs and Tregs cells contribute to immune suppression by releasing IL-10 and TGFβ, which inhibit T cell activation. MDSCs further exacerbate immune suppression by producing IL-10, ROS, NO, and ARG1, while NK cells are inhibited by IL-15, Hsp70, IDO, and ARG1, reducing their cytotoxic activity. DCs and pDCs are also impaired, with DCs being inhibited by IL-10 and TGFβ and the pDCs interacting with MM PCs by inhibiting the T cell cytotoxic activity. Simultaneously, the interaction of MM cells and other elements of the BM environment also induces MM expansion and migration of PCs, facilitates ECM remodeling, and leads to adhesion-mediated drug resistance. EVs are essential in this interplay by facilitating cell communication and transferring proteins, small non-coding RNAs, including miRNAs and lipids, among other cargo that will influence all the above relations. "Created with BioRender.com".

Bone marrow stromal cells (BMSCs) are a key element in the BM microenvironment, creating a favorable niche for MM growth via direct cell-to-cell contact and through the secretion of cytokines and chemokines, which favor myeloma cell proliferation and inhibit apoptosis[104]. The interaction of BMSCs with PCs via the vascular cell adhesion molecule 1/integrin b1 (VCAM1/ ITGB1), intercellular adhesion molecule 1/integrin b2 (ICAM1/ITGB2), and mucin 1 cell surface-associated (MUC1) axes triggers a bidirectional signaling cascade, leading to activation of NF-κB and MAPK1 pathways in BMSCs and MAPK1 in MM PCs[105,106]. In turn, NF-κB and MAPK1 signaling induces the secretion of proliferative, antiapoptotic, and chemotactic cytokines such as IL-6, CXCL12, insulin-like growth factor 1 (IGF-1), and VEGFA, further supporting myelomagenesis[106]. IL-6 specifically stimulates angiogenesis and causes the activation of anti-apoptotic proteins such as BCL2[104]. Other factors produced by BMSCs include the VEGF, which promotes angiogenesis, and Notch Ligand Jagged-1, which plays a relevant role in MM development

and progression[107]. In addition, BMSCs produce factors such as SDF-1 (Stromal Cell-Derived Factor 1), which binds to the CXCR4 receptor (C-X-C chemokine receptor type 4) on MM cells, essential for the migration and homing of myeloma cells to the BM[108].

Interaction with BMSC alters the levels of factors involved in aberrant bone formation, including increased production of receptor activator of nuclear factor- κ B ligand (RANKL) and reduced levels of osteoprotegerin (OPG), culminating in the reduction of the bone-forming capacity of osteoblasts and favoring both the differentiation and the resorptive activity of osteoclasts, which leads to the characteristic osteolytic lesions associated to MM. Osteoclasts also contribute to MM pathogenesis by secreting IL-6 and osteopontin, thus stimulating MM proliferation and angiogenesis[104,109]. Furthermore, it has been shown that MM-derived EVs may lead to osteolytic lesions. Amphiregulin-rich exosomes produced by MM can enhance OC development by activating the EGFR (epidermal growth factor receptor) pathway, increasing the secretion of RANKL, and decreasing the release of osteoprotegerin [110]. Conversely, MM-derived-EVs (MM-EVs) promote bone resorption by augmenting the expression of critical osteoclastogenic enzymes (TRAP, CTSK, MMP9) and inhibiting the apoptosis of the osteoclast precursors by blocking the caspase-3 activity[110]. The RNA content of MM-EVs also has an essential role in osteolysis in MM. The miR-129-5p, once transferred to BMSCs, inhibited the transcription factor Sp1 and its target alkaline phosphatase, thereby blocking osteoblast differentiation[81]. MM-EVs could also block osteoblast differentiation from BMSC by triggering IL-6 secretion from BMSCs via the Ape1/NF- κ B pathway[111]. Another miRNA, miR-21, produced through the STAT3 pathway and stored in MM-EVs, was shown to be involved in promoting osteoclast differentiation through the imbalance of RANKL/OPG[112]. The presence of the long noncoding antisense RNA LncRUNX2-AS1 in a human myeloma cell line-derived exosomes impaired osteogenic differentiation of MSCs by negatively regulating RUNX2 expression, an essential transcription factor regulating osteoblast differentiation[113].

The **BM microenvironment is hypoxic**, resulting in elevated expression of HIF-1 α and VEGFA and increased angiogenesis in MM patients. Also, aberrant expression of HIF-2 by MM PCs and elevated levels of VEGFA, hepatocyte growth factor, and syndecan-1 have all been shown to promote angiogenesis in MM[25]. The hypoxia-resistant MM cells produce more EVs than their parental cells. Chronic hypoxia appears to have caused increased EV production in MMCs, with upregulation of miR-135b content that directly downregulated factor-inhibiting HIF-1 in endothelial cells, resulting in increased angiogenesis[82]. A study examining both naive MM-EVs and MM-EVs treated with bortezomib revealed that MM-EVs contain pro-angiogenic factors that promote migration and proliferation when internalized by endothelial cells (EC). Bortezomib treatment modified the levels of pro-angiogenic and inflammatory proteins in MM-EVs. Notably, the expression of VEGF-R2 and several growth factors, including VEGFA, PDGF-BB (platelet-derived growth factor-BB), angiogenin, and urokinase plasminogen activator receptor, was significantly reduced in bortezomib derived EVs compared to naive MM-EVs[114].

More recently, MM EVs were found to contain the small silencing piwi-interacting RNA piRNA-823, which, when transferred to endothelial cells (ECs), promoted proliferation, invasion, and **angiogenesis** by enhancing expression of VEGF, IL-6, and ICAM-1. piRNA-823-transfected ECs enhanced tumor growth when co-inoculated with MM cells

in a xenograft model, as well as restricted the apoptosis of the recipient EC through the inhibition of the caspase-3 activation, downregulation of the Bcl-2-associated X protein (BAX) expression, and the production of ROS and NO[115]. ECs also generate EVs, which influence tumor invasion and angiogenesis. They release EVs containing metalloproteinases (MMPs) that lead to proteolysis, necessary for metastasis and free angiogenesis[116]. Additionally, miR-126 is present in EC-derived EVs and exerts pro-angiogenic actions on the microenvironment and other ECs through the VEGF/PI3K/Akt pathway[117,118]. It was also observed that high levels of miR-340 contained in EVs from ECs inhibited angiogenesis by suppressing the c-Met signaling pathway and that treating MM cells with C6 ceramide prevented angiogenesis by increasing EV-miR-29b levels, which blocked the PI3K-Akt pathway in ECs[100,119].

In addition to modulating angiogenesis, different mechanisms have been described to show how EVs play an essential role in the pathogenesis of MM[120,121]. The content of the EVs includes increased pro-proliferative protein levels (namely IL-6, junction plakoglobin, and fibronectin), creating a cytokine-rich niche that supports the survival of MM cells[122]. It was found in proliferation-inducing BMSC-derived EVs decreased levels of miR-15a and augmented levels of long-non-coding RNAs (lncRNAs)—LINC00461. miR-15a is a regulator of the BCL-2 expression, a well-known apoptosis suppressor, whereas LINC00461 has a down-regulating ability on miR-15a by direct binding[123]. Moreover, the EVs with an augmented content of miR-21 and miR-146a promote the proliferation and transformation of BMSC into cancer-associated fibroblasts (CAFs) by inducing the expression of IL-6, SDF-1, fibroblast-activated protein (FAP), and α -smooth muscle actin (α -SMA) gene[124]. BMSCs also release miR-10a inside their EVs that induce E3 ubiquitin-protein ligase (β -TRC) within the MM cells. β -TRC is necessary for activating the cell cycle-regulating proteins, such as cyclins and transcription regulators, thus promoting proliferation[125]. Interestingly, MM can also stimulate its proliferation and survival in an auto-stimulatory way via the internalization of the CD147-positive EVs, associated with increased angiogenesis, tumor growth, and matrix metalloproteinase production, leading to extracellular matrix degradation[126].

The **immune system** plays a pivotal role in the MM bone marrow microenvironment, acting as a critical regulator that oscillates between dormancy and development of MM in a process known as immunoediting[127]. Moreover, MM PCs induce a permissive microenvironment, allowing immune evasion and facilitating its own proliferation, survival, migration, and drug resistance. Patients with MM experience profound immunosuppression, characterized by significant alterations in the composition of immune cells and cytokine profiles. These modifications arise from the complex interplay among the various elements of the bone marrow microenvironment, highlighting the relevant roles of immune components in the impaired anti-MM immune responses and disease development[127-129].

As discussed, BMSCs can produce high numbers of pro-inflammatory cytokines and growth factors such as IL6, IL10, and TGF- β (Transforming growth factor beta) that promote the differentiation of naïve T cells to **T helper (Th) 17 cells**, an immunosuppressive CD4⁺ T cell subset which, as positive feedback, secreted different cytokines enduring MM PCs expansion and growth[130,131]. **Regulatory T cells (Tregs)**, known for modulating immune responses against tumor cells, play a significant role in the immune suppression and evasion mechanisms of MM

PCs[132]. It was observed that MM patients have a higher Treg percentage in comparison to healthy donors, which suggests that myeloma cells escape from immune surveillance at least partially through the increase of this population[133,134]. Regarding **cytotoxic T cells**, there is a depletion in the CD8+T cell subset, which is more abundant in the premalignant conditions MGUS and SMM, compared to symptomatic MM[135]. The activity of cytotoxic T is also modulated by the TGF- β that inhibits IL-2-dependent proliferation and maturation of T cells and prevents naïve T cells from acquiring effector functions, which are further diminished by the immunosuppressive actions of adenosine [136,137]. The circulating T cell population is characterized by an exhausted phenotype with decreased expression of immune checkpoint receptors, including programmed death-1 (PD-1) and cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), and has limited capacity for self-renewal and activation[138]. Increased levels of PD-1 in T CD4+ have been observed in higher levels in rMM compared to MGUS and even ndMM and interacts with PD-ligand 1 (PD-L1) in MM PCs and dendritic cells (DCs) being correlated with disease progression[139]. Recently, it was observed that MM-EVs promoted apoptosis and inhibited the viability of CD4+ T cells derived from both healthy controls and MM patients. Similarly, MM EVs promoted proliferation, inhibited apoptosis, and decreased perforin expression in CD8+ T cells from healthy controls. Despite the increase in the quantity of CD8+ T cells, their function was impaired, further confirming the inhibitory effect of MM EVs on CD8+ T cells. Additionally, the viability of Treg cells from healthy controls increased, indicating that MM EVs alter the T-cell phenotype towards a more suppressive profile [140].

Natural killer (NK) cells from MM patients exhibit a diminished expression of activating receptors alongside a concurrent upregulation of PD-1 receptors, contributing to the suppression of NK cell cytotoxicity by the MM cells that express elevated levels of PD-L1, thereby facilitating an immunosuppressive environment[141]. MM-EVs can stimulate the NK cell response. Two studies showed that treatment of MM cells with low doses of genotoxic drugs such as melphalan induced a higher secretion of MM-EVs, triggering the NK activation, proliferation, and release of interferon γ (IFN γ) via binding of HSP (Heat shock protein)-70 to TLR2 (Toll-like receptor 2) and release of IL-15R α [142,143].

Plasmacytoid dendritic cells (pDCs) from MM patients have impaired T-cell stimulation capacities with subsequent dysregulated cytokine signaling and delayed or ineffective immune activity[127,128]. Although T cell numbers increase, a shift towards memory T cells and a reduction in the naïve T cell population is observed due to thymic degeneration[144,145]. In addition, pDCs excrete high levels of IL6 and IFN- γ , as well as upregulate kynurenine-3-mono-oxygenase, shifting the balance between tryptophan and kynurenine metabolites, abolishing the immune anti-tumoral response in MM[146,147]. MM-EVs also serve as anti-tumor immune response inducers and boosters. As tumor antigen transporters, namely mucin 1 and survivin, they can activate the CD8+ T-cell response mediated by DCs and PBMCs [148].

B cell lineage is significantly disrupted in MM patients, notably seen by the decrease in CD19+ B cells, a decline that inversely correlates with the progression of the disease, and by the diminished capacity for secreting polyclonal immunoglobulins and for differentiating into antibody-secreting plasma cells, ending with a deficient antigen presentation [149,150]. Additionally, **B regulatory (Bregs)** cells increase in the transition from MGUS to

symptomatic MM, supporting an immunosuppressive BM milieu by reducing NK-mediated lysis of MM cells, producing interleukin-10 (IL-10) and TGF- β , and promoting the transition of naive Th cells into Tregs[151-153]. This will enhance the activity of tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells, directly promoting tumorigenesis and angiogenesis[154,155].

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells that can suppress the activation, proliferation, and cytotoxic capacity of effector T and NK cells[127,128]. MDSCs have been hypothesized to contribute to myelomagenesis by exerting an antiproliferative effect against lymphocytes via increased nitric oxide (NO) production, L-arginine depletion, and IL-10 secretion, and second, by differentiating into osteoclasts in the context of RANKL-rich milieu[156,157]. Moreover, it has also been shown that MDSCs from MM patients can induce higher Treg differentiation than those from healthy age-matched donors. In turn, MM PCs promote the survival of MDSCs through Mcl-1 upregulation, secretion of IL-6, and accumulation of high levels of ADO[158-160]. EVs can play an essential role in this immune modulation by exerting a direct effect on the MDSCs. It was demonstrated that MM- and MM BMSC-derived EVs induced MDSCs expansion and activation through pStat1 and 3 signaling in vitro and in vivo. Treating healthy mice with MM EVs increased the prevalence and activity of MDSCs, leading to inhibition of T-cell proliferation[161]. Moreover, EVs isolated from the BM plasma of patients with MM contained increased levels of the ectoenzymes CD39 and CD73, resulting in increased levels of adenosine, which can activate MDSC and inhibit T-cell function[162].

Tumor-associated macrophages (TAMs) are an abundant element within the MM microenvironment, promoting the survival of MM cells and their resistance to treatment through various mechanisms[127,128]. In the MM BM microenvironment, TAMs acquire a secretory profile, being a significant source of IL-6, IL-10, TNF- α , and IL-1 β , and proangiogenic factors, such as VEGF, MMPs, and cyclooxygenase-2, providing an optimal milieu for myeloma cell growth[163,164]. Furthermore, TAMs mirror characteristics of an M2-like macrophage subset, exhibiting minimal cytotoxicity against tumor cells due to their reduced production of NO, low pro-inflammatory cytokines, and diminished capacity for antigen presentation[165]. It has been shown that M2 macrophages significantly increase in the BM of MM patients compared with MGUS and SMM, suggesting that MMPCs recruit and polarize TAMs to the M2 phenotype[166,167]. EVs cargo also plays a vital role in TAM modulation. EVs from MM cells, specifically with del13, induce M2 polarization of peripheral blood monocytes via the NF- κ B pathway. These EVs contained reduced levels of miR-16, which was found to inhibit inhibitory Kappa B Kinase α/β (IKK α/β) and increase the levels of IL-10, IL-8, and TNF- α [168]. Under hypoxia, MM cells secrete amounts of EVs that contain miR-1305, which, when transferred to THP1 monocytes, induces M2 polarization[169]. In addition, MM PCs can stimulate the polarization of PBMC-derived macrophages into M2 macrophages by transferring miR-let-7c[170].

1.5. Natural history of MM, evolution dynamics, and clonal architecture

1.5.1. Natural history of MM

The trajectory towards MM can be understood as a development pathway within a clinical spectrum that extends from an initial pre-malignant stage to a highly aggressive extramedullary presentation of MM.

Monoclonal gammopathy of undetermined significance (MGUS) is an asymptomatic plasma cell disorder that precedes the onset of MM and other lymphoproliferative disorders. The prevalence of MGUS is 5% among individuals over 70 years of age and is considered to precede nearly all cases of symptomatic MM[171,172]. Nonetheless, only 10% of patients with symptomatic MM were previously diagnosed with MGUS, as the course of MGUS is primarily asymptomatic[173]. The annual risk of progression from MGUS to symptomatic MM is 1%, being higher for patients with non-IgM MGUS, abnormal serum free light chain (FLC) ratio, and monoclonal protein concentrations exceeding 15 g/L, reaching 2%[174,175].

Smoldering Multiple Myeloma (SMM) is an intermediate stage in the evolution of MGUS to symptomatic MM, characterized by a higher disease burden than MGUS but without the myeloma-defining events. SMM represents a biological heterogeneous group with a highly variable risk of progression to symptomatic MM[176-178]. The annual risk of progression from SMM to MM is 10% per year for the first 5 years, 5% per year during the subsequent 5 years, and 1% per year after 10 years[179]. Considering the SMM risk score, the low-risk one appears to behave clinically as early MGUS-like cases with a slow progression subtype and a low mutation burden, requiring more time for additional genetic events for progression. In contrast, the high-risk group behaves as MM-like cases rapidly progressing and carrying genetic abnormalities typically found in MM[180]. The analysis of paired samples at the SMM stage and after progression to MM showed that high-risk SMM shares aggressive genetic features with MM, such as MAPK/DNA repair pathway alterations, MYC aberration, del 1p, and APOBEC activity[178]. This suggests that high-risk SMM only needs time to evolve and fulfill the clinical diagnostic criteria for symptomatic MM.

The diagnosis of MM is based on the presence of a monoclonal component, the evidence of one or more myeloma-defining events, and having either 10% or more clonal plasma cells on bone marrow examination or a biopsy-proven plasmacytoma. These events consist of established CRAB (hypercalcemia, renal failure, anemia, or lytic bone lesions) features as well as three specific biomarkers: clonal BM PCs $\geq 60\%$, sFLC ratio ≥ 100 (provided involved FLC level is ≥ 100 mg/L), and more than one focal lesion on magnetic resonance imaging (MRI) [181]. These new biomarkers, capable of accurately identifying patients with SMM at high risk of developing symptomatic and imminent end-organ damage, triggered the International Myeloma Working Group (IMWG) criteria revision. In more than two independent studies, each new biomarker is associated with an approximately 80% risk of progression to symptomatic end-organ damage[182-185]. The revised criteria also facilitated the use of advanced imaging technologies to diagnose bone disease and helped refine various other diagnostic requirements[186]. They were also supported by the improvements in MM therapy and data suggesting that early intervention in the

monoclonal gammopathies spectrum may improve overall survival[181]. These criteria represented a paradigm shift in the MM approach since they allow early diagnosis and therapy initiation before end-organ damage.

Acquisition of the ability of MM PCs to thrive and grow independently of the bone marrow microenvironment determines further evolutionary stages of MM, resulting in a high-risk disease associated with increased proliferation, evasion of apoptosis, and treatment resistance. This progression includes extramedullary disease (EMD), which may affect the central nervous system, soft tissues, lymph nodes, internal organs, and body cavities, as well as plasma cell leukemia (PCL), distinguished by the presence of plasma cells in the peripheral blood and usually a terminal event in the evolutionary landscape of MM[187,188] (Figure 2).

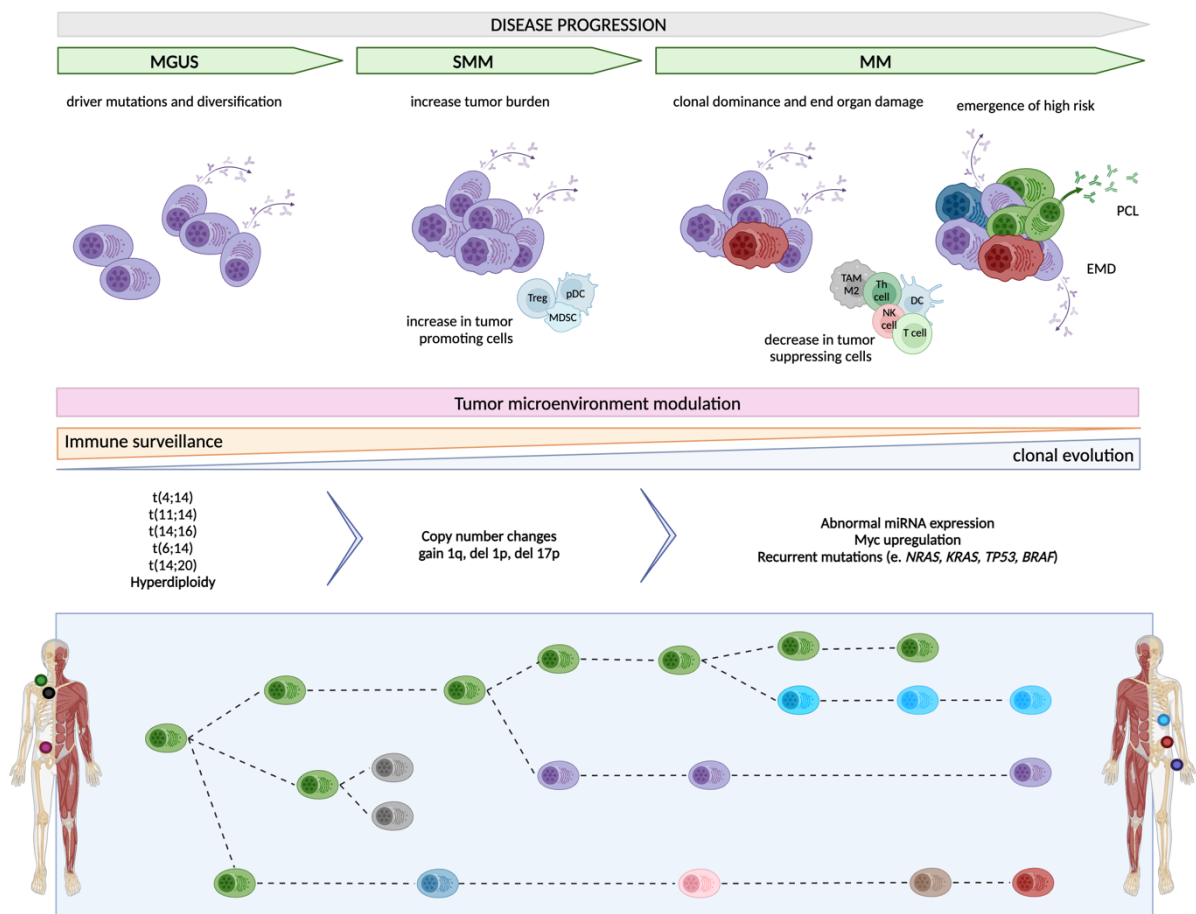


Figure 2. Natural history and evolution dynamics of Multiple Myeloma. MM develops through a complex, multi-step process within the bone marrow, beginning with precursor states such as MGUS and SMM. The progression starts with MGUS, characterized by driver mutations and cellular diversification, although it remains asymptomatic. As it advances to SMM, there is an increase in tumor burden and the number of tumor-promoting cells while remaining asymptomatic. In the final stage of this spectrum, MM, the clonal dominance leads to significant end-organ damage and diverse clinical manifestations, ending with the emergence of high-risk conditions such as PCL and EMD. Throughout this progression, tumor microenvironment modulation and its components are crucial, mainly focusing on immune evasion. Concurrently, several genetic abnormalities emerge. Early genetic events include translocations like t(4;14), t(11;14), and t(14;16), along with hyperdiploidy, which set the stage for subsequent disease evolution. As the disease progresses, clonal evolution becomes more pronounced, driven by copy number changes such as gain 1q, del1p, and del17p. These genetic alterations are accompanied by abnormal microRNA expression, MYC upregulation, and recurrent mutations in genes such as NRAS, KRAS, TP53, and BRAF. As a result, different clones emerge and evolve, promoting temporal and spatial heterogeneity, which contributes to disease progression and impacts clinical manifestations. "Created with BioRender.com".

1.5.2. Evolution dynamics and clonal architecture

Time-course analyses of MM PCs have uncovered a complex genomic architecture of the disease as simultaneously, laboratory and clinical features emerge in a pattern of multiple remissions and relapses[189]. In MM, different clonal changes were demonstrated and categorized as stable, linear, and branching evolution of myeloma clones. In clonally stable MM, the mutational spectrum remains relatively constant throughout the disease course. In contrast, linear evolution is characterized by gains of additional genetic aberrations on top of existing mutations, and branching evolution is marked by the emergence of clones with new, and sometimes entirely distinct, sets of genetic aberrations over time[180,190,191] (Figure 2).

On average, between 3 and 6 subclones are consistently observed at each stage of the disease, with ancestral clones present as early as the MGUS stage[192,193]. For example, in some patients, the driver mutation was detected only in a minor subclone at diagnosis and becomes dominant later or at relapse[193,194]. Also, the type of driver mutation may affect the clonal evolution of MM. MM cells carrying high-risk chromosome abnormalities, such as t(4;14), acquire more copy number abnormalities than those with standard-risk abnormalities, such as t(11;14), during disease progression[195,196]. Clonal diversity arises in the very early phases of the disease, whereas clonal competition may affect the rate and characteristics of disease progression[189].

It was observed that relapsed clones carried only some genes from the set of mutations observed at diagnosis, with or without additional genetic abnormalities. Clones harboring novel mutations, especially in tumor suppressor genes, often emerge during the relapse or at the end of the disease, particularly during the plasma cell leukemic phase[180,197]. This is a common pattern of clonal evolution in MM. Nonetheless, current research supports that the progression of MM appears to be principally driven via branching patterns, reminiscent of the theory of natural selection proposed by Charles Darwin, rather than a linear multistep process[189,191,192]. The branching evolution is a result of the fitness of different clones to the bone marrow microenvironment as well as their evasion of immune surveillance. For example, a clone expressing the receptor for IL-6 could survive and expand in response to IL-6 produced by BMSCs, or a PD-L1-expressing MM clone could escape the attack of immune cells, such as cytotoxic T-lymphocytes and NK-cells and survive in the immune microenvironment[190,191,198].

Moreover, drug treatments frequently serve as selective forces altering the clonal architecture of MM, enabling drug-resistant or high-risk clones, which may be present at undetectable levels at diagnosis, to outgrow other clones, leading to disease progression and relapse[199]. A study in which whole exome sequencing (WES) was performed on diagnosis/relapse samples from a subset of MM patients receiving or not lenalidomide maintenance from the large myeloma treatment study (Myeloma XI trial), observed a change in the mutational spectrum and an increase in the mutation load in most relapse samples, whereas only a subset of patients showed a stable mutational landscape. Patients achieving a good response to initial therapy seemed to go through a clonal bottleneck, leading to a branched clonal evolution upon relapse, whereas most patients with incomplete responses showed a linear evolution or stable clonal patterns. Likewise, lenalidomide maintenance did not increase the number of new genetic aberrations or the overall mutational load compared to the non-maintenance treated

patients[200]. This demonstrates the relation between the depth of response and the pattern of clonal evolution in MM patients, with dormant new clones contributing to relapse in high-risk patients who initially responded well to therapy[199].

In addition to the intraclonal diversity and the temporal evolution of the clonal architecture, marked by the extinction of older clones and the rise of new or previously minor clones that withstand therapeutic intervention, spatial variability is critical to understanding the MM heterogeneity[190,191]. Local differences in clonal compositions were observed by simultaneous analyses of biopsy specimens from the iliac crest, and positron emission tomography-computed tomography (PET-CT) visualized several focal lesions. Spatial genomic heterogeneity was observed in more than 75% of patients, including the inactivation of the tumor suppressor genes CDKN2C and TP53 and mutations affecting MAP kinase family members. This spatial heterogeneity was correlated not only with the size of focal lesions, suggesting the regional outgrowth of advanced clones with higher growth potential but also with a distinct behavior of local diseases, supporting the hypothesis of an independent course of clonal evolution in different locations of the disease[201] (Figure 2).

The progression of MM over time is characterized by phenotypic transitions that rely upon acquired lesions to modify the biology of tumor cells, enabling them to surpass various critical behavioral boundaries. This defines the clinical stepwise progression of MM characterized by multiple phases of remission and relapse. Additionally, this heterogeneity evolves and changes focally. This complexity underpins the dynamic nature of MM progression, underscoring the need for a better understanding of the disease biology to target its evolution and manage it effectively.

1.6. Clinical spectrum and prognosis

1.6.1. Clinical spectrum

MM clinical spectrum is highly heterogeneous, with most patients exhibiting signs or symptoms associated with infiltrating plasma cells into the bone or other organs or experiencing manifestations driven by the high levels of monoclonal protein production. The acronym CRAB encapsulates the main clinical manifestations of MM, representing hypercalcemia, renal impairment, anemia, and bone disease. Sometimes, patients have no clinical symptoms at the diagnosis, and MM is discovered incidentally by detecting a spike in serum protein electrophoresis while assessing another disease. In contrast, there are instances where the disease manifests with exuberant and diverse clinical symptoms[1,3,4,202].

Anemia, which is present in about 70% of patients at diagnosis, is usually related to medullary plasmacytosis, suppression of erythropoiesis induced by cytokines such as IL-6, hemodilution due to hyperproteinemia, and decreased secretion of erythropoietin in cases of renal failure. Also, the expression of Fas-ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors on the surface of MMPCs plays a role in the apoptosis of erythroid progenitors. **Bone involvement** is present in almost 80% of patients newly diagnosed. It is mainly

attributed to plasma cell infiltration, which leads to the activation of osteoclasts and inhibition of osteoblasts, particularly through the secretion of DKK-1 (Dickkopf-related protein 1). Bone pain is present in 70% of patients at diagnosis, primarily targeting the axial skeleton, which often necessitates the use of analgesics and significantly hampers the functional abilities of MM patients. **Renal impairment** occurs in up to 50% of patients with newly diagnosed MM, requiring hemodialysis in 5 to 10% of the cases. The most common cause is myeloma cast nephropathy (about 75% of renal impairments), characterized by the precipitation of casts formed by light chains of immunoglobulins and Tamm-Horsfall proteins in the distal segment of the nephron. Pre-renal involvement is also common and often related to dehydration, hypercalcemia, and the use of nephrotoxic medications. Glomerular damage is rarer and occurs as a secondary complication of myeloma, such as the development of AL Amyloidosis or cryoglobulinemia. **Hypercalcemia** is found in less than 20% of cases at diagnosis, with several mechanisms possibly involved. The osteoclastic activation by MMPCs causes bone lysis and, subsequently, high calcium levels. To a lesser extent, a decrease in the glomerular filtration rate and an increase in the tubular reabsorption of calcium also contribute to the development of hypercalcemia in these patients. At diagnosis, approximately 7% of MM patients exhibit **extramedullary disease**. Less frequent manifestations in fewer than 5% of cases include paresthesias, hyperviscosity syndrome, thrombosis, hepatomegaly, splenomegaly, lymphadenopathy, and fever[1,3,4,202].

1.6.2. Prognosis and risk stratification

The prognosis of patients with MM has changed over the last twenty years, although it has been highly heterogeneous, as is the biology of MM itself. With modern therapy, the median overall survival exceeds eight years and ranges from a few months in patients with ultra-high-risk features to more than 15 years in patients with lower-risk characteristics[203,204]. Four essential prognostic pillars determine the outlook for MM patients. First, staging considers specific disease features and plays a significant role in estimating the overall outcome. Secondly, patient factors, including age and overall health condition, impact outcomes. Thirdly, the biology of MM itself, encompassing characteristics such as plasmablastic morphology and the presence of extramedullary disease, is critical to determining the natural evolution of the disease. Lastly, the availability and response to therapy influence the sequencing treatment plan and the overall prognosis.

The Revised International Staging System (R-ISS) is the most used staging system in MM, having supplanted previous models such as the Durie-Salmon staging system, the original International Staging System (ISS), and the proposed one by the IMWG in 2014[205-207]. In the ISS, serum albumin and β 2-microglobulin levels were used as the main factors, giving insights into tumor burden, rate of cellular turnover, and presence of renal impairment, providing a more comprehensive understanding of the impact of the MM[206]. Disease biology appears to be best reflected based on the molecular subtype of MM and the presence or absence of secondary cytogenetic abnormalities[208]. In 2014, the IMWG consensus statement proposed well-defined and easily applicable risk categories using the ISS and high-risk cytogenetics such as t(4;14), del17p13, and amp1q21 by interphase fluorescence in situ hybridization (FISH) to serve as a framework to rationalize the use of therapies[205]. This was the foundation for the R-ISS, which added to the ISS the serum lactate dehydrogenase (LDH) and the high-risk

cytogenetics del(17p), t(4;14), or t(14;16) by FISH[209]. The R-ISS demonstrated improved ability in identifying patients with MM with high-risk profiles and, in subgroup analysis, established its prognostic significance independently of the age and treatments received[210,211]. Despite being validated across various clinical trials, underscoring its robustness and applicability in diverse clinical settings, choosing specific treatments in real-life clinical practice based on R-ISS stages lacks standardized guidelines, indicating the need to refine these staging systems to help in therapeutic decision-making, reflecting the diversity of MM[212].

In 2022, a second revision of the ISS system was implemented and named the R2-ISS system[213]. The R2-ISS system, which encompasses the ISS stage, lactate dehydrogenase, and the 1q gain/amplification (1q+) beyond the del(17p) and t(4;14) already used, assigns a weighted score to each parameter and ultimately establishes a well-balanced 4-stage classification system. Compared with the R-ISS, the R2-ISS showed an improved discriminating capability, especially in the large group of patients with intermediate-risk newly diagnosed multiple myeloma[213]. Furthermore, R2-ISS offers a dynamic, additive scoring system that can be readily updated to incorporate new prognostic factors as they emerge in MM. Interestingly, several factors, such as microRNAs, circulating plasma cells, TP53 mutations, 1p32 deletion, lambda light-chain translocations, extramedullary disease, and Myc deregulation, have been independently linked to poor outcomes and could potentially be integrated into baseline risk stratification strategies[47,61,214,215]. Also, data from imaging studies, next-generation sequencing (NGS), gene-expression profiles, and inputs from using novel biomarkers as liquid biopsies may have prognostic significance when included in clinical staging system scores. Regarding treatment response as a predictive tool, achieving a negative measurable residual disease (MRD), evaluated with high sensitivity, has been shown to mitigate the adverse outcomes associated with initial prognostic risk factors[216]. Combining R2-ISS scores with MRD status would be an opportunity to develop strategies to tailor risk and MRD results, potentially benefiting many patients.

Despite several studies that have validated the R2-ISS system in different clinical settings, there is currently a lack of validation cohorts specifically focusing on transplantation and MM[217]. Although some validation cohorts include non-transplant-eligible patients, they are underrepresented in the R2-ISS[213]. Moreover, validating this system in real life and patients with new treatment combinations, such as triplets and quadruplets, including monoclonal antibodies and T-cell redirecting therapies, and an extended follow-up will help to validate it as a more robust predictive model affecting overall survival[218,219]. In addition, the value of the R2-ISS score within a population of patients with rrMM should also be explored to verify whether this score can effectively stratify patients in trials that enroll participants following initial treatment[213].

2. CURRENT LANDSCAPE OF MULTIPLE MYELOMA TREATMENT

2.1. Options for the treatment of MM

The treatment landscape for MM has undergone a substantial transformation over the last two decades with the approval of innovative treatments and their combinations, both for newly diagnosed patients and those in the relapsed/refractory setting. This was possible due to the growing understanding of the MM pathophysiology and role of the immune and microenvironment with the emergence of different classes of drugs and the possibility of combining them to overcome the MM complexity and achieve the deepest possible response in all phases of the disease (Figure 3).

Historically, for an extended period of time, the cornerstone of MM treatment was cytotoxic agents such as the alkylating agents melphalan or cyclophosphamide and corticosteroids such as prednisolone or dexamethasone[220]. Subsequently, the advent of high-dose therapy featuring melphalan followed by autologous stem cell transplantation (ASCT) marked a significant improvement in the care of these patients, offering enhanced overall survival rates, mainly for younger patients relative to those receiving standard chemotherapy[221].

Over the first decade of this century, new drugs such as thalidomide, bortezomib, and lenalidomide were included in the treatment of MM, representing a groundbreaking shift in the treatment paradigm for MM both in transplant and non-transplant-eligible patients[222,223]. The upfront use and optimization of combinations of these drugs have led for the first time to an enhancement in the overall survival rates of all patients, with the median survival rate being 2.5 years before 1997, which increased to more than five years in the subsequent decade[224,225]. However, resistance to these agents has been observed, and MM patients who became refractory to both first-generation immunomodulators (IMiDs) and proteasome inhibitors (PIs) have significantly worse outcomes[226]. Furthermore, next-generation IMiDs such as pomalidomide and PIs such as ixazomib and carfilzomib were developed and rapidly introduced in the therapy of relapsed and refractory patients[227,228]. There has also been a shift toward a risk-adapted approach to induction and maintenance regimens to achieve better response rates and longer remission plateau phases[229].

Another landmark in the MM approach was the incorporation of monoclonal antibodies (moAbs), such as the anti-CD38 moAbs, into the induction and relapse/refractory regimens, which has led to more prolonged progression-free survival, above five years in some regimens, and higher rates of measurable residual disease negativity[230-233]. More recently, in the relapsed setting, the emergence of B-cell maturation antigen and other targets directed therapy, including antibody-drug conjugates (ADC), bi-specific monoclonal antibodies (Bi-MoAbs), T-cell engagers (TCE), and chimeric antigen receptor T-cell therapy (CAR-T), has produced profound and durable responses in heavily pretreated patients, changing the landscape and treatment paradigms in the MM treatment[231,234-236].

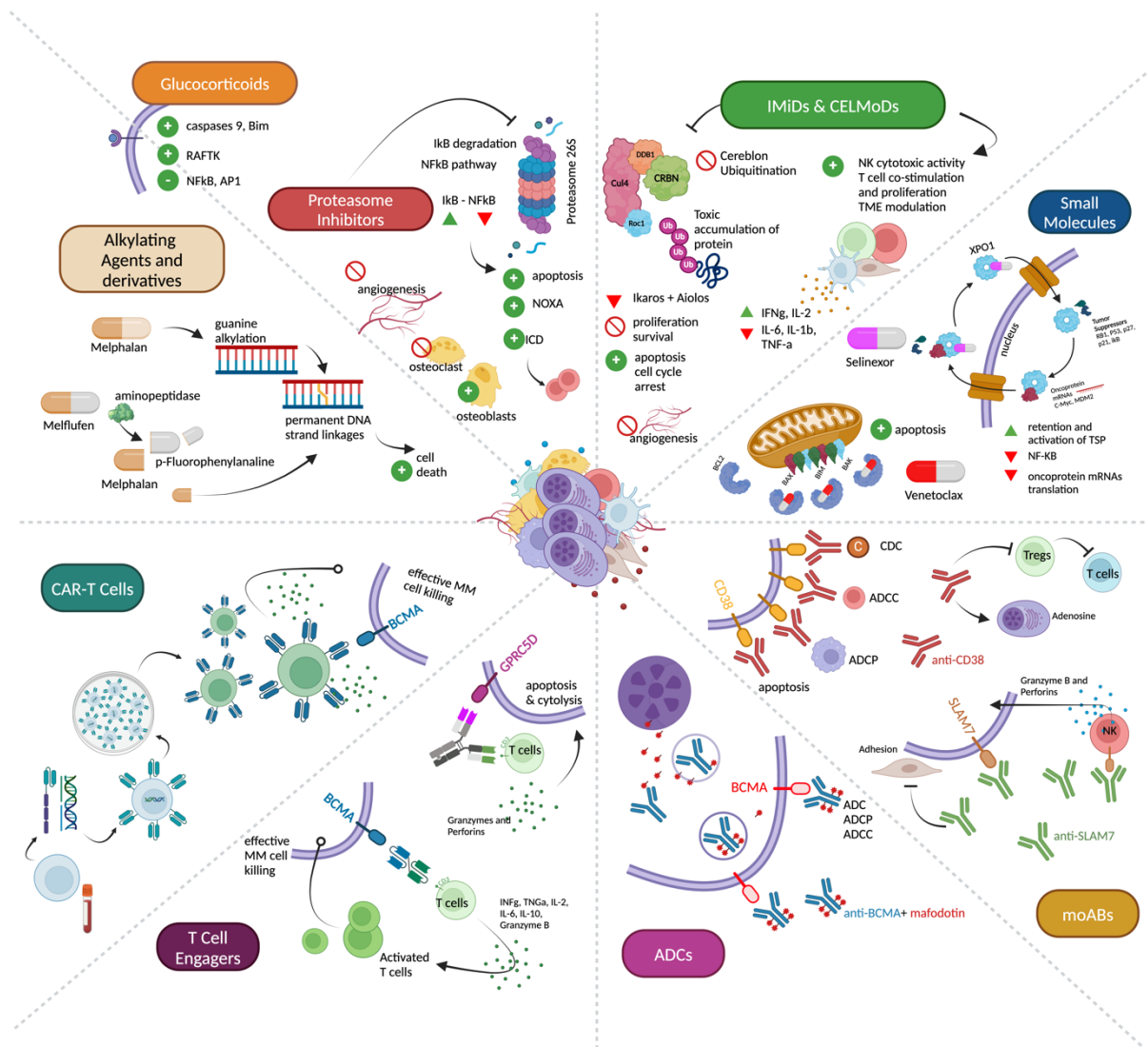


Figure 3. Therapeutic options to counteract MM. Current treatments used for MM include a wide range of options such as glucocorticoids, alkylating agents, proteasome inhibitors (PIs), immunomodulatory drugs (IMiDs), and cereblon E3 ligase modulators (CELMoDs), small molecules with innovative mechanisms, monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), T cell engagers and CAR-T cells. Classical alkylating agents such as melphalan target highly proliferating cells, including malignant PCs, by permanently intercalating their DNA, causing cell death. PIs such as bortezomib, carfilzomib, and ixazomib block the IκB or pro-apoptotic proteins degradation in MM PCs proteasome, overcoming their resistance to apoptotic stimuli. IMiDs as thalidomide, lenalidomide, and pomalidomide, and CELMoDs as iberdomide, avadomide, and mezigdomide target the cereblon protein of the E3 ubiquitin ligase complex blocking the ubiquitination process in malignant PC leads to a toxic accumulation of proteins and cell death, as well modulate the inflammatory environment of the BM inhibiting the progression of MM. Exportin 1 (XPO1) inhibitors such as selenixor act on malignant PCs by blocking the export of tumor suppressor proteins from the nucleus through the XPO1 pump while retaining many oncoprotein mRNAs within the nucleus. Venetoclax targets BCL-2, promoting apoptosis in MM PCs. mAbs, such as the anti-CD38 daratumumab and isatuximab, bind to specific antigens on the surface of malignant PCs. This will, in turn, induce MM plasma cell death by antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or antibody-dependent cellular phagocytosis (ADCP). Anti-SLAM mAbs additionally engage NK cells to target and kill multiple myeloma cells. ADCs, such as belantamab mafodotin, target specific antigens on MM cells, such as BCMA, delivering cytotoxic drugs directly to the cancer cells and inducing apoptosis through various immune mechanisms (ADCP, ADCC). T cell engagers like teclistamab, elranatamab, and talquetamab connect T cells via CD3 and with MM PCs via surface antigens like BCMA and GPRC5D, facilitating T cell-mediated killing of the tumor cells. Finally, CAR-T cells use engineered T cells to target and kill MM PCs specifically by expressing specific targets such as BCMA, resulting in effective tumor cell eradication. "Created with BioRender.com".

At the same time, new small molecules with different mechanisms of action have been developed, diversifying the arsenal of treatments available. These include selinexor (an inhibitor of the nuclear export compound exportin-1), melflufen (derived from the classical melphalan), and venetoclax (a BH3-mimetic antagonist of the BCL-2 anti-apoptotic protein), as well as a novel class of immunomodulatory drugs known as Cereblon E3 Ligase MoDulators (CELMoDs)[237-240].

2.1.1. Proteasome inhibitors

Proteasome inhibitors (PIs) represented a significant breakthrough in the treatment of MM by disrupting the natural protein degradation by the proteasome and leading to the apoptosis of malignant cells. PIs exert several effects in the MM cells, including the inhibition of NF- κ B signaling, the accumulation of misfolded and unfolded proteins, resulting in ER stress and leading to the unfolded protein response, the downregulation of growth factor receptors, suppression of adhesion molecule expression, and inhibition of angiogenesis[241-243]. To date, three PIs are approved and are used in clinical practice, namely bortezomib, carfilzomib, and ixazomib, and several others are under development, such as marizomib, oprozomib, and delanzomib[242].

Bortezomib was the first PI approved for the treatment of ndMM patients, as well as those with rrMM, and it continues to be one of the most essential drugs used in MM[244]. Bortezomib targets the β 5 and β 1 proteasome subunit, blocking the degradation of inhibitor kappa B (I κ B), the inhibitor of nuclear factor B (NF κ B), suppresses the NF κ B signaling pathway, preventing the activation of numerous anti-apoptotic genes involved in MM progression[244,245]. It also leads to the upregulation of NOXA, a pro-apoptotic member of the BCL-2 protein family, and inhibits osteoclasts and stimulates osteoblasts, thereby increasing bone formation[244,246]. Moreover, bortezomib was shown to induce immunogenetic cell death (ICD) by several mechanisms, including by down-regulation of the cell-surface expression of HLA class I and by enhancing NK cell-mediated lysis of myeloma[247]. Also, bortezomib induces ICD by activating the calreticulin pathway, in which calreticulin is released from the ER lumen to the plasma membrane surface, initiating the phagocytosis of MM cells by dendritic cells through the “eat-me.” In addition to calreticulin, STING (stimulator of interferon genes) pathway activation by bortezomib can enhance cancer antigen presentation, contributing to the priming and activation of T cells and facilitating the trafficking and infiltration of T cells into the tumor to eliminate MM cells[248]. Carfilzomib is a second-generation PI approved for the treatment of patients with rrMM. It binds irreversibly to the proteasome, targeting the β 1 proteasome subunit, leading to potent inhibition of proliferation and induction of apoptosis even in bortezomib-resistant MM cell lines and patient samples[249]. Ixazomib, targetting the β 5 and β 1 proteasome subunit, is the first oral PI approved for treating rrMM. As with the other PIs, ixazomib promotes caspase-dependent induction of apoptosis and inhibition of the cell cycle, inhibits the NF- κ B pathway in MM cells, and inhibits tumor-associated angiogenic activity[250].

2.1.2. Immunomodulators and Cereblon E3 Ligase Modulators

Immunomodulator drugs (IMiDs) such as thalidomide, lenalidomide, and pomalidomide are backbone drugs in several MM regimens, enhancing outcomes both in ndMM and rrMM. IMiDs bind to a primary protein target termed cereblon (CRBN), which belongs to the CRL4 E3 ubiquitin ligase (CRL4CRBN) complex. Therefore, inhibition of the ubiquitination by IMiDs leads to the toxic accumulation of proteins and MM cell death[251,252]. Also, cereblon has been identified to be associated with other downstream targets, participating in the binding, ubiquitination, and degradation of Ikaros (IKZF1) and Aiolos (IKZF3), two essential transcription factors that regulate the expression of other genes, such as IRF4 and MYC, and is essential for the proliferation and survival of MM cells[253-255]. Accordingly, MM cells lacking cereblon become highly resistant to IMiDs[256]. Moreover, IMiDs have several effects on the immune system by modulating its components, enhancing immune surveillance, changing the inflammatory BM microenvironment, and improving the anti-myeloma immune response[257].

Thalidomide disturbs the interactions between MM cells and the BM microenvironment by inhibiting the expression of IL-6, interleukin 1 β (IL-1 β), basic fibroblast growth factor (bFGF), and vascular VEGF, all of which are essential for MM cell growth[258,259]. Thalidomide modulates T-cell activity, which leads to a reduction in the levels of the tumor necrosis factor- α (TNF- α), another cytokine important for MM cell growth and survival. Also, these IMiDs impact other immune cells and inhibit de novo IgM antibody synthesis[259]. Furthermore, thalidomide enhances T-cell proliferation by secreting interferon-gamma (IFN- γ) and IL-2, vital for immune response modulation[258]. Lenalidomide and pomalidomide are more potent and effective than thalidomide in modulating the immune system, inhibiting the production of pro-inflammatory cytokines and the adhesion of MM to BMSCs while co-stimulating T-cell proliferation and enhancing the NK-cell activity against MM cells, expansion, and antibody-dependent cellular cytotoxicity (ADCC). Additionally, they are more potent angiogenesis inhibitors [259,260]. To produce its effects, cereblon must be present in the MM cells[256,261,262].

The combination of IMiDs with moAbs is particularly important. It was observed that loss of Ikaros or Aiolos induced by IMiDs resulted in an upregulation of CD38 surface expression on MM cells, priming for daratumumab-induced NK cell-mediated antibody-dependent cellular cytotoxicity[263]. By contrast, it was seen that dexamethasone, essential in almost all anti-MM cells due to its effective inhibition of cell growth and synergistic to IMiDs, appears to reduce induced immunomodulation of T and NK cell function. In this context, early discontinuation of dexamethasone or use of low-dose dexamethasone should be used[264].

After the CRBN was identified and explored, new immunomodulator drugs emerged called CELMoDs. These drugs have some chemical structures different from the classical IMiDs that determine the interaction with CRBN and new CRL4CRBN E3 substrates.[252] CELMoDs, compared to IMiDs, present a higher affinity for CRBN and cause a stronger degradation of Ikaros and Aiolos. They are associated with a more potent anti-MM and immunomodulatory effect, explaining their more significant activity at lower levels of CRBN or in cases of resistance to IMiDs due to mutations in CRBN[238,265]. CELMoDs bind specifically to CRBN, influencing the activity of E3 ubiquitin ligase and targeting specific substrate proteins, resulting in the degradation of some transcription factors

that are proteasome-mediated transcriptional repressors. Ultimately, this action results in immunomodulation, including the activation of T lymphocytes and the degradation of proteins essential for the proliferation of MM cells [252,266]. Ixerdomide, mezigdomide, and avadomide represent this new class of immunomodulator drugs being actively evaluated for treating rMM.

2.1.3. Monoclonal Antibodies

In addition to PIs and IMiDs during the last decade, naked monoclonal antibodies significantly changed the treatment landscape of MM[267]. Monoclonal antibodies (moAbs) bind to specific antigens on the surface of cells, inducing tumor cell death by antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis (ADCP). Most mAbs are associated with cell death mediated by Fc gamma receptor (FCyR) crosslinking of tumor-bound antibodies and target antigen enzymatic activity modulation. Daratumumab and isatuximab, both CD38-targeting antibodies and elotuzumab, a SLAMF7 (Signaling lymphocytic activation molecule 7)-targeting antibody, were the first moAbs introduced in the clinic to treat MM[267-269].

CD38 is highly expressed in normal and malignant PCs, as well as in specific subsets of immune cells. It is also expressed at lower levels in other tissues of non-hematopoietic origins, such as airway-striated muscle cells, prostatic epithelial cells, and pancreatic islet cells. CD38 functions as a receptor for CD31 and also has ectoenzymatic activities (NADase)[268]. CD38-targeting moAbs have direct on-tumor activities as described but also have immunomodulatory effects, including the elimination of CD38-positive regulatory T-cells, regulatory B-cells, and myeloid-derived suppressor cells, which is followed by an increase in T-cells with enhanced killing capacity[270,271]. Currently, daratumumab, a fully human IgG1-kappa antibody, and isatuximab, a humanized IgG1-kappa antibody, are approved for treating MM. Daratumumab induces superior CDC, while isatuximab has more potent direct anti-MM activity. Regarding ADCC, ADCP, and cell death in the presence of FC receptor crosslinking, both anti-CD38 moAbs are comparable[272,273]. Both daratumumab and isatuximab have single-agent activity with an overall response rate of 30% in heavily pretreated patients, including activity in patients with a median of 4 prior therapy lines[274,275]. Nonetheless, CD38-targeting moAbs were identified to be more potent when partnered with IMiDs in vitro, leading to several clinical studies that evaluated the combination of a CD38-targeting antibody in combination with the most used IMiDs, lenalidomide or pomalidomide, in rMM and ndMM with impressive results[263,276]. Likewise, the efficacy of CD38-targeting antibodies can be enhanced by the already-used PIs, bortezomib or carfilzomib[276,277]. It was observed that response to daratumumab is influenced by CD38 expression levels, with reduced CD38 levels conferring resistance[270]. To overcome this resistance, the addition of all-trans retinoic acid at the time of resistance to daratumumab resulted in a temporary increase in CD38 expression on the MM cell surface, but so far, this combination has had limited clinical activity[278]. Also of significant debate is the retreatment with a CD38-targeting antibody after developing CD38-targeting antibody-refractory disease, with switching of the partner drug. Some retrospective studies suggest efficacy, but prospective studies are needed to investigate whether a treatment-free interval is required to allow for NK cell recovery and restore CD38 expression[279,280].

Elotuzumab, a humanized IgG1-kappa monoclonal antibody, targets the SLAMF7, a glycoprotein highly expressed on the MM cell surface and subsets of normal immune cells (such as NK cells and T-cells). Elotuzumab targets MM cells via ADCC and, through the action of NK cells, reduces the MM cell binding to the bone marrow stroma and promotes tumor cell death [281,282]. Decreased NK cell numbers induced by CD38 antibodies may impair the efficacy of elotuzumab after daratumumab treatment[283]. Elotuzumab when combined with lenalidomide or pomalidomide and low-dose dexamethasone, elotuzumab demonstrated clinical efficiency, with superior outcomes compared to the IMiDs alone, supporting the use of this moAb in rrMM[284,285].

2.1.4. Antibody Drug Conjugates

Antibody-drug conjugates (ADCs) are highly selective humanized or human moAbs chemically linked to a potent cytotoxic compound (payload)[286-288]. The antibody is usually internalized via endocytosis and then processed by natural cellular processes, leading to cleavage of the linker and release of the payload by degrading the antibody in the lysosome and, ultimately, killing the malignant cell[287]. Although the efficacy of ADCs essentially depends on the optimal selection of target membrane protein and successfully delivering toxic payloads to target cells, additional anti-tumor mechanisms leveraged by the ADCs, common to moAbs, include ICD, ADCC, ADCP, and CDC[289].

The B-cell maturation antigen (BCMA), also known as CD269 or Tumor necrosis factor receptor superfamily member 17 (TNFRSF17), has become one of the most prominent targets among all potential options for ADCs, CAR-T cells, and bispecific T-cell engagers. BCMA is highly expressed on the surface of late B-lymphocytes and malignant plasma cells, with low-level expression on hematopoietic stem cells and no expression in non-hematopoietic cells, which makes it an attractive target. BCMA has two known ligands: BAFF (B-cell activating factor) and APRIL (A Proliferation-Inducing ligand). Activation of BCMA leads to activation of NF kappa B pathways, creating a prosurvival signal, resulting in the proliferation, differentiation, and more prolonged survival of plasma cells. Also, BCMA interacts with the bone marrow environment and osteoclasts[290].

The first-in-class ADC approved for MM patients is the Belantamab Mafodotin (BelaMaf). This is a BCMA-directed humanized afucosylated monoclonal IgG1 antibody conjugated to the microtubule inhibitory drug monomethyl auristatin F (MMAF), also called mafodotin. Once this ADC is internalized, in addition to inducing direct cytotoxic cell death after the release of MMAF, which inhibits microtubule polymerization and induces G2/M arrest and apoptosis, its Fc fragment is defucosylated and facilitates other effects typical for moAbs, such as ADCC, ADCP, and ICD[291,292]. BelaMaf showed important single-agent activity with an overall response reaching 30% and longer progression-free survival (PFS) in patients with good response in heavily pre-treated patients with rrMM. Even though the therapy is targeted, off-target effects of BelaMaf can occur, namely keratopathy. Combining BelaMaf with other anti-MM agents can enhance its efficacy, allowing less frequent dosing and mitigating toxicities.[293,294].

2.1.5. T-cell engagers antibodies / Bispecific antibodies

Bispecific antibodies (BsAbs) are a T-cell-based therapy designed to simultaneously bind to a target on tumor cells and to T cells *in vivo*. BsAbs recruit and activate T-cells by binding to the T cell receptor C3, then specifically recognize an antigen target that is ideally exclusive to MM cells and absent in other tissues, thereby reducing the risk of on-target off-tumor toxicity. In this way, an immune synapse is built between the MM and the T cells from the patient, leading to T-cell (mainly CD81 T cells) activation and proliferation, which induce MM cell lysis by release of granzymes, perforins, and different cytokines such as interleukin (IL)-6, IL-2, interferon-gamma (INF-g), IL-5, and monocyte chemoattractant protein-1 (MCP-1)[236,295-297]. Therefore, the fitness of patients' T cells is an essential factor that correlates with the therapy outcome. Several studies have demonstrated poorer patient outcomes associated with a lower CD4/CD8 ratio and reduced proportions of memory-like T cells, alongside increased numbers of PD1+ T-cells, MHC class II HLA-DR1 activated T cells, immunosuppressive regulatory T cells, and bone marrow stromal cells[298,299]. There are two BsAbs classes of T-cell engagers in MM; the first one is a bispecific T-cell engager (BiTE) containing two single-chain variable fragments linked with each other, and the other one is DuoBody consisting of two different antigen-binding fragments and a functional constant region fragment (Fc), which can extend the half-lifetime of the molecule. Beyond BCMA, several MM-specific targets have been investigated: G-protein-coupled receptor family C group 5 member D (GPC5D), Fc receptor-homolog 5 (FcRH5), CD38, CD138, CD19, and signaling lymphocytic activation molecule family-7 (SLAMF7), being some of them already in the clinical practice[236,295-297].

Similar to ADCs, BCMA has also been chosen as an optimal candidate for BsAb therapy due to its prominent expression in MM cells and critical involvement in the pathogenesis of MM. Teclistamab and elranatamab are two BCMAxCD3 BsAbs approved for treating rrMM with triple class-exposed disease, with a rapid achievement of deep hematologic response[300,301]. Real-world evidence with patients out of clinical trials demonstrated similar results to the clinical trial, highlighting the role of this BsAb in the treatment algorithm of rrMM and warranted to evaluate a potential role of teclistamab in less advanced treatment lines or ndMM[302,303]. Currently, several trials are ongoing exploring the association of these BsAbs with anti-Cd38 mAbs, IMiDs, and PIs in rrMM and patients, which may improve response by overcoming the immunosuppressive BM microenvironment[295].

GPC5D is a transmembrane orphan receptor of the G protein-coupled receptor family. It is highly expressed in the BM and on the surface of MM cells, with a lower expression on B and plasma cells and no expression on another hematopoietic cell. It is also expressed in the hair follicle, explaining the skin-related events when targeted[236]. Although its functions are poorly characterized, GPC5D overexpression has been associated with the high-risk cytogenetic events del(13q14) and t(4;14)[296,304]. Talquetamab is the first IgG4 GPC5DxCD3 BsAb in MM, which is approved for patients with rrMM who have previously received at least 4 prior lines of therapy, including a proteasome inhibitor, an immunomodulatory agent, and an anti-CD38 antibody[305]. In preclinical studies, talquetamab, beyond causing lysis of MM cells, also induced T cell activation-related cytokines, including IFN-g, TNF-a, IL-2, and IL-10, but its anti-MM activity was adversely affected by Treg, BMSC, or T cells expressing a high level of PD1 or HLA-DR[306]. Combining it with teclistamab, daratumumab, or pomalidomide enhanced its MM

cell lysis activities[236,295,296,301]. Compared with BCMAxCD3 bsAbs, GPRC5D-targeting bsAbs induce a much lower B-cell depletion and, subsequently, a lower grade of hypogammaglobulinemia and less infectious complications[307,308].

FcRH5 is a surface molecule predominantly expressed in B cells and malignant plasma cells, with the function of FcRH5 also yet to be fully understood. It appears to be more expressed in MM cells rather than healthy plasma cells, especially MM cells with 1q21 copy number abnormalities[236,295]. Cevostamab is an IgG-based BsAb FcRH5xCD3 being explored in the rrMM population after preclinical studies showed a robust T-cell activation and proliferation, as well as complete depletion of B cells and bone marrow plasma cells[301,309]. Moreover, the blockade of PD-1/PD-L1 by an anti-PD-L1 agent enhanced the activity of cevostomab to explore possible combinations further[236,295].

2.1.6. Chimeric Antigen Receptor T Cells

In recent years, Chimeric Antigen receptor T cell (CAR-Ts) therapy has revolutionized cancer treatment and emerged as a highly promising personalized treatment based on the specific type of cancer and the patient's requirements, changing the treatment landscape of hematological malignancies. This approach involves genetically modifying the patient's own T cells, equipping them with the ability to recognize and attach to tumor-specific antigens. After the modified T cells undergo proliferation, they are infused back into the patient to target and eliminate cancer cells[234].

To generate CAR-Ts, patient-derived T cells are genetically engineered to express a chimeric antigen receptor (CAR) that targets tumors. This CAR encompasses an antigen-binding domain, typically sourced from the single-chain variable fragment of an antibody, which is a fusion protein of the antibody's light and heavy chains' variable regions, facilitating the attachment of CARTs to specific cells, fused with hinge and transmembrane segments, along with intracellular signaling domains that trigger the T cell activation, enabling the killing of malignant cells independent of the HLA system[234,310,311]. CAR-T cells interact with tumor cells, forming an immune synapse that leads to target cell killing through various pathways, including the secretion of cytotoxic granules containing perforin and granzymes, the induction of apoptosis via the Fas/Fas ligand molecular pathway, and the production of pro-inflammatory cytokines like IFN- γ and TNF- α [311,312]. While CAR-T cell design is based on achieving tumor lysis via direct T cell-tumor cell interactions, cytokine production by activated CAR-T cells could further enhance their anti-tumoral capabilities, stimulating lymphocyte proliferation and activating other immune cells[312].

In the context of MM, as for ADCs and BsABs, several antigens such as CD19, CD38, CD138, BCMA, Kappa light chain, SLAMF7, NKG2D, and GPRC5D have been identified as effective targets for CAR- T cell therapy. Currently, BCMA is the most successful target used for CAR-T cell therapy in MM, and anti-BCMA CAR-T cell therapy has achieved unprecedented responses in rrMM patients, including those with extramedullary disease. So far, two anti-BCMA CAR-T cell products, idecabtagene vicleucel (ide-cel) and ciltacabtagene autoleucel (cilta-cel), are approved for the treatment of rrMM patients triple class exposed. Many other clinical trials are currently ongoing in rrMM, both in

monotherapy and in combination with anti-MM drugs, reflecting a widespread effort to develop new CARs and optimize existing ones, exploring new targets, co-stimulatory domains, or vector transfection[311].

2.1.7. Small molecules with distinctive mechanisms of actions

Exportin-1 (XPO1) is a karyopherin responsible for the nuclear export of numerous cargo proteins, including nearly all tumor suppressor proteins and several oncoproteins[240,313]. In MM, XPO1 is overexpressed, resulting in enhanced transport of tumor suppressor proteins from the nucleus and allowing cancer cells to evade immune surveillance, dysregulation of growth signaling, and escape from cell-cycle regulation. Moreover, when XPO1 is complexed with the mRNA cap-binding protein, eukaryotic translation initiation factor 4E (eIF4E) transports many oncoprotein mRNAs such as c-Myc, cyclin D1, and MDM2 to the cytoplasm, leading to their downstream effects in cancer promotion[313,314]. Selinexor is the first-in-class oral selective inhibitor of XPO1 (SINE) that reversibly binds to cysteine 528 in the cargo binding pocket of XPO1, forcing the nuclear retention and activation of tumor suppressor proteins, trapping I κ B α in the nucleus to suppress NF- κ B activity, and reducing oncoprotein mRNAs translation by blocking the eIF4E– mRNA complex transport. Ultimately, it causes a selective induction of apoptosis in malignant cells, sparing normal cells[240,314]. In addition, selinexor has synergistic effects with dexamethasone and directly inhibits osteoclastogenesis and bone resorption with little effect on osteoblasts and bone marrow stromal cells by blocking RANKL-induced NF- κ B and NFATc1 (nuclear factor of activated T cell c1) [315,316]. Selinexor has shown superior therapeutic effects in doublet, triplet, and quadruplet regimens in rMM, especially those with high-risk cytogenetic abnormalities such as 17p deletion[313,317].

Melphalan flufenamide (melflufen) is a first-in-class peptide-drug conjugate that targets intracellular aminopeptidases and rapidly and selectively releases alkylating agents into tumor cells[239,318]. Melflufen is quickly taken up by MM cells due to its high lipophilicity, overcoming the development of transporter-associated resistance. Inside the cell, aminopeptidases cleave melflufen into melphalan and p-fluorophenylalanine, achieving higher intracellular concentrations of the drug with an equipotent alkylating effect. Melphalan accumulates in myeloma cells and, within the nucleus, triggers robust and irreversible DNA damage and induces apoptosis, resulting in potent antitumor activity in myeloma cells, including those with resistance to melphalan, bortezomib and dexamethasone[319,320]. Although its cytotoxic activity is not dependent on the activation of p53 function, unlike melphalan, melflufen increases p53 levels, explaining its activity in melphalan-resistant cells[321]. Melflufen demonstrated preclinical activity as an anti-angiogenic agent but also inhibitory action on myeloma cell migration and capacity to overcome the cytoprotective effects of the bone marrow microenvironment, providing a framework for different clinical trials.[318]

Venetoclax is a selective anti-apoptotic BCL2 that directly targets the BCL protein, modulating the mitochondrial apoptotic pathway and inducing tumor cell death. Venetoclax mimics BH3 and inhibits BCL2 by interfering with its binding to pro-apoptotic BH3-only proteins like BIM (BCL2 Interacting Mediator of cell death). This enables activation of the apoptotic effectors BAX and BAK and induces the permeabilization of the mitochondrial outer membrane. The resultant release of the protein cytochrome c – usually locked inside the mitochondria – activates

the apoptosome complex and causes apoptosis in the affected cell[322,323]. In MM, 20% of patients present the t(11;14) with activation and overexpression of BCL2. Nonetheless, so far, there is no known biological function for cyclin D1 in the apoptotic pathway regulated by the BCL2 protein family [324,325]. It was also observed that MM cells might evade apoptosis by uncontrolled transcription of BCL-2 protein, facilitated by gene deletions or the amplification of the miR-17–92 cluster[326,327]. While the efficacy of venetoclax as a monotherapy in MM treatment remains limited, combination therapy with other drugs such as dexamethasone, PIs, IMiDs, and moABs has proven to yield better outcomes for MM patients[323,325]. Dexamethasone has been shown to shift the binding of BIM toward BCL2, increasing the sensitivity of venetoclax, and bortezomib downregulates MCL1 by inducing NOXA, which neutralizes MCL1, a significant cause of venetoclax resistance[328,329]. The diverse responses to venetoclax across various MM subtypes could be due to the different patterns and levels of BCL-2 expression. Biomarker analyses from trials of venetoclax in MM and in vitro data have suggested that high BCL-2 expression, both in absolute terms and relative to the other antiapoptotic family members, Bcl-XL and MCL1, as well as a more B-cell-like MM cell phenotype, maybe more precise markers of venetoclax sensitivity than a t(11;14) translocation[330,331].

2.2. Drug resistance mechanisms

The natural history of MM is characterized by multiple relapses after different lines of treatment with different deepness of response at each line, becoming ultimately refractory. The emergence of drug resistance is appointed as the leading cause for this pattern of evolution, which is dramatically associated with an unfavorable prognosis[332]. Drug resistance in cancer may result from intrinsic mechanisms, in which malignant cells are resistant to therapies even before treatment or acquired during treatment, reflecting the “selective pressure” induced by treatment[333]. To date, the reason why patients relapse and how drug-resistant MM clones alter their dominance and persist after therapies are not fully understood. Due to the heterogeneous nature of MM, many causes are reported to be involved in MM drug resistance (Table 2)[332].

Table 2. Mechanisms of drug resistance of the most used classes of drugs in MM.

Agents	Type of resistance	Mechanism of Resistance
Proteasome Inhibitors (bortezomib, carfilzomib and ixazomib)	Abnormal drug transport	Upregulation of P-gp (mainly carfilzomib) [334,335]
	Escape from apoptosis, autophagy activation and dysregulated signaling pathways	Upregulation of pro-survival proteins (Mcl-1, Bcl-2, Bcl-XL); constitutive activation of the NF- κ B pathway; activation of the aggresome and autophagy pathway; low levels of the UPR transcription factor XBP1 and autophagy-inducer activating transcription factor 4; increase in heat shock proteins (Grp78, Hsp90, Hsp70, Hsp8) [336-343]
	Metabolic alterations	High expression levels of genes related to OXPHOS; high glycolytic rate[344,345]
	Persistence of Cancer Stem Cells	Stem cell-like phenotype with increased levels of multidrug transporters (ABCG2/BCRP) and ALDH1A1 enzymatic activity; Activation of Hedgehog, Wnt and Notch pathways; upregulation of BTK receptors and RARa2[332,341,346-349]
	Microenvironment	Proliferation and cell survival signaling such as IL6/JAK/STAT3, MAPK, PI3/AKT, IGF-1; increase production of VEGF leading to angiogenesis, cell proliferation and migration; Increase of pro-inflammatory TNF- α ; increase of cell adhesion molecules (LFA1, VLA4, ICAM1, VCAM1); activation of SDF1/CXCR4 axis; increase expression of MARCKS in adhesion and metastatic spread; EVs cargo and cell communication (miR-214-3p, miR-5100, miR-15a, miR-146, lncRNA PSMA3-AS1, acid sphingomyelinase, MYH4, CD166, CD44, ANXA2, and FN1)[350-359]
	Genetic abnormalities	Mutations of gene TP53; mutation of gene MAF, t14;16) and t(14;20); point mutations of the gene PSMB5 with overexpression of B5 subunit; upregulation of the proteasomal system; overexpression of the gene MYC; 1q21 gain; modification or loss of 8p21 [360-365]
	Epigenetics	Abnormal regulation miR-15a, miR-137, miR-202, miR-631, miR-29b-3p[366,367]
Immunomodulators (thalidomide, lenalidomide, pomalidomide) Cereblon E3 Ligase Modulators (iberdomide, avadomide, mezigdomide)	Abnormal drug transport	Upregulation of P-gp (mainly lenalidomide)[368]
	Others	Upregulation of others CRBN substrates[265]
	Persistence of Cancer Stem Cells	Stem cell-like phenotype with increased levels of multidrug transporters (ABCG2/BCRP) and ALDH1A1 enzymatic activity; Activation of Hedgehog, Wnt and Notch pathways; upregulation of BTK receptors and RARa2[332,346-349]
	Microenvironment	Increase of cell adhesion molecules (CD44 thought the Wnt/B-catenin signaling); Upregulation of IL-6/STAT3 pathway; EVs cargo and cell communication (miR-214-3p, miR-5100, miR-15a) [369,370]
	Genetic abnormalities	Mutations in cereblon and genes in the cereblon-pathway (IFK1 and KPNA2)[254]
	Epigenetics	Abnormal regulation miR-9-5p[366,367]
Monoclonal Antibodies (daratumumab, isatuximab, elotuzumab,)	Reduced target expression	Reduced expression of CD38 and SLAMF7[371,372]
	Complement resistance	Increased expression of CD46, CD56 and CD59 blocking anti-body-induced CDC[371,373,374]
	Microenvironment	Competition by the extracellular soluble forms of CD38 and SLAMF7 [268]
	Neutralization	Anti-idiotype antibodies neutralizing the activity of the therapeutic monoclonal antibodies; Stromal cell protecting MM cells for moAbs induced apoptosis by the upregulation of survivin and MCL1.[374,375]
	Genetic abnormalities	Point mutations disrupting the epitopes on CD38 [372]
Antibody drug conjugates (belantamab-mafodotin)	Reduced target expression	Reduced expression of BCMA[376]
T-cell engagers antibodies / Bispecific antibodies (teclistamab, elranatamab, talquetamab, cevostamab)	T cell stemness	Increase of terminally exhausted T cells and senescent cells; upregulated expression of certain T-cell checkpoint molecules such as LAG3, TIGIT, and PD1 [298,299]
	Reduced target expression	Reduced expression of BCMA, GPR5CD, FcRH5[377]
	Microenvironment	immunosuppressive cytokines, T regulatory cells, and expression of the programmed cell death ligand 1 (PDL-1)[378]
Chimeric Antigen Receptor T Cells	T cell stemness	Increase of terminally exhausted T cells and senescent cells; upregulated expression of certain T-cell checkpoint molecules such as LAG3, TIGIT, and PD1 [378]
	Reduced target expression	Reduced expression of BCMA[377]
	Activation, expansion, and persistence of CAR-T cells	Inhibition by PI3K; elimination of CAR-T cells by tumor cells[379,380]
	Microenvironment	Immunosuppressive cytokines, T regulatory cells; expression of the PDL-1[379]
	Metabolism	Arginine-deficient metabolic environment[381]
Exportin 1 inhibitors (selinexor)	-	-
BLC2 inhibitors (venetoclax)	Abnormal drug transport	Upregulation of P-gp [382,383]
Alkylating agents (melphalan, cyclophosphamide)	Abnormal Drug Transport	Upregulation of P-gp; increased ABCG2 expression[384]
	Persistence of Cancer Stem Cells	Stem cell-like phenotype with increased levels of multidrug transporters (ABCG2/BCRP) and ALDH1A1 enzymatic activity; Activation of Hedgehog, Wnt and Notch pathways; upregulation of BTK receptors and RARa2[346-349]
	Microenvironment	Increase of cell adhesion molecules (VLA4)[385]
	Epigenetics	Abnormal regulation miR-221/222[386]
Steroids (dexamethasone, prednisolone, methylprednisolone)	Reduced target expression	Functional defect of the glucocorticoid receptor [332]
	Microenvironment	Increase secretion of pro-survival cytokines [387]
	Genetic abnormalities and epigenetics	Overexpression of the gene MYC and FGFR3; epigenetic inactivation of RASD1; abnormal regulation miR-149[332,366,367]

2.2.1. Abnormal drug transport

One of the most frequent causes of cancer drug resistance is an abnormal drug efflux, mediated by several ATP-binding cassette (ABC) efflux pumps, causing a reduction in the intracellular drug levels and the therapeutic effects of drugs that are substrates of these proteins [388-390]. One of the most studied efflux pumps is P-glycoprotein (P-gp, also known as MDR1 or ABCB1), a product of the MDR gene which has been frequently observed to be overexpressed in hematological malignancies and strongly associated with drug resistance[388,391,392]. In MM, P-gp was found to be weakly expressed at diagnosis (around 6%) but overexpressed following chemotherapy (more than 40%), reducing the success of treatment[393]. Curiously, high P-gp expression was directly correlated with low intracellular accumulation of melphalan in MM patients without evidence of induced P-gp expression after treatment[384]. Other drugs used in MM, such as lenalidomide, carfilzomib, and venetoclax, are known substrates to P-gp, being susceptible to the development of drug-resistant cells by this mechanism[335,368,382,383,394]. Thalidomide and bortezomib, also used in MM, were identified as weak P-gp substrates[384]. Nonetheless, bortezomib activity is affected by P-gp expression, and conversely, the expression of P-gp affects the ability of bortezomib to act as a P-gp substrate. It was also found that bortezomib reduces the expression and function of P-gp and is enhanced using a P-gp inhibitor[334].

2.2.2. Escape from apoptosis, autophagy activation and dysregulated signaling pathways

Apoptosis is a programmed cell death process mediated by proteins involved in several signaling pathways, such as PI3K/AKT, NF- κ B and the ubiquitin/proteasome system[395]. MCL1, a pro-survival protein, is associated with MM cell survival. Its inhibition rapidly induced apoptosis in MM cells, and its overexpression contributed to relapse and disease severity across all stages. MCL1 levels were found enhanced in MM cell lines, MM patient cells, and in all stages of the disease in response to IL-6, upon activation of the JAK/STAT3 pathway in the presence of VEGF[396-398]. High levels of the BCL-XL protein contributed to apoptosis inhibition, also through activation of the JAK/STAT3 pathway by IL-6, with higher levels in relapsed patients when compared to newly diagnosed[399]. Indeed, both MCL1 and BCL-XL may be more critical for plasma cell survival than BCL2, with higher sensitivity to venetoclax found in patients with high BCL2/BCL-XL or MCL1 ratios[400]. The TNF-related apoptosis-inducing ligand/Apo2 ligand (TRAIL/ApoL) caused apoptosis in MM cells of patients with either sensitivity or resistance to melphalan and dexamethasone[336]. Interestingly, its overexpression reverted bortezomib resistance in some MM cells by increasing apoptosis[401]. NF- κ B is a family of transcription factors that regulate cell growth, differentiation, and apoptosis, contributing to malignant cells' survival and playing a significant role in drug resistance. In MM, NF- κ B is constitutively active, and drug-sensitive MM cells presented lower NF- κ B activity when compared to drug-resistant cells[244,337,338]. In addition, higher levels of NF- κ B were found in MM cells derived from relapsed patients compared to newly diagnosed MM patients [244,337,338].

The unfolded protein response (UPR) pathway is triggered by the accumulation of misfolded or unfolded proteins at the ER of cancer cells[402,403]. UPR ameliorates ER stress, leading to inhibition of protein synthesis and increasing the transcription of heat shock proteins (HSP) folding chaperones[403]. The remaining misfolded proteins are

targeted for degradation by the proteasome and autophagy systems[402]. The increased expression of major elements of the UPR pathway, such as Ire1 α and its major substrate XBP1, has been observed in several hematological malignancies, including MM. In most cases, the activation of the Ire1 α -XBP1 pathway resulted in cell proliferation, reduced apoptosis, and, ultimately, drug resistance[404]. MM is characterized by high levels of misfolded or unfolded proteins in the ER, becoming extremely dependent on the UPR system for survival and being more sensitive to proteasome inhibitors. For instance, bortezomib causes the accumulation of misfolded proteins within the ER by inhibiting proteasome activity, leading to apoptosis in malignant plasma cells[339]. Also, in MM, a decrease in the UPR activity could predict resistance to proteasome inhibitors [340]. Correlations were found between higher levels of XBP1 and sensitivity to bortezomib and lower levels of the UPR regulator activating transcription factor 6 (ATF6), XBP1 inactivation, and bortezomib resistance[340,341].

Autophagy is an adaptative response to stress stimuli characterized by a lysosomal degradation pathway for the clearance of cellular materials, such as misfolded or unfolded proteins[405-407]. In MM cells, the induction of autophagy is not only necessary to collaborate with the UPR signaling but is also an important survival mechanism that cells use to degrade misfolded proteins and to survive [406-408]. In MM, it was observed that upregulation of the ATF4 induced resistance to bortezomib or carfilzomib, and this could be overcome by combining these proteasome inhibitors with autophagy inhibitors[342,343,409]. The heat shock proteins HSP70 and HSP90, players in chaperone-mediated autophagy, were associated with various MM survival pathways, stabilizing proteins involved in antiapoptotic signals, including AKT, STAT3, and IL-6 receptors. HSP90 has been identified as a target of both JAK/STAT3, MAPK/ERK pathways, and PI3K/AKT pathways[410,411]. In vitro studies and orthotopic in vivo models have shown promising results with combinations of HSP90 inhibitors and bortezomib or AKT pathway inhibitors[410,412].

2.2.3. Metabolic alterations

Metabolic reprogramming is an emerging hallmark of cancer, essential for tumor cells adaptation to the microenvironment to maintain their survival and proliferation, and works as an adaptive mechanism of drug resistance through changes in metabolic pathways mediated by alterations in metabolic enzymes, hypoxia, and regulatory signals[413-415]. Multidrug-resistant (MDR) cells with P-gp overexpression have different metabolic profiles when compared to the profile of drug-sensitive counterparts, with increases in glutathione metabolism and glycolysis, decreases in the pentose phosphate pathway and oxidative phosphorylation rate, and alterations in the methionine/S-adenosylmethionine pathway[416]. The Warburg effect describes aerobic glycolysis in which tumor cells prefer glycolysis to mitochondrial oxidative phosphorylation (OXPHOS), even when in an oxygen-rich environment, promoting drug resistance through epigenetic and genetic variations to activate survival pathways and escape apoptosis[414,417,418]. In MM, not only higher expression levels of genes related to OXPHOS were found in patients with poor response to bortezomib, but it was also observed that, under hypoxia, higher glycolytic rates reduced bortezomib sensitivity, while LDHA enhanced sensitivity to bortezomib and carfilzomib in the same conditions[344,345].

2.2.4. Immunotherapy specific resistance

Passive immunotherapy represented a major step in the treatment of MM, with monoclonal antibodies (moAbs) being widely used in clinical practice, and thus, the emergence of resistance is becoming inevitable[372,376,419]. Abnormalities in the surface antigen regarding its expression and density are involved in most cases of drug resistance to moAbs[372,376]. For example, in MM pre-treatment, CD38 levels were higher in patients who achieved response when compared to those who did not, and CD38 was highly expressed before treatment and significantly decreased over time, with very low levels of CD38 at progression/relapse[371]. Many explanations are hypothesized for these abnormalities, such as the “antigen shaving” by effector cells that expressed FCyR, the internalization of the antigen into lysosomes after the monoclonal antibody binding, and the appearance of point mutations in the antigen gene[372,376]. In addition, soluble forms of CD38 and SLAMF7 may neutralize the efficacy of daratumumab and elotuzumab through the extracellular binding to the moAbs, thereby reducing their specific binding to plasma cells[268]. Likewise, developing anti-idiotypic antibodies can neutralize the activity of moAbs before reaching their specific targets[420]. Similar patterns of CD38 were identified in PB and BM-derived extracellular vesicles (EVs), suggesting that PB EVs may reflect MM BM plasma cells following daratumumab treatment. In addition, EVs expressing high levels of CD147, CD55, and CD59 may have diagnostic and prognostic value, while EVs with high levels of PDL1 may indicate a response to daratumumab[373]. Furthermore, upregulation of cell surface complement inhibitory proteins CD46, CD55, and CD59 by the tumor was found in cases of resistance to daratumumab in MM cells, blocking the antibody-induced complement-directed toxicity[371,374]. Additionally, it was observed that stromal cells can protect MM PCs from daratumumab-induced apoptosis by upregulating anti-apoptotic proteins such as survivin and MCL1[375].

Resistance to TCE and CAR-T cells may develop by multiple mechanisms involving the T cells themselves, the tumor cells, or the microenvironment[379,380,421,422]. The activation, expansion, and persistence of CAR-T cells depend on the stemness of the effector T cells, being promoted by overexpression of IL6 (through the STAT3 signaling), IL15, and IL7, whereas PI3K inhibits its expansion[378]. In TCE, the functionality of the patient's T cells may be an important factor that correlates with the therapy outcome. It was observed that MM patients responding to CAR-T cells or BsAbs therapy displayed a higher CD4/CD8 ratio and higher proportions of memory-like T cells (T memory stem cells and central memory T cells) in comparison to patients resistant to T-cell-based immunotherapies[298]. Moreover, these resistant patients showed increased percentages of terminally exhausted T cells and senescent cells with upregulated expression of certain T-cell checkpoint molecules such as LAG3 (Lymphocyte-activation gene 3), TIGIT (T cell immunoreceptor with Ig and ITIM domains), and PD1[299]. As with moAbs, tumors can become resistant to TCE and CAR-T cells by eliminating the target antigen or masking the epitope, reducing the effector cells' efficacy[377]. The microenvironment also presents major challenges to TCE and CAR-T cells since immunosuppressive cytokines, T regulatory cells, and expression of the PDL-1 can suppress CAR-T cell activation and function[379,423]. Elimination of CAR-T cells can also be mediated by an interaction between the apoptosis-inducing ligand, such as FasL expressed in the tumor cells, and Fas expressed in the CAR-T cells [424]. Furthermore, CAR-T cells can also be suppressed by an arginine-deficient metabolic environment induced by the tumor, limiting cell expansion and proliferation[381].

2.2.5. Persistence of cancer stem cells

Cancer stem cells (CSCs), also called tumor-initiating cells, are an important subset of cells in the tumor heterogeneous niche contributing to drug resistance[346-348]. CSCs share unique characteristics explaining their roles in drug resistance, such as the potential for self-renewing ability, multi-differentiation potency and ability to remain quiescent, low levels of reactive oxygen species (ROS), enhanced DNA damage repair, enhanced anti-apoptotic and defective pro-apoptotic signaling, overexpression of MDR efflux pumps, evasion from the immune response, adaptation to the TEM and greater cellular plasticity.

A population of MM cancer stem cells (MM CSCs) seemed to arise from clonotypic B-cells, expressing regular B cell markers (CD19, CD20, and CD27) and no CD138[349,425]. CSCs express higher levels of ABC transporters, mediating active and persistent efflux of drugs[346-348]. In fact, in MM, the ABCG2 (ATP-binding cassette sub-family G member 2) transporter is overexpressed in the CSCs and in the side populations, being associated with resistance to dexamethasone, as well as with the persistence of disease. It was observed that ABCG2 inhibition, with cyclosporine analogs or verapamil, reduced the fraction of CSCs and re-sensitized tumor cells to those drugs[426]. Several signaling pathways, namely Hedgehog, Wnt, and Notch, are highly activated in CSCs, being essential for their stemness and persistence, preventing their apoptosis as well as mediating the activation of drug efflux pumps, such as ABCG2[346-348]. The inhibition of the Hedgehog pathway resulted in differentiation of the CD138- plasma cells in MM, and resistant subpopulations were depleted through downregulation of Akt and BCL2 expression[427,428]. In CSCs, the ROS levels are maintained very low, avoiding cell death, by the enhanced expression of aldehyde dehydrogenase (ALDH)[349,429]. This enzyme is involved in the reduction of oxidative stress, as well as the metabolic detoxification of a variety of compounds, such as ethanol and active metabolites of cyclophosphamide, becoming another major mediator of drug resistance in CSCs[349,429]. ALDH is not only upregulated in MM CSCs, such as the CD138- cells, but is also highly expressed in MM cells with rapid proliferation features, high tumorigenicity, and chromosomal instability linked to treatment resistance[430,431].

2.2.6. Tumor microenvironment

The complex and infinite interactions between tumor cells and the microenvironment, besides the fundamental role in the pathophysiology and persistence of the tumor, also play a significant role in drug resistance. This type of resistance is referred to as “soluble factor-mediated” drug resistance (SFM-DR) and “cell adhesion mediated” drug resistance (CAM-DR)[432-437]. SFM-DR is mediated by cytokines and growth factors from the tumor microenvironment, and CAM-DR is mediated by the adhesion of tumor cells to stromal cells, such as fibroblasts, BMSCs, or extracellular matrix (ECM) proteins, involving adhesion molecules like B1 integrins[433].

Diverse cytokines or ligands, like IL-6, IGF-1, VEGF, BAFF, FGF, SDF1 α , and TNF- α , are secreted reciprocally by mesenchymal stem cells or tumor cells, disrupting the normal signaling pathways[432-435]. IL6 is secreted by BMSC or by MM cells in an autocrine fashion, being implicated in resistance to various apoptotic signals (including Fas/Apo-1) and controlled through the JAK/STAT signaling pathway[350,438]. IL-6 overexpression is associated

with resistance to several anti-MM drugs, including bortezomib[350,439,440]. It was also observed that MM cells with autocrine IL6 signal were more resistant to dexamethasone when compared to those without this signal[387]. IGF-1, produced by MM cells and present in the BM environment, promoted proliferation and bortezomib resistance by activating the MAPK and PI3/AKT pathways[351,441]. Enhanced VEGF levels in the microenvironment promoted angiogenesis and contributed to MM cell proliferation, migration, and drug resistance[352]. Some VEGF inhibitors and antiangiogenic agents, such as lenalidomide, showed synergistic effects with melphalan and bortezomib. These inhibitors increased MM cellular apoptosis in the presence of BMSCs by decreasing IL-6 and VEGF secretion[353].

In MM cell lines, the adhesion of MM cells, via VLA-4 and ICAM1, to the ECM component fibronectin prevents apoptosis and contributes to doxorubicin and melphalan resistance[385,442]. On the other hand, bortezomib overcame CAM-DR through the downregulation of VLA-4 expression [385,442,443]. Integrin B1 played an important role in CAM-DR characterized by G1 cell cycle arrest, increase in p27kip1 protein levels, decrease in the enzymatic activity of cyclin A and cyclin E, upregulation of p21Cip1/Waf1 or downregulation of BIM[444,445]. Moreover, adhesion through integrin-induced resistance to apoptotic signals such as Fas/Apo1 in various hematopoietic cancer cell lines[446]. Concerning SDF-1/CXCL12, it is constitutively expressed and released by BMSCs and fibroblasts, while its receptor CXCR4 is found in MM cells. The activation of the SDF1/CXCR4 axis promotes trans-endothelial migration, bone marrow homing, migration, and adhesion of MM cells. The CXCR4 expression is correlated with bortezomib resistance in cell lines, and the use of CXCR4 inhibitors may enhance the sensitivity of MM cells by disrupting their adhesion to the BMSCs[354]. Another potential cause for drug resistance in MM is the activation of the myristoylated alanine-rich c-kinase substrate (MARCKS) membrane protein, which is essential in cell adhesion and metastatic spread. MARCKS is activated by the phosphorylation of protein kinases C and is associated with bortezomib resistance. Inhibition of MARCKS phosphorylation enhanced, in vitro, the sensitivity of resistant MM cells to bortezomib[355].

Intercellular communication between tumor cells and the microenvironment is, in part, mediated by EVs[447]. EVs are secreted by normal and tumor cells, containing, among others, a powerful cargo of microRNAs, long non-coding RNAs, and proteins, playing essential roles in regulating cell proliferation, differentiation, and apoptosis[448-450]. Also, EVs can transfer drug resistance between tumor cells horizontally[451,452]. In MM, EVs obtained from BMSCs enhanced tumor cell resistance to bortezomib by activating several survival pathways, such as c-Jun N terminal kinase, p38, p53, and Akt[453]. More importantly, BMSC EVs could induce bortezomib resistance by increasing Bcl2 levels. BMSC EVs also contained caspase3-cleaved Bcl-XL, which was essential for EV uptake and delivered antiapoptotic signals to the recipient MM cells [454].

Further investigation into the content of BMSC EVs revealed that miR-573, miR-544, miR-545, miR-382, miR-16, miR-23b-3p, miR-27b-3p and miR-125b-5p (all down-regulated), and miR-10a, miR-214-3p and miR-5100 (all up-regulated) were able to distinguish MM patient BMSCs from MGUS and healthy donors[356,357]. Of these, only miR-214-3p and miR-5100 were incorporated by MM cells, which induced MM cell proliferation and drug resistance via downregulation of PTEN[356]. Interesting results showed that EVs released by primary cultures of MSCs from

relapsed or refractory MM patients presented downregulated levels of miR-15a, a tumor suppressor miRNA, when compared with EVs from primary BM-MSCs obtained from healthy donors, justifying a potential contribution of MSCs to MM disease progression[455]. Additionally, LINC00461, present in BM MSC exosomes, was found to act as a sponge for miR-15a/16 upon transfer into MM cells, thereby releasing Bcl-2, leading to enhanced MM survival[123]. Also, lenalidomide-sensitive MM cells were able to acquire resistance when cocultured with resistant MM cells. Lenalidomide resistance was correlated with the overexpression of SORT1 and LAMP2 genes that are responsible for a higher EV release, although with unknown cargo and cell adhesion ability[369].

BMSCs are not isolated from mediating the acquisition of drug resistance in MM. Stem cell-derived EVs contain the lncRNA PSMA3-AS1, which is involved in the positive regulation of the $\alpha 7$ proteasome subunit PSMA3 in MM cells. Upregulation of both PSMA3 and PSMA3-AS1 mRNA is associated with PI resistance and, therefore, shorter PFS and OS. Targeting PSMA3-AS1 reduced tumor burden in a xenograft model and synergized with carfilzomib treatment [358]. It was further demonstrated that bortezomib-resistant MM cells could transfer resistance to sensitive cells via exosomal HSP70 delivery. Treatment with polyclonal intravenous immunoglobulin, which contains various immunoglobulins, including antibodies against HSP70, induced apoptosis in MM cells. This interruption of the HSP70 loop enhanced the sensitivity of MM cells to bortezomib in both in vitro and in vivo studies[456].

Curiously, MM cells can also autologously induce drug resistance. It has been reported that MM cells can produce exosomes in high quantities in response to anti-myeloma drugs such as melphalan and bortezomib. These exosomes, named chemo-exosomes, present an altered proteome profile enriched in proteins that regulate the cell cycle. Transfer of these exosomes to other MM cells enhanced proliferation and survival signals, including pERK[457]. The surface of chemoexosomes is enriched with heparanase. When these chemoexosomes interact with other MM cells, they transfer heparanase, leading to the activation of the ERK pathway, increased production of TNF- α , and degradation of the extracellular matrix. These effects collectively contribute to the development of drug resistance in MM patients[458]. There is an increase in acid sphingomyelinase expression in MM cell lines treated with melphalan or bortezomib, as well as in their exosomes. Exosomes with a high content of ASM can transfer the drug-resistant phenotype to chemosensitive cells, thereby suggesting a tumor-protective role for ASM[459]. MM cells can stimulate BMSCs to transform into CAFs via EVs communication, establishing a mutual feedback loop. These CAFs express fibroblast-specific protein 1 (FSP1) and, when activated by cancer cells, also express α SMA and FAP[460]. MM cells can transfer miR-146a via EVs to BMSCs, triggering through the NOTCH pathway enhanced cytokine secretion, including IL-6 and CXCL10. This, in turn, stimulated MM growth and migration. Mir-146a transfer led to the upregulation of α SMA and FAP in MSCs[461]. MM EVs further increased the expression of miR-27b-3p and miR-214-3p in MM by transferring exosomal WWC2, which regulates the Hippo pathway. These miRNAs modulated MCL1 via FBXW7 and PTEN and enhanced the expression of the CAF markers α SMA and FAP[72]. All this highlights the key role of MM cells in modifying the bone marrow microenvironment by reprogramming the marrow fibroblasts' behavior. Moreover, MM EVs can impact healthy BMSCs, inducing their proliferation, migration, and adhesion via the transfer of migration/adhesion-related proteins (i.e., MYH4, CD166, CD44, ANXA2, and FN1), inducing drug resistance[359].

2.2.7. Genetic Abnormalities Influencing Drug Resistance

The cytogenetic alterations implicated in MM pathogenesis are closely linked to drug resistance and eventual relapse[332]. Initial events, such as IGH translocations involving chromosome 14q32, are pivotal in MM onset. These translocations, notably t(4;14), t(14;16), and t(14;20), result in the upregulation of various oncogenes like FGFR3, MMSET, c-MAF, and MAFB, contributing to MM pathogenesis [462]. Notably, the t(4;14) translocation is associated with treatment failure due to overexpression of FGFR3 and MMSET, leading to high relapse rates. Elevated FGFR3 levels aid in tumor establishment, whereas MMSET intensifies cell proliferation by reducing cell cycle arrest and apoptosis while enhancing cell adhesion[463,464]. Also, in t(14;16) and t(14;20), drug resistance is associated with the upregulation of CCND2 genes mediated by MAF. This upregulation targets PCs to be more responsive to external stimuli and strengthens adhesion signaling to the stroma. Notably, overexpression of MAF correlates with diminished effectiveness of bortezomib and carfilzomib treatments[360,361]. On the other hand, t(11;4) upregulates CCND1, potentially enhancing bortezomib therapy response, although CCND1 amplification may lead to multidrug resistance in some studies[362,465].

Secondary cytogenetic events, notably deletions, further influence MM prognosis. Deletion of chromosome 17p13.1 (del17p) confers a dismal prognosis in MM, associated with advanced stages of MM and drug resistance. This is essentially due to the loss of the TP53 and consequent dysregulated control of cell cycle and apoptosis[466]. So far, all classes of treatments appear both at diagnosis and relapse appear not to overcome the treatment resistance associated with this cytogenetic event[467,468]. Deletion of chromosome 13(q14q21) is associated with an intermediate prognosis but may benefit from bortezomib therapy[469]. Abnormalities in chromosome 1, particularly deletion of the 1p21 region and gain of the chromosome 1q arm have been associated with shorter survival in MM. The deletion of 1p implicates target genes such as FAM46C and CDKN2C, contributing to reduced remission rates and overall survival, particularly in patients treated with ASCT[363,470]. Gains of 1q also affect the outcome of patients who underwent ASCT or PIs-based therapy, conferring bortezomib resistance[363,471,472]. However, abnormalities in chromosome 1 do not seem to affect the efficacy of IMiDs in some studies[472,473]. Furthermore, the combination of carfilzomib or pomalidomide and isatuximab shows promise in overcoming the adverse prognosis associated with 1q gain[474]. Loss of the chromosome region 8p21 is not an event common in MM, but when present, it is associated with poor response to bortezomib. The 8p21 deletion confers resistance to bortezomib by upregulating the decoy TNF-related apoptosis-inducing ligand (TRAIL) receptor, causing resistance of the abnormal PC to Apo2L/TRAIL-mediated apoptosis. The use of IMiDs, such as lenalidomide, may counteract this resistance mechanism[364]. Overexpression of the MYC oncogene at the 8q24 chromosome occurs at a later stage of the MM pathogenesis and is associated with poor prognosis. MYC abnormalities, together with its co-localization with super-enhancers, are associated with melphalan and bortezomib resistance through several mechanisms, namely inducing higher serum PDGF-BB receptor levels and upregulating the IRF4[475-477].

Other abnormalities may be found in the latter and aggressive stages of MM, mainly gene mutations involving gain of function of oncogenes such as NRAS, KRAS, BRAF, and CCND1, loss of function of tumor suppressors like TP53, RB1, DIS3, CDKN2A, and CDKN2C, and mutations in the NF-κB and STAT3 pathways[44,478,479]. These mutations

play a significant role in oncogenesis, promoting tumor progression and drug resistance. For example, inactivation of p53 is frequent due to loss of heterozygosity due to mutations in the p53-DNA domain or through overexpression of the MDM2 gene[480,481]. This loss of TP53 activity is frequent in all hematological malignancies and contributes to persistent replication and resistance to multiple drugs[480].

Resistance due to mutations in the drug targets is also observed in MM, mainly related to alterations in proteasome conformation in the case of PIs or cereblon activity in the case of IMiDs. Mutations in proteasome subunits conferring resistance to PIs have been identified, resulting in overexpression of the proteasome subunit or changes in the spatial arrangement in the proteasome inhibitor binding pocket. The most common mutations are in the proteasome subunit beta 5 (PSMB5) gene, which encodes for the B5 subunit of the proteasome, the main target for bortezomib and carfilzomib, and is responsible for their chymotrypsin-like proteasome activity[365]. Regarding IMiDs, mutations in cereblon were identified, leading to their downregulation and resistance. Moreover, some mutations were identified only in refractory and relapsed disease, probably due to clonal selection during long-term use of IMiDs[482]. Other mutations related to cereblon function were also identified, leading to low levels of the cereblon-binding protein IKZF1 and high levels of another cereblon-binding protein, the karyopherin subunit α 2 (KPNA2), which was correlated with lack of response to pomalidomide[483].

2.2.8. Clonal heterogeneity and evolution

The impact of clonal heterogeneity on disease progression and drug resistance is widely recognized as a significant problem for the effective treatment of hematological malignancies[484-486].

According to the most consensual approach to clonal evolution, preexisting drug-resistant clones are selected by therapy and eventually will replace drug-sensitive clones, which are eliminated successfully by treatment[484-486]. Nevertheless, the emergence of heterogenous subclones results from multiple and complex biological processes[484]. In MM, the pattern of clonal evolution appears to be related to the type of response to treatment rather than the treatment itself[484,487,488]. In patients achieving partial response, a model of the stable subclonal structure was observed, with stable mutational and structural profiles over time, reflecting suppression of a sensitive clone while the refractory one remains stable and thus becomes proportionally dominant[487,488]. On the contrary, patients achieving a complete response, and mainly those with negative measurable residual disease, presented predominantly a branching or linear model, leading to relapse, characterized by acquired structural abnormalities, a higher mutational burden, altered mutational profile, and bi-allelic inactivation of tumor suppressor genes[487,488].

Following relapse, the NF- κ B and RAS/MAPK pathways are commonly changed, with a prevalence of 45-65% each, as well as the TP53 pathway with 45% of mutations and the MYC pathway with 10%[489]. Alterations conferring resistance to the immunomodulators (mutations in CRN, CUL4B, and NR3C1), proteasome inhibitors (mutations in PSMB5, PSMB8, PSMB9, MSMD1, OSMG2, XBP1), and moAbs (point mutations disrupting the epitopes on CD38 that interact with anti-CD30 moAbs) were identified in rMM[489,490].

The persistence of residual disease works as a reservoir of drug-resistant subclones, representing the founder population for future clinically overt relapse. Indeed, the genotypic and phenotypic characterization of residual clones showed overexpressed integrins, cytokine receptors, adhesion molecules, and others involved in the interaction with dendritic cells, underlying the importance of the TME[491].

2.2.9. The role of epigenetics and microRNAs

Epigenetic modifications have been identified as important contributors to the appearance of drug resistance through hypomethylation of DNA, hypermethylation of tumor suppressor genes, and histone modifications, promoting cell survival and cell cycle progression, and abnormal miRNAs expression, which disturbs several pathways involved in MM pathogenesis[332].

In MM, hypomethylation is associated with disease progression, poor prognosis, and drug resistance by upregulation of the ABCG2 gene and, consequently, increased drug efflux[492]. On the other hand, in rrMM patients, mainly following treatment with bortezomib, there is lower global methylation and hypermethylation of NFKB1, CDKN2A, CDKN2B, TNF, and RB genes. The same study also found that resistance of MM cells to dexamethasone was associated with hypermethylation of the RASD1 gene and that sensitization of these cells was possible with the hypomethylating agent 5'-azacytidine[493].

Epigenetic control by miRNAs also plays a significant role in developing drug resistance in hematological malignancies[366,494]. Indeed, miRNAs may regulate the expression of drug-resistance-related genes or influence pathways that mediate cell survival[367,494-496]. The aberrant expression of some miRNAs may affect the expression of multiple target mRNAs, altering the expression levels of proteins linked to drug resistance and impacting the sensitivity of cancer cells to treatment[447,495]. For example, depending on their mRNA target, miRNAs may influence drug resistance by controlling the levels of expression of ABC transporters, drug targets, cell cycle checkpoints and cell signaling, cell death/apoptosis pathways and autophagy, DNA repair mechanisms, the TME or drug-metabolizing enzymes (Figure 4)[366,367,494,495,497,498].

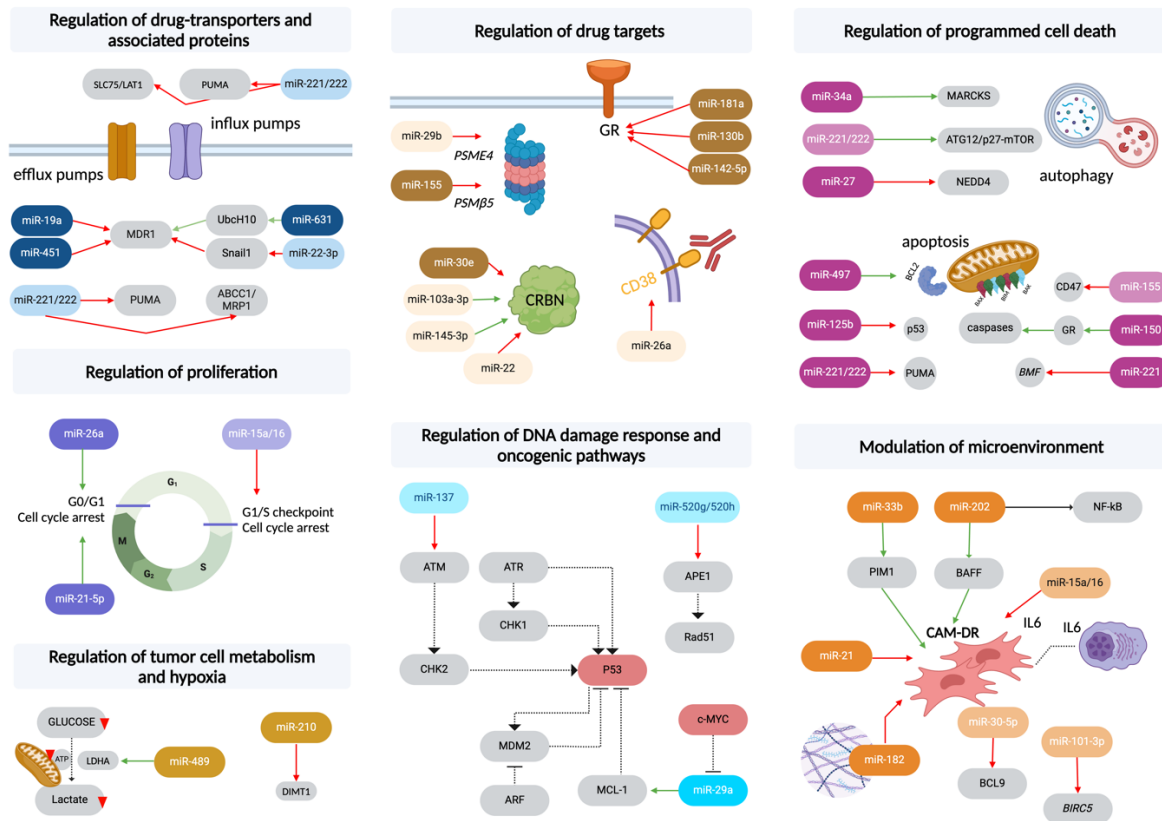


Figure 4. MicroRNA-driven drug resistance pathways in Multiple Myeloma. The aberrant expression of some miRNAs may affect the expression of multiple target mRNAs, altering the expression levels of proteins linked to drug resistance and impacting the sensitivity of cancer cells to treatment. MicroRNAs such as miR-19a, miR-451, and miR-221/222 are involved in the regulation of drug transporters and associated proteins, influencing drug resistance by modulating the expression of efflux and influx pumps such as MDR1 and ABCB1/MDR1. Other microRNAs, such as miR-29b, miR-130, and miR-30e, influence drug-specific targets such as the PSME4, GR, and CRBN, respectively, thereby impacting the effectiveness of therapeutic agents. In the context of programmed cell death, microRNAs, including miR-34a, miR-140-5p, and miR-125b, regulate apoptosis and autophagy pathways by targeting key components like MARCKS, caspases, and NEDD4, promoting drug resistance. Furthermore, microRNAs such as miR-26a, miR-15a, and miR-21-5p play crucial roles in controlling cell cycle progression, contributing to the proliferation of MM PCs. MicroRNAs are also crucial in regulating tumor cell metabolism and responses to hypoxia, with miR-210 and miR-489 modulating glucose metabolism, lactate production, and adaptation to low oxygen conditions. MicroRNAs like miR-137 and miR-29a influence the DNA damage response by targeting components of the ATM/ATR and p53 pathways, affecting genomic stability and the oncogenic potential of MM PCs. Modulation of the microenvironment is another essential influence by microRNAs, such as miR-33b and miR-202, which target PIM1, BAFF, and NF- κ B, increasing IL-6 expression and contributing to the creation of a supportive niche for MM PCs survival and resistance to therapy. "Created with BioRender.com".

Regulation of drug transporters and associated proteins

The miRNAs can post-transcriptionally regulate the expression of some drug transporters of the ABC family, including P-gp/MDR1, MRP, and BCRP. For example, patients with high expression of miR-19a respond poorly to bortezomib treatment, mainly due to upregulation of the drug efflux transporter MDR1 [499,500]. Indeed, miR-19a acts as an oncogene in MM by promoting cell proliferation and inhibiting apoptosis, and it was identified that BCL2 and MDR1 were upregulated after elevated expression of miR-19a. Accordingly, miR-19a may be used as a potential biomarker to predict response in MM treatment [501]. In addition, it was described that miR-221/222 inhibition modified melphalan sensitivity in MM cells by increasing PUMA, a BH3-only BCL2 member, and by modulating the expression of influx/efflux transporters resulting in upregulation of the influx SLC7A5/LAT1 transporter and a strong

downregulation of the efflux ABCC1/MRP1 transporter [74,386]. It was also shown that in those circumstances, cells from the MM.1S cell line were more resistant to dexamethasone [74,502]. Another example is miR-451, which regulates the stemness of MM CSCs and targets the tuberous sclerosis 1 (TSC1) gene, activating the PI3K/Akt/mTOR signaling in MM cells. This miRNA inhibits the efficacy of anti-MM drug agents, including bortezomib and melphalan, by decreasing cell death and increasing MDR1 mRNA expression [503]. In addition, drug efflux may be indirectly modulated by miRNAs targeting proteins closely associated with drug transporters. For example, miR-631 targets UbcH10, a major inhibitor of MDR1 ubiquitination [504]. Overexpression of miR-631 inhibits UbcH10 expression, thus targeting MDR1 for ubiquitination with the consequential decrease of MDR1 protein levels, overcoming bortezomib resistance in some MM cell lines [504]. Similarly, miR-22-3p is also linked indirectly to bortezomib resistance through the Snail1/miR-22-3p/P53 pathway, upregulating the transcription of the intracellular MDR1 gene and inhibiting the p53 protein expression. By increasing MDR1 and reducing p53, Snail1/miR-22-3p triggered resistance to bortezomib, while its silencing enhanced cell sensitivity to bortezomib treatment [505].

Regulation of drug targets

The miRNAs can decrease the expression of drug targets, leading to a drug-resistance phenotype [506]. Glucocorticoids are an essential part of all MM treatment protocols and can also be used in monotherapy for cytoreduction and control of symptoms [507]. GCs mediate their functions via the activation of a glucocorticoid receptor (GR), leading to the induction of apoptosis by the upregulation of proapoptotic genes in cancer cells [507,508]. It was shown that reduced expression of GR is associated with insufficient response to GCs, and restitution of GR in MM cell lines lacking the GR led to recuperation of GCs response. Consequently, GR expression is essential to GCs response and reduced expression, or mutation of GR is involved in GCs resistance [508,509]. Indeed, overexpression of miR-130b was associated with resistance to GCs in the MM.S1 cell line (sensitive to CGs) by downregulating the GC receptor protein and its downstream effector, thus promoting cell survival. In cell lines, it was observed that GR is also regulated by miR-142-5p and miR-181a [508]. Indeed, miR-142-5p and miR-181a mimics inhibited GR expression, and miR-142-5p and miR-181a inhibitors enhanced its expression, showing the importance of those two miRNAs. Moreover, triptolide in association with dexamethasone, by suppressing miR-142-5p and miR-181a, enhanced apoptosis and, subsequently, GCs sensitivity in MM.1S cells, causing a significantly higher proportion of apoptotic cells when compared to these drugs used separately [509].

The miR-29b is a tumor suppressor miRNA found to be significantly reduced in bortezomib-resistant cells as well as in cells resistant to new proteasome inhibitors (PIs) such as carfilzomib and ixazomib [510,511]. The miR-29b targets PSME4, which encodes the proteasome activator PA200. It was observed that the expression of synthetic miR-29b impaired the growth of myeloma cells, both in cell lines and tumor patient cells, by decreasing the PA200 association with proteasomes and disrupting the aggresome/autophagosome formation. This highlighted the potential of synthetic miR-29b, synergistically enhancing the anti-myeloma effect of bortezomib [510]. Also, regarding bortezomib resistance, a negative correlation was found between miR-155 and the mRNA coding the proteasome subunit gene PSM β 5, resulting in bortezomib resistance [512].

IMiDs such as lenalidomide, thalidomide, and pomalidomide became active by binding and modulating the E3 ubiquitin ligase substrate recruiter cereblon and, therefore, stimulating the degradation of hematopoietic transcription factors IKZF1 and IKZF3 [513]. It was observed that AGO2, which plays a key role in miRNA maturation and function, is a cereblon binding partner and that steady-state concentration of AGO was determined by cereblon [514]. Interestingly, irrespective of cells sensitivity to IMiDs, by silencing AGO2 in MM cells, there was a marked reduction in the levels of AGO2 and several miRNAs, such as miR-103a-3p and miR-145-3p, accompanied by an extensive cell death, supporting the hypothesis of using AGO2 as a drug target to overcome IMiDs resistance in MM [514]. In another work, it was found that cereblon mRNA levels were inversely correlated with miR-30e and that overexpression of this miRNA significantly reduced cereblon mRNA levels in lenalidomide-resistant MM cell lines [515]. Additionally, BLIMP mRNA and protein levels were also reduced in miR-30e overexpressed cells, leading to XBP1 expression while suppressing cellular myelocytomatosis oncogene (c-MYC), influencing the cell differentiation axis and cell growth, respectively [515]. Indeed, MYC activation is involved in IMiDs resistance, although a definite mechanism is unclear. It was observed that MYC inhibits the expression of the tumor suppressor miR-22, and a low level of miR-22 expression is related to IMiDs resistance in MM patients by promoting cell survival and escape from NK cytotoxic activity. It was also verified that miR-22 mimics reduced MYC oncogenic activity and that the combination of lenalidomide and miR-22 mimics could restore IMiDs sensitivity in patients with poor response to this class of drugs [516].

Regarding monoclonal antibodies, namely daratumumab (anti-CD38), it was verified that the low expression of the tumor suppressor miR-26a could hamper treatment success [87]. In MM-sensitive cells, although CD38 persists at a low level during daratumumab treatment, a high-quality response is assured. Contrarily, and by a mechanism not yet clarified, in daratumumab-resistant MM cells that lose CD38, the restoration of CD38 levels did not correlate with daratumumab-induced cell death. Interestingly, in a MM xenograft mouse model, overexpression of miR-26a reduced CD38 expression but was sustained over time via direct targeting of CD38, triggering cell apoptosis and inhibiting cell proliferation. This could be therapeutically explored to maintain a low and persistent expression of CD38 in MM cells with increased treatment response [87].

Regulation of programmed cell death

In MM cells from patients and cell lines, the tumor suppressive miR-497 was found to be downregulated, and the anti-apoptotic protein Bcl-2 was upregulated. Overexpression of miR-497 in MM suppressed proliferation and induced apoptosis by directly targeting Bcl-2 [517]. In addition, miR-497 modulated the expression of Bcl-2 downstream apoptosis-related proteins, increasing the sensitivity of MM cells to the bortezomib [517]. With these results, it was suggested that a combination of miR-497 and bortezomib should be explored to potentially overcome drug resistance and become a new approach to treating MM [517].

The proapoptotic p53 was confirmed as a target of miR-125b, and its expression was augmented when miR-125b expression was inhibited by anti-miR-125b [518]. It was observed that p53 transactivated the proapoptotic miR-34a, which suppressed the sirtuin1 deacetylase, leading to persistent acetylation and inactivation of p53 [518].

Using antisense miR-125b enhanced the expression of p53, repressed expression of SIRT, and significantly improved the dexamethasone-induced apoptosis in MM cells [518].

The miR-221/222 family not only regulates MDR1 as previously reported but also has a major impact in modulating apoptosis [74,502]. In MM cell lines resistant to dexamethasone, increased miR-221/222 expression was associated with the downregulation of PUMA as a proapoptotic target [502]. Also, using a miR-221/222 vector, it was observed that MM.S1 cells became resistant to dexamethasone, while anti-miR-221/222 partially restored dexamethasone sensitivity in the MM.R1 (resistant to CGs) [502]. Moreover, increased levels of miR-221/222 and diminished PUMA expression were observed in MM cells from patients at relapse compared to controls, suggesting that miR-221/222 could be a good therapeutic target for these patients [502]. The miR-221 synthesis is regulated by the RelB-p52 complex of the NF- κ B pathway, to which resistant cells appear to be addicted [519]. RelB-p52 induces the expression of miR-221 and represses the expression of the proapoptotic gene BMF, also identified as a target of miR-221, by impairing the translation of BMF mRNA [519]. These findings revealed that one of the potential mechanisms of drug resistance induced by miR-221/222 is the promotion of anti-apoptotic pathways [502,519].

On the other hand, several miRNAs are associated with an augmented apoptotic effect of dexamethasone. For example, a combination treatment consisting of low-dose GCs and a synthetic vector of miR-150 had a synergistic apoptotic impact [520]. In fact, miR-150 showed additional effects through indirect mRNA regulation of GR-interacting transcription factors and hormone receptors, unfolded protein stress, and molecular chaperones, decreasing the likelihood of GCs resistance [520].

The miR-155 not only has an impact on bortezomib target resistance, as previously discussed but also on cell death drug resistance [512,521]. The miR-155 is present at small concentrations in drug-resistant MM cells and regulates CD47 (an integrin-correlated receptor) [521]. Overexpression of miR-155 is accompanied by a reduction of CD47 expression, leading to stimulation of programmed cell death and increased phagocytosis of MM cells by macrophages [521]. Ultimately, this allows to re-sensitize bortezomib-resistant cells, causing cell death by targeting the tumor necrosis factor- α -induced protein 8 (TNFAIP8), a negative controller of apoptosis [521].

The Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) protein is implicated in the progression of MM and drug resistance via the promotion of autophagy [355,522]. The miRNA-34a regulates MARCKS translation and is underexpressed in drug-resistant MM cells [522]. Indeed, overexpression of miR-34a decreases MARCKS expression, reduces cell survival in drug-resistant MM, and sensitizes resistant cells to anti-MM drugs [522]. In vitro studies showed that inhibition of MARCKS phosphorylation can overcome drug resistance in MM cell lines. Moreover, MARCKS phosphorylation induces the downstream activation of protein kinase B (PKB), and even when MARCKs is suppressed, the PKB/ mammalian target of rapamycin (mTOR) survival pathway is strongly aberrantly expressed in drug-resistant cells, promoting a pro-survival autophagic pathway mediated via a PKB-independent mechanism [522]. This suggests that bortezomib resistance could be only overcome with combined treatment, including MARCKS inhibitors (like miR-34a mimetics) and an autophagy antagonist (like chloroquine) [355,522].

Expression levels of miR-221/222 are inversely correlated with dexamethasone sensitivity. Dexamethasone treatment reduces the expression of miR-221/222 by targeting the autophagy-related gene 12/p27kip-mTOR (ATG12/p27-mTOR) autophagy regulatory axis, ultimately promoting autophagy and inducing cell death in dexamethasone sensitive MM cells [523]. Conversely, it was observed that miR-221/222 overexpression inhibits autophagy by negatively targeting ATG12 and p27, promoting dexamethasone resistance in MM cells [523].

Several other miRNAs are related to autophagy-mediated drug resistance. For example, miR-140-5p upregulation is associated with the suppression of autophagy in melphalan-resistant MM cells [524]. Additionally, miR-27 upregulates Notch1 expression by targeting the developmentally downregulated protein 4 (NEDD4), promoting the development of MM by inhibiting cell autophagy, stimulating proliferation and invasion of MM cells, and, subsequently, drug resistance [525].

Regulation of proliferation

Tumor suppressors miR-15a and miR-16 are known to directly control the cell cycle regulators targeting Cyclin D1 and D2 (CCND1 and CCND2), myeloid cell leukemia-1 (MCL-1) and Wnt family member-3 (WNT3) mRNAs in several hematological malignancies [526,527]. These miRNAs are often downregulated in MM. Indeed, the secretion of IL-6 by MM-BMSCs suppresses the expression of miR-15a and miR-16, reducing the sensitivity of MM cells to bortezomib [528,529]. Interestingly, miR-15a-transfected MM cells were arrested in the G1/S phase of the cell cycle, resulting in decreased growth and survival [528,529]. Thus, IL-6 secreted by BMSCs promotes drug resistance by suppressing miR-15a expression, thus protecting MM cells from bortezomib-induced apoptosis [529].

Expression of miR-27a-5p, also a tumor suppressor, is downregulated in bortezomib-resistant MM cell lines compared to normal MM cells and is known to target CDK5 [530,531]. MM cells transfected with miR-27a-5p have increased sensitivity to bortezomib activity, demonstrated by an increase in apoptosis levels [530,532].

Some miRNAs can overcome MM drug resistance [506]. For example, it was found that miR-26a inhibited CDK signaling in MM cells by targeting CDK6, resulting in cell cycle arrest at the G0/G1 phase [533]. Overexpression of miR-26a also enhanced the cytotoxic action of doxorubicin in MM L1 and U266 cell lines [533]. This could be used as a new approach for anti-MM treatment, mainly in combination with other drugs.

High expression of S-Phase Kinase Associated Protein 2 (SKP2) is associated with tumor cell growth and drug resistance in MM, mediating the ubiquitination of cyclin kinase inhibitor p27Kip1, a crucial negative regulator of the cell cycle [534]. The miR-21-5p was found to be inversely correlated to SKP2 [534]. Overexpression of miR-21-5p induced cell cycle arrest in MM drug-resistant cells by downregulating SKP2 gene expression, overcoming drug resistance in these cells [534]. Like miR-26a, a miR-21-5p mimic could be combined with bortezomib as a promising therapeutical approach to overcome bortezomib resistance in MM.

Regulation of DNA damage response

MiR-137 and miR-197 were found to be significantly lower in MM patients, while their overexpression is associated with enhanced apoptosis and suppression of tumorigenicity by targeting MCL-1 in MM cells [535]. The low expression of miR-137 on MM was due to epigenetic modification of regulatory sequences in miR-137 CpG islands [536]. Ectopic expression of miR-137 increased sensitivity to bortezomib and epirubicin and reduced tumor size in a xenograft model, suggesting its role as a potential tumor suppressor in MM [536]. Interestingly, this ectopic expression of miR-137 decreased the incidence of chromosome 1q21 gains and 1p22.2, 14q, 17p13 deletions, characteristically common in MM and associated with advanced disease and adverse prognosis [536]. Furthermore, Aurora kinase A (AURKA) was described as the direct target of miR-137, and in the absence of this miRNA, the AURKA protein upregulates the p-ATM/Chk2 levels, resulting in drug resistance [536]. AURKA also phosphorylated p53 at the serine 315 residues to facilitate MDM2-mediated degradation, promoting drug resistance [537]. Indeed, overexpression of miR-137 enhanced the expression of p53 and p21 but decreased p-ATM/Chk2 and p-BRCA1 expression and cleaved caspases -9, -7, -3 levels, modifying DNA damage repair pathways [536]. These findings suggest that aberrant miR-137 expression may have a significant impact on the AURKA/p53/ATM/Chk2 signaling pathway and contribute to drug resistance and chromosomal instability in MM patients.

One of the main deregulated DNA repair pathways in MM drug resistance is homologous recombination, which is critical for bortezomib-related resistance [538]. Rad51, a protein associated with homologous recombination, was shown to be low expressed in MM after treatment with bortezomib, suggesting an anti-DNA repair function of bortezomib [538,539]. The miR-520g and miR-520h are downregulated in bortezomib-resistant MM cell lines, and their upregulation decreased the expression of Rad51 by targeting the apurinic/apyrimidinic endonuclease 1 (APE1), a major DNA repair-related protein [540]. Combined overexpression of miR-520g and miR-520h reversed bortezomib resistance in MM through suppression of DNA repair [540].

Regulation of tumor cell metabolism and hypoxia

LDHA mediates aerobic glycolysis, promoting the growth and proliferation of MM cells. LDHA was described as the target of miR-489, and a potential negative correlation between miR-489 and LDHA in MM cells was identified. Overexpression of miR-489 inhibited aerobic glycolysis through the decrease of glucose uptake and reduction of lactate and ATP production, limiting MM tumor growth via the LDHA-mediated glycolytic metabolism [541]. Gain-of-function tests to overexpress miR-489 decreased MM cell viability, proliferation, and aerobic glycolysis by targeting LDHA, suggesting that miR-489 is a potential molecular target to prevent MM proliferation and overcome drug resistance when combined with other drugs [541].

The hypoxia in the MM microenvironment is responsible for the MM cell's quiescence, resistance to apoptosis, and glycolytic metabolic pattern. In fact, hypoxic MM cells acquired an anti-apoptotic phenotype and drug resistance by downregulation of IRF4, confirming that MM cells are not dependent on this oncogene. The hypoxia-inducible miR-210 downregulates the expression of ribosomal RNA methyltransferase DIMT1, suppressing IRF4 [542]. MYC and BCL2 are not repressed by the downregulation of DIMT1, suggesting that this methyltransferase regulates selective oncogenic targets [542].

Modulation of microenvironment

The influence of BMSCs among many players in the bone marrow microenvironment is critical for the progression of MM as well as for tumor growth, migration, and drug resistance. The interaction between BMSCs and MM cells reduced miR-15a/16 expression and promoted cell survival, a process mediated by the negative influence of IL-6 produced by BMSCs [528,529]. As described previously, miR-15a transfected MM cells were arrested in the G1/S phase of the cell cycle and secreted less VEGF than control cells [529]. Via suppressing miR-15a/16 expressions, IL-6 secreted by BMSCs promoted drug resistance in MM cells, mainly to bortezomib [528,529].

BMSCs downregulated miR-101-3p and upregulated survivin in MM cells. Overexpression of survivin increased the viability of MM cells in the presence of anti-myeloma drugs, leading to BMSC-induced drug resistance. Also, the miR-101-3p overexpression, or survivin silencing, prompted apoptosis and sensitized MM cells to bortezomib or carfilzomib in the presence of BMSCs, overcoming the stroma-induced drug resistance [543].

The interactions between MM cells and BMSCs downregulated miR-30-5p, a tumor suppressor, and enhanced the expression of B-cell lymphoma 9 (BCL9), a transcriptional coactivator of the oncogenic Wnt/ β -catenin signaling pathway, involved in MM cells proliferation, survival, migration, and drug resistance [544]. An inverse correlation was found between miR-30-5p and BCL9 levels in MM cell lines and in BM biopsies from MM patients. Indeed, H929 cells co-cultured with BMSCs presented miR-30-5p downregulation, with increased expression of BCL9, promoting MM progression and drug resistance [544]. Ectopic expression of miR-30-5p in MM cells downregulated the expression of CD44 in MM cells, essential in CAM-DR, restoring apoptosis and resensitizing the H929 cells to dexamethasone [544]. The findings confirmed that it is possible to modulate the bone marrow microenvironment and overcome CAM-DR.

Overexpression of miR-33b decreased MM PCs viability while increasing apoptosis and sensitivity to ixazomib [96]. The miR-33b blocks PIM-1, a protooncogene that promotes cell proliferation and survival, as well as homing and migration, through modification of cell cycle regulators, apoptosis mediators, and chemokine receptors, contributing to the overall antitumor activity of miR-33b in MM cells [96]. Notably, the upregulation of miR-33b can be triggered by ixazomib in the presence of BMSCs, overcoming the drug resistance mediated by BMSCs [96].

MM cell adhesion to BMSCs can also cause drug resistance when, for example, miR-202 is overexpressed in BMSCs, or miR-21 is upregulated in MM cells [545,546]. The miR-202 in BMSCs affects the growth and adhesion of MM by negatively regulating the BAFF [545]. It was found that BMSCs transfected with miR-202 had lower expression of adhesion molecules, including ICAM-1 (CD54) and VCAM-1 (CD106), inhibiting their adhesion to MM cells, losing their protective effect on MM cells, and suppressing CAM-DR [545]. Additionally, miR-202 inhibited the NF- κ B pathway, improving the response of MM to bortezomib treatment (probably because of its inhibitory effect on the reactivation of NF- κ B signaling) [545]. In turn, miR-21 upregulation induced by MM cell adhesion to BMSCs conferred an apoptotic resistance to anti-MM drugs [546]. Bortezomib induced miR-21 down-regulation in MM cells that adhered to BMSCs, and its use combined with dexamethasone and doxorubicin inhibited myeloma cell

survival, being more effective than drug treatment alone [546]. It was also observed that the RhoB gene is one of the targets of miR-21, and it is also involved in CAM-DR [546]. Indeed, miR-21 overexpression or inhibition may regulate RhoB expression in MM cells, conferring resistance to apoptosis or promoting a reduction in the invasiveness of MM cells, respectively [546].

Adhesion of MM cells to ECM fibronectin induced miR-182 expression and, consequently, decreased programmed cell death 4 (PDCD4) gene expression in MM cells [547]. Overexpression of miR-182 suppressed doxorubicin-induced cell death in H929 and MM.1S cell lines, whereas ectopic inhibition of this miRNA increased doxorubicin-induced cell death and partially eliminated CAM-DR in both cell lines [547]. Moreover, ectopic expression of miR-182 and PDCD4 downregulation enhanced AKT phosphorylation at Ser473 in both H929 and MM.1S cells, suggesting that PDCD4 down-regulation is required for CAM-DR [547].

Regulation of oncogenic pathways

Several miRNAs regulate different oncogenic pathways, such as MYC, AKT/ERK, and JAK/STAT3, which are crucial for the emergence of drug resistance in MM [548-553]. The proto-oncogene and transcription factor c-MYC has an essential effect on MM tumorigenesis and in drug resistance [548]. There is growing evidence that MYC and miRNAs interact significantly, with MYC controlling the expression of several miRNAs (with widespread miRNA suppression) and MYC being controlled by miRNAs (with sustained MYC activity and the accompanying MYC downstream pathways) [549,550]. While c-MYC repressed several miRNAs, including miR-15a/16-1, miR-26a, miR-29, miR-34a, and miR-151, and their function in regulating c-MYC mediated responses (apoptosis, survival, cell cycle arrest, metabolic alterations) have been thoroughly studied in lymphomas, it is still unclear how these miRNAs can regulate drug resistance in MM [550]. In MM, among many possible mechanisms, c-MYC may act via interaction with different targets, such as MCL-1 and survivin, both inhibitors of apoptosis, to cause drug resistance [550]. Indeed, it was observed that the tumor suppressor miR-29a is inversely correlated with MYC expression and regulates the p53 reactivating drug (PRIMA-1Met) response in hematological malignancies [550].

The miR-21 also plays a pivotal role in modulating intracellular signaling pathways involved in drug resistance. It was verified that upregulation of miR-21 targeted the tumor suppressor PTEN in MM cells and abrogated AKT/ERK signaling [552]. This suggested a role for miR-21 in MM drug resistance and the possibility of using miR-21 inhibitors to overcome drug resistance [552]. Moreover, miR-21 also promoted the oncogenic potential of JAK/STAT3 signaling pathway [553]. Indeed, in IL-6-dependent MM cells, miR-21 was induced by IL-6 in a strictly STAT3-dependent manner, and ectopic expression of miR-21 reduced MM cell apoptosis, even in the absence of IL-6, contributing to drug resistance [553]. In contrast, miR-197-3p increased drug sensitivity in bortezomib-resistant MM cells through inhibition of acetylation-mediated expression of IL-6 and inactivation of the JAK/STAT3 signaling pathway [554].

2.3. Response Assessment in Myeloma

The current MM response criteria were established in 2016 by the IMWG, updating the previous ones from 2006, to reflect the progress made in understanding the disease biology, improved diagnostic standards, use of sensitive and specific tools for predicting disease outcomes, increasingly effective treatment strategies, and better supportive care[555]. The assessment response criteria in MM have 3 objectives: to identify patients with primary resistance to treatment and greater risk of disease progression, to refine patient prognostication according to the depth of response, and to compare the efficacy of different therapies under investigation in clinical trials.

Assessing MM response is a complex task that requires expertise. It involves understanding multiple response levels and interpreting their implications for prognosis and treatment decisions. Traditionally, the evaluation of response in MM was based on the measurement of monoclonal protein concentrations present in serum and urine. As treatment continued to improve, new methods were needed to detect and quantify the level of residual disease beyond the detection of the clinical response criteria, strengthening the role of the measurable residual disease in the context of MM, using sensitive techniques such as NGS and NGF and immediately included in the last criteria revision[555]. Also, the understanding of the importance of extramedullary disease made possible the incorporation of new imaging techniques, such as PET and MRI, to be used to detect MRD along the NGS/NGF[555,556].

2.3.1. Primary refractory MM definition and its impact

According to the IMGW, refractory myeloma is defined as a disease that either does not respond during primary or salvage therapy or progresses within 60 days of the last treatment. Nonresponsive disease is characterized by either the inability to achieve a minimal response or the development of progressive disease (PD) during treatment. Refractory myeloma can be subclassified into "relapsed-and-refractory myeloma" and "primary refractory myeloma"[555,557].

Relapse-and-refractory myeloma is defined as a disease that is nonresponsive while on salvage therapy or progresses within 60 days of the last therapy in patients who have achieved minimal response (MR) or better at some point previously before then progressing in their disease course. In turn, primary refractory myeloma is defined as a disease that is nonresponsive in patients who have never achieved a minimal response or better with any therapy. It includes patients who never achieve MR or better in whom there is no significant change in M protein and no evidence of clinical progression, as well as primary refractory (called "non-responding-nonprogressive"), and those PD where patients meet criteria for true PD ("progressive")[555,557].

The impact of a lack of response to initial treatment on survival outcomes has also been studied in several clinical trials and real-world experiences, with different inclusion criteria and combinations of drugs. Earlier protocols based on bortezomib, associated with alkylating agents, showed 12% to 26% of induction failure with bortezomib, cyclophosphamide and dexamethasone, and bortezomib, melphalan, and prednisolone, respectively[558]. Additionally, a retrospective study found comparable rates of induction failure in patients treated with PIs and

IMiDs as first-line therapy, with 16% experiencing failure, compared to 25% in those who did not receive at least one of these agents[559]. Despite the lack of specific robust data on this population, results using more potent triplets and quadruplets incorporating moAbs appear enthusiastic. A recent retrospective study from the Mayo Clinic showed a rate of 6-7% of patients being primary refractory, with 7.4% for bortezomib, lenalidomide, and dexamethasone, 4.4% for carfilzomib, lenalidomide, and dexamethasone, and 2.5% for patients treated with a protocol including moAbs[560]. Indeed, over the years, it has observed better response rates for patients treated with moAbs in addition to the PI/IMiDs backbone or in alkylating-containing protocols and decreased odds for suboptimal responses.

Observations from these studies suggest that patients who do not respond to induction therapy are not a homogeneous population regarding prognosis or clinical features[559,561]. Factors predisposing to primary refractory MM remain undefined. While high-risk cytogenetics predicted survival outcomes, it was not associated with primary refractoriness[559]. This is consistent with the results of the ability of novel agents and different combinations to overcome traditional sources of resistance[332]. Several risk factors, including aneuploid clones and mutations of specific oncogenes and cell signaling molecules, have been associated with first-line treatment failure. Nonetheless, many of those were identified before the everyday use of novel therapies, and whether they continue to hold prognostic value in the current era requires additional investigation[559,561].

In several studies, induction failure was found to be an independent adverse prognostic factor for PFS and OS, and it retained its significance after adjusting for other prognostic factors in multivariate models (including high-risk cytogenetics, compromised performance status, extramedullary disease, advanced ISS/RISS, renal failure, and elevated lactate dehydrogenase). Although the percentage of nonresponders has substantially decreased over the years, reflecting better frontline treatment, the impact on survival continues to be pronounced, with a median PFS of less than 18 months and median OS of less than 40 months[560,562].

Currently, there are no definitive guidelines for the management of patients who fail induction in both transplant-ineligible ndMM and transplant-eligible patients. These primary refractory patients are frequently excluded from clinical trials, and studies targeting this population are highly anticipated. CART-T cells could be considered in earlier lines of therapy, especially for patients with primary refractory disease who could be refractory to many drugs from the first line. Recent findings from the CARTITUDE-2 trial, which reported a 12-month PFS rate of 90%, demonstrated the promising effectiveness of cilta-cel in patients receiving only one prior line of therapy[563].

2.3.2. From Complete Response to Negative Measurable Residual Disease

In MM, complete response (CR) is defined as negative immunofixation on the serum and urine, along with the disappearance of any soft tissue plasmacytomas and <5% plasma cells in bone marrow aspirates. An upgraded level was defined as stringent CR (sCR) with a normal sFLC ratio and absence of clonal cells in bone marrow biopsy by immunohistochemistry[555].

The association between CR and PFS has been more consistent than between CR and OS. This association appears to be dynamic and with several new inputs over the years due to knowledge of disease biology, risk stratification, different treatment strategies after reaching a CR, and the proper depth of response beyond the conventional approaches. Initially, the statistical correlation between CR and improved outcomes was predominantly observed in patients who underwent high-dose therapy followed by ASCT, in which sCR and CR were more frequently attained in these patients than those treated with conventional chemotherapy[564]. Indeed, in this setting of patients, a meta-analysis reported a significant correlation between achieving a CR and improving OS in eight out of ten studies[565]. However, with the introduction of new agents, the frequency of CR increased, and it became possible to show the prognostic impact of CR achievement in patients treated with nonintensive therapy. Furthermore, combinations of new agents with or without high-dose therapy followed by ASCT, consolidation, and prolonged maintenance therapy increased the proportion of patients achieving CR, translating into improved long-term outcomes compared with lesser profound responses [566-568].

The same trend was observed in patients achieving more profound responses, defined by sCR[568]. Nonetheless, the impact of sCR is debatable when analyzing only the patients who achieve CR, as the superiority of stringent CR over conventional CR still needs to be confirmed in larger prospective studies. It was shown that response assessment according to the sCR criteria for MM patients in CR does not predict different outcomes, highlighting the limitations of immunohistochemistry when polyclonal normal PCs mask low numbers of clonal PCs and showing that the sFCL test is insensitive to the monoclonal or polyclonal nature of light chains and can be influenced by the oligoclonal bands that emerged in the context of immune regeneration[569,570].

Moreover, most patients who achieve CR will relapse, reflecting the persistence of residual cells that remain undetected by conventional techniques and the suboptimal sensitivity of the standard or stringent CR criteria. Introducing more sensitive methods to detect and quantify measurable residual disease (MRD) was necessary to obtain a better prognostic correlation. The level of MRD, undetected by conventional methods, became the most important feature contributing to the link between the depth of response and long-term outcomes[232,571-575]. In the IMWG 2016 consensus criteria, there are four definitions of MRD negativity (flow, sequencing, imaging, and sustained), and independent of the method used to define it, MRD-positive patients consistently have an inferior PFS than MRD-negative ones[555].

Bone marrow examination has been the cornerstone of disease assessment in the absence of a measurable monoclonal component in the serum or urine, as such, to define CR to treatment (CR or sCR). To increase the

accuracy of the detection of MM cells, sensitive assays have emerged for the evaluation of bone marrow aspirates, including multiparametric flow cytometry (MFC) such as basic MFC or next-generation flow (NFG), and molecular methods such as allele-specific oligonucleotide (ASO)-qPCR, droplet digital PCR (ddPCR), and next-generation sequencing (NGS) of VDJ sequences[576,577]. NFG and NGS became the most used methods of examining hundreds of thousands to millions of bone marrow cells (or the corresponding amount of DNA) per assay and can provide a quantitative assessment of any residual tumor cells in the BM[555,578].

Multicolor flow cytometry (MCF) uses an antibody panel to detect and quantify immunophenotypically aberrant and clonal plasma cell populations in BM aspirate samples. The immune profile of the aberrant plasma cells is stable over time with possible minor alterations from clonal evolution[579]. The typical immunophenotypic profile of MM PCs includes the combined high expression of CD38 and CD138, which are traditionally combined to identify the MM PCs. Many other markers show aberrant expression in MM PCs: CD19, which is typically absent; CD27, CD45, and CD81, which generally exhibit lower or negative expression compared to normal/reactive PC; and CD56, CD28, and CD117, which are overexpressed in 60-75%, 15-45%, and 30-32% of cases, respectively. Cytoplasmic κ and λ light chains are also used as clonality markers[580,581]. The diversity of flow-based protocols for detecting MRD in MM leads to significant variability in the reported results. The EuroFlow group designed, optimized, and validated a monoclonal anti-body panel for MRD monitoring in MM, together with a new sample processing protocol that allows for the analysis of a higher number of cells cost-effectively, known as “next-generation flow cytometry” (NGF). This panel combines the analysis of 10 markers (CD38, CD138, CD45, CD19, CD27, CD56, CD81, CD117, cytoplasmic Ig κ , cytoplasmic Ig λ) in two independent tubes analyzed by 8-color MFC[582]. With this approach, MRD with a sensitivity of 10^{-6} can be detected in most samples when at least 10 million events are examined, and the IMWG currently recommends it as the reference immunophenotypic method for the evaluation of MRD in MM[555]. The assay includes markers for the distinction between normal and clonal PCs but also allows the detection of mast cells, erythrocytes, and B-cell precursors to assess sample quality and hemodilution as internal controls[583,584]. MFC can be used at any time point, without the need for a baseline profile, and has a quick turnaround time and can, therefore, be used to inform clinical decision-making in real-time. However, MFC needs direct processing, usually within 24 hours, to preserve viable cells, and for optimal sensitivity, 10 million cells need to be analyzed[584-586]. Additionally to MDR, it can provide a comprehensive immune profile of the bone marrow microenvironment, including T cells, NK cells, B cells, and macrophages[587]. Recently, patients receiving anti-CD38 moAbs present an additional challenge, as anti-CD38 moAbs can interfere with MCF analyses and plasma cell identification. To address this, several assays, including EuroFlow, are developing solutions such as alternative CD38 multi-epitope and nanobody conjugates and other plasma cell markers like CD229, CD319, and VS38c[588-590].

Next-generation sequencing (NGS) takes advantage of the unique V(D)J sequences created during B-cell development. As there are approximately 10^{11} possible variations of IGH rearrangements, it is highly unlikely that there would be an identical V(D)J sequence independently in the malignant cell clone and any normal plasma cell in the same bone marrow sample. Also, The V(D)J sequence is preserved throughout the disease course and clonal evolution, making it ideal for monitoring MRD over time[591-593]. Several protocols have been developed to sequence Ig receptors using this technique, including ClonoSEQ by Adaptive and LymphoTrack by Invivoscribe,

consisting of a consensus multiplex PCR where combinations of IGHV and IGHJ primers allow the amplification of (virtually) all rearrangements present in the sample. The PCR is followed by next-generation sequencing and sophisticated bioinformatic computational analysis[592,594]. NGS allows for the detection of clonality at baseline. Typically, a threshold of 5% of identical sequences among all the other detected sequences is used to define clonality. After therapy, resistant tumor cells can be traced using the patient-specific clonotype, and tumor burden can be quantified using a spike-in strategy[595,596]. NGS assays are highly sensitive for MRD tracking (10^{-6})[591-593,596]. In addition to its high sensitivity, NGS does not require live cells, can be run on stored samples, and can be used to explore the complex MM genomic landscape for research purposes and individual risk assessment[576,577,597]. However, knowledge of the baseline sequence is necessary, and there is a longer turnaround time compared to NGF, usually 5–7 days[576,577].

As mentioned, NGF and NGS have advantages and disadvantages regarding utility and sensitivity. Nonetheless, as long as the sensitivity is high, the prognostic impact of MRD negativity has been corroborated regardless of the method used. Medina et al. compared EuroFlow NGF versus NGS (LymphoTrack) and found a high level of concordance, $R^2 = 0.905$, and similarly, Ho et al. found a 93% concordance between NGS and high-sensitivity MCF[598,599]. Recently, a head-to-head comparison between NGF and NGS MRD was prospectively evaluated in the phase II FORTE trial, with a biological agreement of 87% at the 10^{-5} and 83% at the 10^{-6} cut-offs and with a remarkable prognostic concordance, hazard ratios in NGF-MRD and NGS-MRD-negative vs -positive patients were 0.29 and 0.27 for PFS and 0.35 and 0.31 for OS, respectively[600].

A meta-analysis with more than 8000 patients with MM confirmed the prognostic benefit of MRD negativity in improving long-term survival[601]. The benefit of MRD negativity retained its independent value in different clinical contexts, being predictive irrespective of the disease setting (ndMM, rMM, ASCT, and non-ASCT), the sensitivity thresholds, the cytogenetic risk, and the treatment protocol. The PFS and OS benefit with MRD negativity was observed in patients who achieved MRD negativity at any sensitivity level compared to MRD-positive patients, with increased benefit with the higher sensitivity level of MRD negativity. Indeed, the most significant benefit in PFS and OS was seen in patients who were MRD-negative at the 10^{-6} sensitivity level. In the meta-analysis, the benefit of MRD negativity in long-term outcome survival was also significant in patients with relapsed/refractory disease[601]. This is encouraging as many novel therapies, such as bispecific antibodies and CAR-T cell therapy, induce deep responses in the relapse setting with MRD negativity above 25%[602-604]. Notably, the same meta-analysis and other studies showed that MRD negativity was able to overcome the unfavorable prognosis associated with high-risk cytogenetics. Nonetheless, the achievement of MRD negativity, as well as of sustained MRD negativity, is less frequent in high-risk patients than in standard-risk patients [601].

This meta-analysis also showed that MRD status impacted the outcome of patients, irrespective of serological responses, with the three-year PFS being similar in patients who achieved VGPR and CR and were MRD-negative. This suggests that MRD assessment should also be considered in patients in VGPR as modern combination therapies and novel agents can quickly clear the bone marrow, while the serological response, due to factors such as the longer half-life of paraproteins, may take longer to achieve [601]. The opposite and unexpected discordance

has also been described, in which the M protein persists in patients with undetectable MRD[575]. Several explanations were postulated, including lower sensitivity of some MRD methods, inadequate sampling of tumor burden in a single BM aspirate, long half-life of immunoglobulins, oligoclonal responses after treatment or IVIG supplementation, interference of therapeutic monoclonal antibodies, immature clonotypic cells secreting identical immunoglobulins, and patient reclassification as achieving less than CR because of unavailable measurements in clinical trials[605-608]. It was also noticed that discordances persist with high-sensitivity NGF or NGS and survival of patients with negative MRD, but persistent M protein is similar to that of the remaining patients with undetectable MRD, highlighting that the probability of false-positive detection of M protein is greater than that of a false-negative MRD test[575].

The analysis of two phase 3 studies on daratumumab plus standard-of-care regimens for the treatment of transplant-ineligible ndMM showed that negative MRD was associated with longer PFS, and that benefit is further enhanced in patients who achieve durable MRD negativity. The data highlight the prognostic value of MRD durability lasting ≥ 12 months, in line with IMWG criteria, and support the clinical relevance of assessing MRD durability of shorter duration (≥ 6 months). MRD negativity and durability were linked to improved PFS across all treatment regimens, with daratumumab-based therapies more effective in achieving and maintaining MRD negativity for ≥ 6 and ≥ 12 months. It suggests that daratumumab-based therapies may induce longer MRD negativity and more profound responses, possibly due to continuous exposure to daratumumab alone or in combination with lenalidomide[609]. Another recent study evaluating MRD dynamics during ixazomib maintenance showed that MRD status is dynamic, and its prognostic value increased considerably with periodic vs single assessment, in which conversion from MRD-negative to MRD-positive or from MRD-positive to MRD-negative status during maintenance modulates the risk of disease progression. Patients who lost MRD-negative status had a poor PFS similar to patients with persistent MRD-positive[610]. Higher circulating tumor cell levels, ISS 3, and longer time to first MRD negativity were found to be predictors of MRD resurgence and/or PD in patients with MM. These predictors of resurgence are easily measurable, suggesting that more frequent MRD assessments are indicated in these patients and may aid in identifying patients who are eligible for treatment cessation once MRD negativity is reached. Employing MRD to guide clinical decisions in MM is promising, and different approaches in this field have already been investigated. Several prospective clinical trials incorporated MRD in fundamental clinical decision-making time points, such as the duration of induction therapy, upfront ASCT, and the intensity and continuation of maintenance therapy[611].

However, current NGF and NGS techniques for assessing MRD only focus on bone marrow, potentially missing residual disease located elsewhere. These methods do not consider the spatial heterogeneity of myeloma, which is conferred by the patchy infiltration of bone marrow plasma cells and the possible presence of extramedullary disease[612,613]. In this context, whole-body imaging techniques such as PET-CT or MRI provide important complementary insights into residual disease after therapy once both are sensitive approaches to detect focal lesions, extramedullary disease, and diffuse tumor infiltration pattern in the BM[612,614].

¹⁸Fluorine-fluoro-deoxyglucose (¹⁸F-FDG) PET/CT is currently the gold standard for evaluating and monitoring the metabolic response to therapy in MM[556,615]. In assessing responses to treatment, several cohorts have been evaluated, showing the prognostic value of PET-CT in MM, with a correlation between a negative result with good responses and a prolonged survival rate. In patients achieving a CR, FDG-PET/CT negativity after ASCT predicted a lower risk of progression or death compared to patients with metabolically active lesions[556,615-617]. However, approximately 10–15% of patients with active MM may have a false-negative PET-CT result since the lack of hexokinase enzyme reduces the ¹⁸F-FDG avidity of plasma cells, and alternative markers (choline, methionine, thymidine, fluciclovine) have been proposed[617,618]. Moreover, different studies confirmed the complementarity of PET-CT and BM MRD techniques. It was observed that patients who were both NFG-MRD negative and PET-CT negative had the best PFS compared to patients who were NGF-MRD negative but PET-CT positive or vice versa[619]. These observations underscore the importance of integrating BM assessments and imaging techniques to fully evaluate MRD in MM.

MRI can detect both MM bone involvement, with the ability to describe the infiltration pattern by plasma cells (normal, focal, diffuse, heterogeneous), and the presence of extramedullary disease. It has been shown to be more sensitive and specific than PET-CT for detecting focal lesions and diffuse infiltration before significant bone destruction is present. Nonetheless, focal lesions may take time to disappear even in responding patients, thus making traditional MRI a less desirable tool for MRD assessment than PET-CT[620,621]. More recently, whole-body diffusion-weighted MRI (DWI-MRI) emerged as a promising option for response assessment with several advantages over PET-CT once it lacks exposure to radioactive agents, has better sensitivity to identify patchy infiltration of abnormal PC in the BM, and it does not depend on the level of hexokinase expression in MM PCs[620,622].

In this light, whole-body imaging techniques (PET-CT and MRI) assume a significant role in evaluating the MRD “outside” BM, helpful in capturing the patchy infiltration of the bone marrow by plasma cells and the presence of extramedullary disease. NGF and NGF consolidate their role in evaluating MRD “inside” BM. Taken together, these techniques should be used in a complementary, rather than alternative, fashion [623-626].

More recently, much attention has been given to mass spectrometry (MS) in peripheral blood in MM patients to evaluate residual disease using monoclonal proteins. Classical PB-based methods, such as serum protein electrophoresis and immunofixation that assess for the monoclonal immunoglobulin product in MM, do not have sufficient sensitivity as the monoclonal protein spike fades into the polyclonal background with lower levels of disease. By contrast, MS can detect the presence of the plasma cell clone at a heightened sensitivity via the mass-to-charge ratio of its secreted paraprotein, making it uniquely suited to assess MRD[627-629]. Currently, two techniques for MS have arisen for analyzing monoclonal immunoglobulins. The top-down MS methods directly detect intact HCs or LCs isolated from M proteins by reduction, which are the main components of M proteins and are regarded as alternative biomarkers of M proteins. These methods are further divided into the matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) with a limit of detection of 0.05g/dL and the liquid chromatography (LC) quadrupole time-of-flight MS, which achieves a higher sensitivity with a limit of detection of

0.005g/dL. In contrast, the bottom-up MS method uses specific peptides of M proteins as surrogate markers for analysis, known as the clone peptide method[628,630,631]. In 2021, the IMWG recommended using MS methods in MRD assessment and assisting in defining the time point for classic MRD assays with bone marrow samples[627]. In the GMMG-MM5 and STAMINA trials, MS positivity after treatment had a significant negative impact on PFS even in CR patients, suggesting that positivity does not just reflect residual circulating monoclonal protein but derives from treatment-resistant tumor cells that constitute a source of disease relapse[632,633]. In the GMMG-MM5 trial, it was also shown that MS is superior to conventional response monitoring, mainly when performed sequentially and combined with baseline disease features and as a complementary assay to the MRD testing from the BM or imaging-based techniques[632]. Additional research is required to validate the role and determine the optimal timing of MS in disease evaluation protocols.

Recently, the use of PB has been an attractive method for MRD to overcome the dependence of the patchy infiltration and tackle the spatial heterogeneity of MM, as well as avoid the invasiveness of repetitive bone marrow aspirations. These prompted the development of several methods for evaluating tumor components of PB as liquid biopsies, which have been explored with promising results.

3. LIQUID BIOPSIES AS A SOURCE OF BIOMARKERS FOR DRUG RESPONSE/RESISTANCE

3.1. Use of biomarkers in MM

Although the MM treatment landscape has substantially changed over the last years, as previously described, treatment decisions are primarily based on clinical trials, in which most patients are treated using the conventional one-size-fits-all therapeutic approach. It is undoubtedly clear that there is extensive heterogeneity, not only between patients but also within patients, resulting from complex pathophysiology, genetics, and clonal evolution over time. This heterogeneity presents numerous opportunities for optimizing risk-adapted therapies, which should be based on clinically applicable biomarkers. Indeed, integrating these biomarkers into clinical practice could enhance the precision and adaptability of therapeutic strategies, ultimately leading to more effective and patient-specific treatment solutions with better outcomes.

A biomarker is classically defined as any characteristic, such as a gene, protein, clinical variable, or imaging feature, objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Disease-related biomarkers assist in diagnosis, prognosis, and response monitoring. Conversely, drug-related biomarkers indicate whether a drug will be effective in a specific patient and how the patient's body will process it[634]. A robust biomarker should identify patients whose diseases are highly likely to be cured with the standard treatment alone, making additional therapy unnecessary despite its potential efficacy. Likewise, when further therapy is required, predictive biomarkers should help to determine which patient subpopulations will benefit significantly from new targeted therapies and which will not[635].

In MM, research on biomarkers is advancing across various domains. Firstly, biomarkers are being developed for patient risk stratification and prognosis, providing guidance on survival outcomes and informing the selection or titration of therapeutic approaches. Secondly, specific biomarkers can distinguish between aggressive and indolent disease evolution, allowing for early intervention in individuals most at risk of progressing from precursor states to MM. Lastly and probably most exciting, biomarkers are being identified to indicate the effects of therapeutic interventions, predict sensitivity or resistance, and correlate with treatment outcomes[636-639].

3.1.1. Established biomarkers in MM

The systemic monoclonal protein is the hallmark of MM and the most straightforward and accessible biomarker. Paraprotein levels are routinely monitored in serum and urine in secretory MM, as previously described, with an elevated level prompting the diagnosis, indicating progressive disease or no response. Although M protein levels greater than 3g/dl or above 500mg/day in the blood and urine, respectively, are used to differentiate between MGUS and myeloma, the quantitative levels of M protein are not an exclusive marker for myeloma as can be seen in others plasma cell dyscrasia[555]. The ratio of free light chains of the monoclonal immunoglobulin is a reliable

prognostic marker for diagnosing and following patients with nonsecretory and oligo secretory myeloma and light chain MM and is an indirect measure of clonality. It was rapidly included in the IMWG response criteria, although normalization of the ratio unequivocally demonstrates superior prognostic value compared with CR[555,569,640]. More recently, heavy/light chain (HLC) assays have added sensitivity to monitoring disease response and eventually detecting residual disease. The HLC ratio reflects the balance between monoclonal and polyclonal immunoglobulins of involved and uninvolved isotypes, considering the polyclonal plasma cell suppression or expansion that occurs with the treatment and could be used as a noninvasive response marker[555,641].

Myeloma cells are characterized by aberrant glycolysis. Elevated levels of lactate dehydrogenase (LDH), an enzyme involved in anaerobic cellular metabolism, are a prognostic marker in MM, in which values ≥ 300 IU/L have been shown to correlate with lower overall survival and failure to respond to conventional MM therapy[636,639]. Although it was included in the risk prognostication score R-ISS, LDH still lacks specificity. The same happens with low albumin and high β_2 -microglobulin included in the ISS and the subsequent revisions of this score once they can be altered accordingly by several other pathological conditions[206,642].

Cytogenetics and FISH biomarkers appear to be the most valuable biomarkers currently used in MM, which can change treatment decisions, even if there are conflicting results. Reasons for these conflicts include retrospective analysis, small sample size, variable techniques, lack of a standard definition of high-risk, and failure to control for other biological or treatment processes that may confound the outcome. Nonetheless, some of these abnormalities are included in the R-ISS and R2-ISS[206,642]. In general, t(14;16), t(14;20), and del(17p) are associated with a poor prognosis, whereas t(11;14), t(6;14), and hyperdiploid MM are considered to impart standard risk, provided there are no additional adverse features such as amp1q21 and del(17p). Trisomies are typically associated with a better prognosis, except when associated with gains of 1q and monosomy of chromosome 13. Gain of 1q has been linked to adverse prognosis in patients treated intensively with or without IMiDs[643]. Some of these biomarkers have treatment-specific prognostic implications and change patient approach; for instance, patients with t(4;14) and del(17p) appear to benefit from PI-based first-line therapy followed by tandem ASCT[644]. Likewise, these high-risk patients could benefit from double strategies in the maintenance phase[645,646]. Additionally, t(11;14) became the first biomarker to prompt a tailored therapy in MM with higher and deeper responses in venetoclax-based protocols[237,647].

Paralleling the improvements in laboratory techniques, significant advances have been made in imaging biomarkers, mainly MRI and PET, as described in evaluating MRD. Compared with the traditional skeletal survey, the significant advantages of both techniques are discrimination between normal and invaded bone marrow, visualization of extramedullary disease and cord involvement, and better sensitivity for lytic bone lesions. It was found that the number of focal lesions at baseline on MRI or 18F-FDG PET/CT negatively impacted PFS and OS[556,615]. Specifically, at baseline, more than three PET focal lesions and more than seven MRI focal lesions (observed in 32% and 36% of ndMM patients, respectively) were associated with shorter PFS and OS[648]. Additionally, the presence of extramedullary disease (EMD) and failure of FDG suppression before the ASCT also adversely affected outcomes[556,615].

3.1.2. Potential novel biomarkers in MM

Biomarkers of bone turnover are attractive noninvasive tools for detecting early bone involvement and evaluating the risk of skeletal morbidity and response to antiresorptive treatments[636,638]. Urinary N-terminal cross-linking telopeptide of type-1 collagen (NTX), C-terminal cross-linking telopeptide of type-1 collagen (CTX), C-terminal cross-linking telopeptide of type-1 collagen generated by metalloproteinase (ICTP) levels are elevated in patients with MM and correlate with advanced osteolytic disease, reflecting osteoclast-mediated degradation of collagen. Furthermore, urinary NTX and serum ICTP correlate with risk for skeletal complications, PFS, and OS[649]. Regarding bone formation, high levels of procollagen type-1 N-propeptide and procollagen type-1 C-propeptide signify new bone formation, as well as elevated levels of RANKL and osteoprotegerin[650,651].

Numerous proteins have been identified as pro-angiogenic factors, such as VEGF, fibroblast growth factor (FGF), angiopoietins, and hepatocyte growth factor (HGF)[637,639]. Out of the above angiogenic activators, VEGF is the most established, studied, and potent factor in association with angiogenesis in MM, which demonstrated a potential diagnostic and prognostic marker[652,653]. The experimental downregulation of VEGF by chemotherapeutic agents was associated with a significant reduction in cellular proliferation and an increase in apoptotic myeloma cells[654,655]. Additionally, high VEGF plasma levels were negative prognostic markers and were associated with a lower OS in MM patients receiving treatment[653]. It was also noted that FGF, HGF, and angiopoietin-2 were correlated with progression from MGUS to MM, which can be added to the established guidelines to improve risk stratification models for MGUS patients[639].

Proteomic analysis using two-dimensional electrophoresis and MALDI-TOF-MS revealed deregulated expression of numerous proteins in MM patients, including haptoglobin, kininogen 1, transferrin, serum amyloid A protein, plasma kallikrein, integrin alpha-11, apolipoprotein A-I, sulfhydryl oxidase 1 and isoform-1 of multimerin-1[656-658]. Notably, the up-regulation of proteins associated with protein folding and proteasome functions, such as proteasome activator complex subunit 1, heat shock protein 90, stress-induced-phosphoprotein 1, and protein disulfide-isomerase, are correlated with refractory response bortezomib-based therapy, while the proteins functioning during inflammation and apoptosis were downregulated in patients not responding to bortezomib-based therapy[659]. On the other hand, the up-regulation of other proteins, such as Zinc-a-2-glycoprotein, amyloid-A protein, and vitamin D-binding protein, plays a role in predicting thalidomide response in MM patients[660,661].

MM patients have longer telomeres than controls, with higher TERT amplification detected and presenting a significant association with poor disease prognosis[662]. For instance, alterations of the telomere structure were specific to different phases of MM and correlated with the aggressiveness of the disease, and several studies linked the risk, outcome, prognosis, disease heterogeneity, cytogenetic status, and OS in MM cases with telomerase activity and telomere length, suggesting the potential diagnostic and prognostic value of telomere in MM[663].

Global gene expression (GEP) integrates the influence of multiple genetic abnormalities on critical cellular pathways associated with proliferation, differentiation, apoptosis, and other biological features in a single signature. The University of Arkansas was the first to define a 70-gene classifier (GEP70) characterizing 7 distinct gene

expression clusters that identified patients with high risk for short PFS and OS[664]. This was validated by other international groups and included in the HOVON65/GMMG-HD4 trial that expanded it to 92 genes (EMC-92-gene signature) capable of distinguishing between patients with high-risk and low-risk for relapse[665]. This GEP provides valuable information regarding disease biology in the context of clinical trials, but more work is required to define a standardized and simpler GEP signature that can be more widely applied as a prognostic tool in clinical practice[666,667].

Over the years, the introduction of several immune-based approaches to treating MM has revolutionized the field, making it one of the most investigated areas for novel MM therapeutics. To achieve this, efficient predictive immune biomarkers are required[668,669]. The pretreatment expression of CD38 was correlated with a better outcome to daratumumab, while lack of response and tumor progression was associated with expression of both CD55 and CD59[670]. High CD64 in MM patients treated with bortezomib, thalidomide, and dexamethasone were associated with an inferior median OS. Also, high-density neutrophils isolated from the PB of ndMM patients showed upregulation of CD64 and downregulation of CD16, which were associated with increased immune suppression[671].

Neoantigens, a group of antigenic products of somatic mutations specific to tumors, are potential immune biomarkers in MM. Recent data suggested that high somatic mutation and neoantigen burden were not only correlated with decreased PFS in MM patients but also increased in rrMM compared to ndMM patients[672,673].

Some of the players in the immunosuppressive microenvironment in the bone marrow in MM are also suggested to have a role as potential biomarkers, especially cytokines and chemokines that have disturbed expression[674]. Elevated levels of Th2 cytokines such as IL-4 and IL-10 while the Th1 associated cytokines IFN- γ were significantly lower, suggesting the polarization of cells towards Th2 response in MM[675]. IL-6 has a significant role in the final differentiation of B cells to mature plasma cells, and it is known to protect myeloma cells against dexamethasone-induced apoptosis by activating protein tyrosine phosphatase. Also, it activates osteoclasts and promotes defective bone physiology, measuring the extent of bone disease in myeloma patients[676]. Nonetheless, these interleukins are not specific to MM, and it is difficult to use them as biomarkers widely[674]. Additionally, PD-1 expression is higher in the NK and T-cells of MM patients, with higher expression in PB found in rrMM patients, suggesting it to be used as a non-invasive biomarker[677].

Still, in the BM microenvironment, various extracellular matrix proteins were specifically expressed at different phases of MM, thus representing a potential diagnostic and prognostic tool. Proteins like ANXA2 and LGALS1 are expressed more in MM, and their abundance is associated with a decreased OS[678]. Similarly, laminin subunit beta-1 (LAMB1) and integrin subunit alpha 9 (ITGA9) are dysregulated in MM and have shown prognostic value and clinical correlation in MM patients[679]. However, further research focusing on validating the sensitivity and specificity of these markers is required[678,679].

3.2. Liquid biopsies in MM

Most of these biomarkers result from the MM manifestations and intricate relations based on its pathophysiology or event genetics background, failing to represent direct the plasma cells or have the clinical impact that MRD evaluated in the BM has achieved. Currently, no minimally invasive testing of MRD is available. An ideal test would directly measure markers of MRD or disease during routine follow-up, be minimally invasive, represent multi-site infiltrates, and allow for simultaneous analysis of disease burden. Known as liquid biopsy, the possibility of probing the molecular landscape of tumors via a blood draw or other non-invasive samples, with significant implications for research and patient care, has attracted remarkable interest[680].

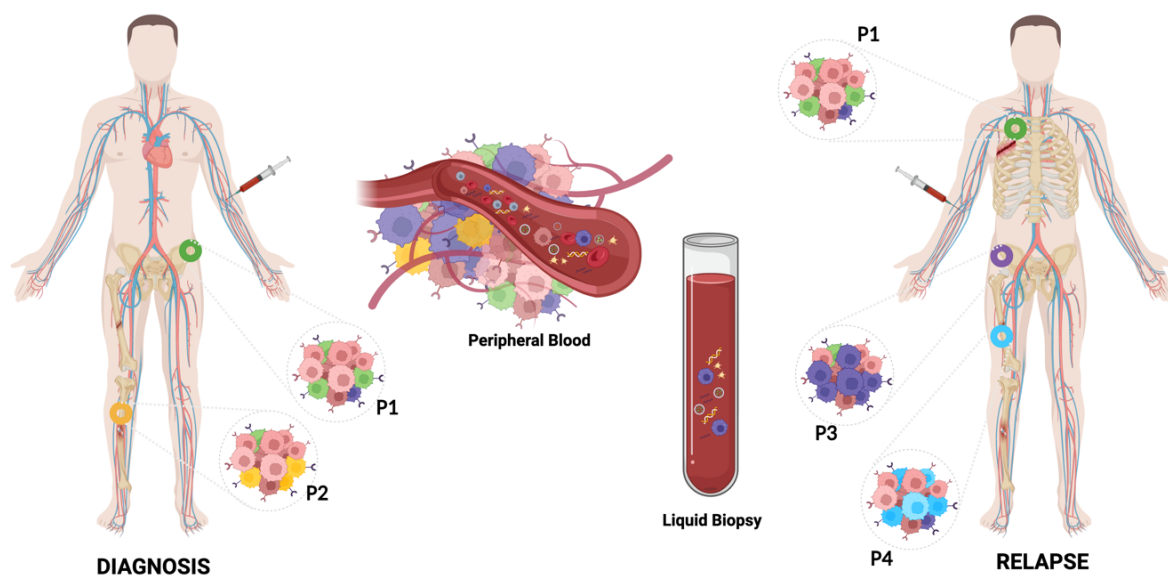


Figure 5. The role of liquid biopsy in tracking disease evolution in MM. At diagnosis, distinct MM clones are present with the bone marrow at different specific sites, identified as P1 and P2. At relapse, new or dominant clones, such as P3 and P3, may emerge, indicating clonal evolution and disease spread. Additionally, original old clones, like P1, may still be present at relapse even if they are differently located compared to diagnosis. As the disease progresses, MM PCs or genetic material from these clones can enter the bloodstream and be detectable through liquid biopsy. This method provides a painless and non-invasive way to frequently repeat sample collection, enabling longitudinal monitoring of MM and overcoming the challenges that surge with the temporal and spatial clonal heterogeneity of MM. “Created with BioRender.com”.

MM is characterized by a spatial heterogeneity marked by a patchy infiltration in the bone marrow and extramedullary sites, in which cytogenetic alterations and mutations can occur differently. Therefore, single-site bone marrow aspirates do not address this spatial heterogeneity[681,682]. Also, MM presents a significant degree of subclonal heterogeneity, including site-specific subclones, harboring different genetic abnormalities conferring therapy resistance and becoming more challenging over time, representing a dynamic evolution of the genetic profile throughout disease progression and/or relapse[192,682,683]. Because of this spatial limitation and dynamic evolution, liquid biopsy as a flexible tool for characterization and follow-up emerges as one of the most essential tools in the MM approach. Moreover, they allow for a non-painful, non-invasive, and frequently repeated sample collection optimal for an MM longitudinal follow-up[680,684-687] (Figure 5).

In MM, a range of tumor components can be isolated from the blood as liquid biopsies. These include circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), tumor-educated platelets (TEP), extracellular vesicles (EVs), and microRNAs[684-693] (Figure 6).

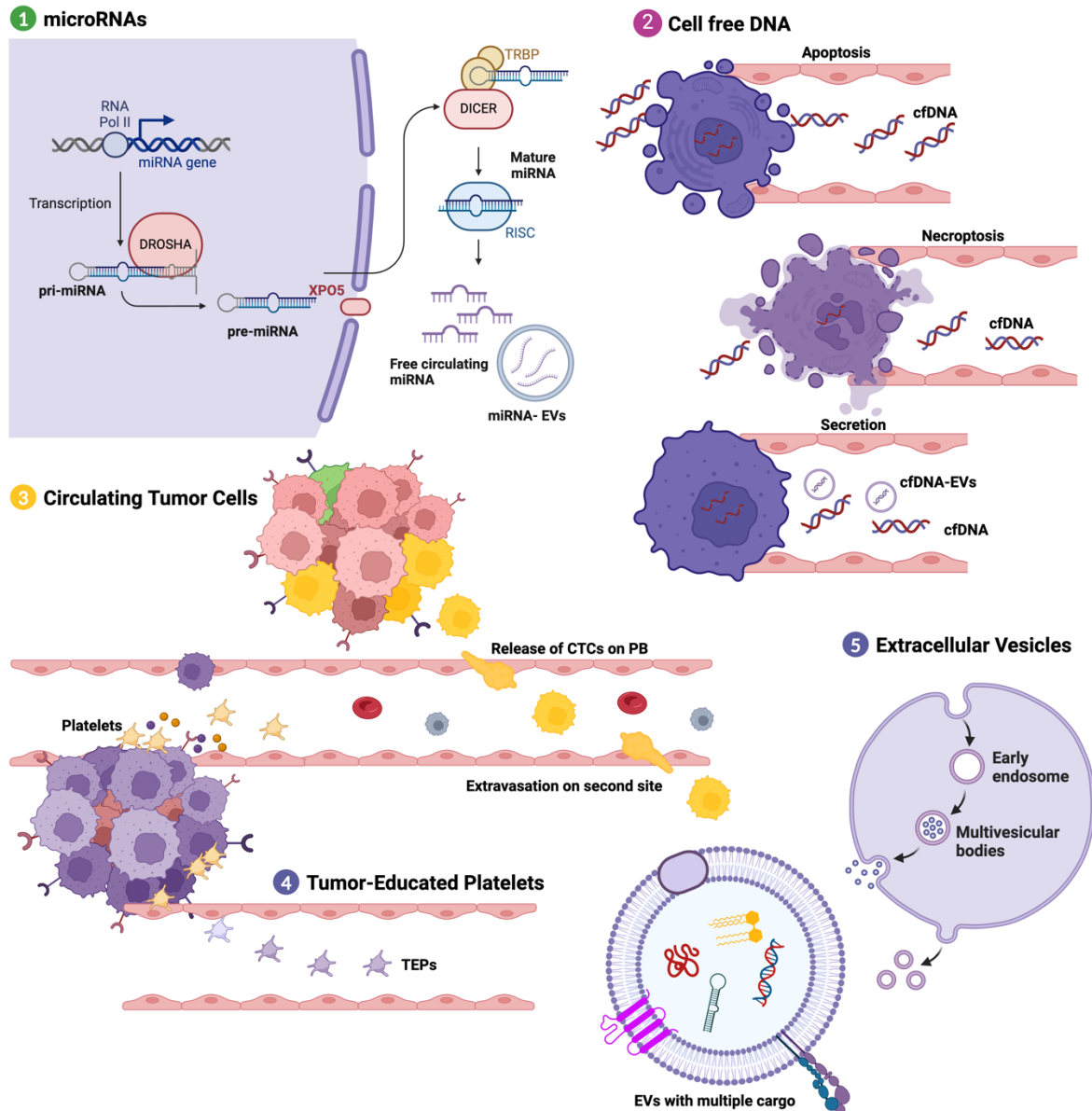


Figure 6. Types of liquid biopsies used in MM. Several tumor-derived components can serve as biomarkers in liquid biopsy for monitoring MM, including microRNAs, cell-free DNA (cfDNA), circulating tumor cells (CTCs), tumor-educated platelets (TEPs), and extracellular vesicles (EVs). 1) MicroRNAs, small non-coding RNAs involved in gene regulation, are processed and released into the bloodstream within EVs or freely, serving as essential biomarkers for diagnosis, prognosis, and monitoring treatment response. 2) CfDNA, released from dying cells through apoptosis, necroptosis, and ferroptosis, carries tumor-specific genetic alterations that provide insights into tumor dynamics. 3) CTCs, cancer cells shed from the primary tumor into peripheral blood, play a crucial role in metastasis and serve as a critical indicator of tumor burden and prognostic surrogate. 4) TEPs, which have altered RNA profiles due to interactions with tumor cells, offer additional diagnostic and monitoring potential. 5) EVs, membrane-bound particles released by cells, carry a variety of cargo, including proteins, lipids, RNAs, small non-coding RNAs (such as miRNAs and long non-coding RNAs), and DNA fragments, making them valuable tools for diagnostic, treatment response, and prognostic analysis in MM based on their content. "Created with BioRender.com".

3.2.1. Circulating Tumor Cells

One of the hallmarks of malignant tumors is their ability to spread beyond the primary tumor site and cause disease dissemination at secondary locations. In MM, CTCs are essential in this process, in which they are discharged from the BM into PB and return later to the BM in different positions or homes in different locations outside BM, similar to a metastatic process[694-697]. They are identified in virtually every MM patient with active disease when using sensitive techniques, and their phenotype should match that of the BM PCs[694,696,697]. MM CTCs exhibit phenotypically immature characteristics with decreased adhesion molecules CD56 and CD117 and expression of the integrins CD11a, CD11c, CD29, CD49d, and CD49e. This phenotype is associated with a reduced ability to interact with the BM environment, favoring the escape into the PB[696-698]. CTCs also appear to act as MM stem cells, overexpressing the stem cell marker CD4 and by being in a quiescent state with increased clonogenic potential, favoring the presence of EMD[699]. It was found that MM CTCs showed overexpression of genes associated with hypoxia and epithelial-mesenchymal transition-related processes, contributing to the egression of CTCs out of their original BM niche and disease spreading[699,700].

Several methods to identify CTCs have emerged over the last few years, either via cytometric techniques or nucleic acid-base approaches. These methods have different targets and markers, making it difficult to standardize the CTC evaluation approach[686,687].

Immunofluorescent-based methods, including diffuse in vivo flow cytometry" (DiFC) and enrichment-free fourplex immunofluorescence associated with single-cell DNA sequencing, confirmed the brief half-life of CTCs in the blood, probably due to the constant detachment of CTCs from BM, as well as that CTCs presents the same genomic modifications that were present in BM plasma cells[701-703]. The combination of FISH and NGS identified a more significant number of CTCs in all phases of MM compared to normal controls, the number of PCs in the NM, the amount of the monoclonal component, and the ISS staging[704]. Using NGF to evaluate aberrant phenotypes identified for highly purified fluorescence-activated cell sorting of CTCs and correlating it with FISH abnormalities, it was found a concordance between BM tumor cells and CTCs was high for chromosome arm-level copy number alterations ($\geq 95\%$), though not for translocations (39%), supporting the ability of CTCs as non-invasive risk-stratification tool of MM patients. In the same study using whole exome sequencing (WES), in which EMD samples were compared to CTCs and BM PCs, it was observed an 87% concordance between the mutation profiles in EMD and CTCs[705]. Furthermore, CTCs might be associated with the development of EM disease, given the high degree of mutational concordance between EMD PCs and CTCs [705,706]. This is of particular value because it is often challenging to conduct an EMD biopsy in locations that are difficult to reach. Other studies showed that CTCs mutations at a single-cell level agreed with BM PCs, mainly when employing broader approaches such as WES, in which CTCs presented up to 93% of genetic alterations identified in BM PCs. In addition, there was a near-100% concordance between clonal mutations detected in BM and CTCs, whereas 16% of the somatic variants were subclonal and only detectable in either BM or CTCs[707].

The low absolute CTCs counts in PB of MM patients and their brief half-life (minutes to hours) make their isolation and genetic approach challenging[708,709]. CTCs from PB are isolated using an immunomagnetic enrichment

method targeting the CD138, CD45, or CD38 antigens. However, CD138 expression in CTCs is lower than the BM PCs, and CD38 and CD45 can have a heterogeneous expression on malignant PCs, representing a limitation to using enrichment methods to capture CTCs[697,710]. Additionally, the phenotype of malignant PCs can change over time following therapy, potentially complicating the detection of CTCs using NGF during relapse[711].

Clinically, the levels of CTCs were identified to be prognostically important, and high numbers contribute to faster progression and inferior survival in MGUS, SMM, and MM patients[712-715]. CTCs can be detected as early as the MGUS stage in 59% of patients, reaching 100% detection in both the SMM and MM stages[716]. The primary difference between these conditions lies in the CTC count, which increases from 0.0002% (0.008 CTCs/ μ L for MGUS) to 0.004% (0.16 CTCs/ μ L for SMM) and 0.04% (1.9 CTCs/ μ L for overt MM)[717]. A cut-off of 0.01% displayed an independent prognostic significance with a hazard ratio of 2.02 in multivariate survival analysis, including lactate dehydrogenase concentrations, ISS, and cytogenetic analysis. MM patients with undetectable CTCs had longer PFS regardless of CR and MRD status, whereas patients with detectable CTCs only obtaining MDR negativity had longer PFS[215]. It was also seen that a high number of CTCs in SMM were correlated with an elevated risk of progression to MM in 2–3 years after diagnosis and that patients with MM with CTCs above 0.01% presented a high risk of progression and relapse[712].

Importantly, CTCs are already included in phase III clinical trials as biomarkers for response to therapy. A 99.6% reduction in CTCs count was observed from diagnosis to post-ASCT. Notably, patients with ISS III showed decreased CTCs after undergoing ASCT, with no significant reduction following induction treatment alone[718]. It is hypothesized that CTCs might contribute to chemoresistance, as they may evade standard therapy and lead to residual disease that remains undetectable with current methods[719].

3.2.2. Circulating tumor DNA

Nucleic acids are discharged into the PB after cellular death due to apoptosis, necrosis, or even spontaneous liberation of DNA/RNA-lipoprotein complexes. It was also suggested the release of cell-free DNA (cfDNA) and circulating-tumor DNA (ctDNA) was associated with extracellular vesicles[720-722]. Most of the cfDNA in PB derives from hematopoietic cells, and ctDNA is usually present in very small quantities, according to the tumor and stage of the disease[723,724]. In most healthy individuals, the concentration of cfDNA in the blood ranges between 0 and 100 ng/mL, while in cancer patients, it typically increases to concentrations between 0 and 1000 ng/mL[725]. It has a short life (4-30 minutes) and is rapidly eliminated by the hepatic and renal systems and circulating DNase[726]. However, plasma cfDNA levels can remain stable for 24 hours when properly stored and, in some methods, up to several days[686,687]. Any condition where there is destruction or death of non-tumoral cells, such as inflammation, infections and trauma, enhances cfDNA concentrations[686]. Serum contains 20 times more cfDNA than plasma, mainly due to the destruction of white cells, inducing background interference, and reducing the cfDNA deriving from the tumor[686,687,708].

Several research groups have investigated the use of cfDNA for mutation profiling in MM using different techniques with different sensitivity, making it difficult to find a standard method. Most studies use targeted gene sequencing,

while droplet digital PCR (ddPCR) and WES are used to a lesser extent[686,687,708,727]. Deep sequencing of ctDNA from MM is an extremely sensitive method that recapitulates patterns corresponding to BM aspirates, while ddPCR is used to analyze mutational status and track MM progression[728,729]. The proportion of variants found in DNA from BM detected in matched cfDNA samples varies between 30% and 100%, with most studies observing a concordance between BM and PB above 80%[686,728,730,731]. Using a hybrid-capture-based LB Sequencing (LB-Seq) technique employed to evaluate all protein-coding exons of BRAF, EGFR, KRAS, NRAS, and PIK3CA in cfDNA samples from MM patients, it was possible to identify ctDNA at incidences as low as 0.25%. This enabled the identification of 49 out of 51 somatic mutations with subclonal hierarchies that reproduced tumor profiling with a concordance of 96%, while other mutations were likely skipped in the BM aspirate[732,733]. It is also noted that nearly all studies report variants that are exclusively present in cfDNA and not detected in matched BM-DNA, which is coherent with the spatial heterogeneity of MM[728-734]. Another study demonstrated major differences in the mutation profiles of BM and EM MM sites and matched cfDNA in a patient with EMD. The cfDNA analysis permitted the detection of variants in BM/EM tissue while revealing unique variants detected in neither of the tumor tissues. Furthermore, ctDNA presented a better concordance to the EM tumor compared to the BM aspirate [735,736]. All this suggests that studying ctDNA enables the understanding of subclonal hierarchies resembling BM samples and allows the capture of variants from distant tumor sites inaccessible by a single-site bone marrow aspirate, representing a strategy for comprehensive mutation profiling in MM patients.

Progression of predecessor MM conditions, such as SMM, presents lower amounts of cfDNA than overt MM. cfDNA levels were higher in the GEP70 high-risk subgroup than the low-risk one and were associated with different classical biomarkers, including beta2-microglobulin and LDH. It was also observed that in serial analyses of cfDNA levels in SMM, there was an increase in allele proportion of mutated KRAS as cfDNA increased, appointing this as a possible tool to predict progression in monoclonal gammopathies[737]. Interestingly, tumor fraction in cfDNA was significantly associated with the clinical stage of the disease, with a higher tumor fraction observed in cases with rrMM and ndMM compared to MGUS and SMM[738].

Additionally, the longitudinal tracking of ctDNA can be informative in detecting MM disease progression/relapse and appears to have prognostic ability in ndMM and rrMM. Changes in ctDNA level precede changes in serum FLC, permitting an earlier detection of disease progression, specifically 3 to 9 months before serological changes. This is particularly important in FLC escape, non-secretory, or oligo-secretory MM, where there is a lack of effective serological markers [728,733,738]. Studying the RAS/RAF pathway and TP53 in BM plasma cells, it was observed that the amount of cfDNA and percentages of genetic alterations were remarkably higher in rrMM than in ndMM patients. Furthermore, patients with more than two mutations and TP53 alterations presented a significantly shorter OS. Also, cfDNA evaluation recognized a greater occurrence of DNA-repair gene-altered subclones than BM examination[739]. A recent trial on patients with rrMM receiving ixazomib, lenalidomide, and dexamethasone showed ctDNA mutations, particularly those in TP53 and KRAS, are stronger prognostic predictors than bone marrow plasma cell mutations in these patients. These findings allowed the authors to create a simplified risk stratification model using ctDNA mutations. It was observed that a higher number of ctDNA mutations (≥ 2

mutations), an increased number of prior therapies (≥ 3 regimens), and high plasma DNA concentration were independently associated with a shorter PFS[734].

Because ctDNA abundance can reflect the entire tumor burden, ctDNA detection and quantification are useful for MRD monitoring[734]. Moreover, analysis of cfDNA is emerging as a promising approach to MRD outside BM by detecting the spatial genetic heterogeneity that hampers the outcome in several patients by being associated with chemoresistance[736,738,740,741]. A study evaluating cfDNA by allele-specific oligonucleotide real-time quantitative PCR (ASO-qPCR) highlighted a relevant connection between the amount of tumor-specific cfDNA concentrations with specific clinical settings such as induction treatment and ASCT. Not only do levels of cfDNA change in reaction to therapy, but the overall distribution of cfDNA fragment lengths also changes, especially in patients with the best response. Moreover, despite the low number of samples with MFC measurements, the MRD status determined using cfDNA also corresponded to results acquired by MFC better than M-protein levels[740]. A different study demonstrated the usefulness of cfDNA in accessing MRD in patients with EMD, in which the concordance of plasma ctDNA to extramedullary tumor concordance was much higher than that of BM aspirates[736]. However, other studies question its strength to be used on MRD. In one study, it was observed that there was a lack of a connection between ctDNA and BM for the MRD by NGS, employing only IGH gene rearrangements and that ctDNA may be missed in more than half of the MM patients with MRD-positive in the BM using NGS or NGF[738,742]. Additionally, it was found that 39% of patients with less than a VGPR showed the presence of ctDNA, indicating that the release mechanisms of M protein and ctDNA into the bloodstream operate independently of each other[743]. This suggests that ctDNA alone may not serve as a routinely applicable marker of disease status in MM in these conditions, although there is increasing evidence that it might be a helpful tool for MM monitoring[686,687,744,745].

3.2.3. Tumor-educated platelets

Platelets are anucleated cell fragments derived from megakaryocytes in the BM. They are released into the bloodstream and are known to play a major role in hemostasis, thrombosis, immunity, inflammation, and cancer. Platelets can influence the development and progression of cancer through several mechanisms - platelets aggregate around tumors, promoting growth and evading immune elimination; platelets facilitate tumor cell adhesion, allowing them to evade killing by the immune response; platelet activation enhances tumor invasion and metastasis by synthesizing lipid products, releasing proteins from alpha granules, inducing epithelial-mesenchymal transition, promoting angiogenesis, and facilitating tumor cell resistance and extravasation[746].

Additionally, platelets can alter their RNA profile following interaction with tumor cells or tumor-associated biomolecules, operating as tumor-educated platelets (TEPs)[691,746]. This can occur directly by transferring tumor-derived RNA via EVs or CTCs or indirectly by releasing signals that regulate mRNA processing or uptake of plasma proteins that play important roles in tumor growth and neovascularization, including VEGF, basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)[691,746,747]. In the presence of tumor cells and tumor microenvironment, these changes lead to the conversion of immature platelet mRNA into mature mRNAs that are

translated into functional proteins, ultimately resulting in the generation of TEPs with tumorigenic function[691,746].

The identification of TEPs in PB of cancer patients rapidly emerged as a potential diagnostic tool, as platelets are one of the most abundant cells within the PB and have been routinely isolated for decades using simple, cost-effective procedures in contrast to CTCs, ct/cfDNA and EVs[686,691,708].

While no results are available from dedicated studies of TEPs and MM, numerous correlations between platelets and MM have been identified. It was demonstrated that platelets isolated from MM patients have differential expression in more than 3000 genes, whose expression was predominately upregulated (2200 genes). Platelets showed increased basal activation, implying that RNA modifications may reflect those seen with platelet activation by conventional agonists and not tumor-specific signals[748]. Significantly higher concentrations of sP-selectin, a marker of platelet activation, are found in ndMM patients compared to healthy volunteers, suggesting it is a valuable marker of response to therapy[749]. In another study, a low mean platelet volume was found to be correlated with worse outcomes in MM subjects and may be employed as a relevant marker prognosis[750].

3.2.4. microRNAs

MicroRNAs (miRNAs) have been detected in various body fluids, including plasma, serum, saliva, urine, and semen, that can be reached without invasive techniques. As a result, circulating miRNAs have been considered potential non-invasive biomarkers for several cancers, including MM, with promising applications in diagnosis, prognosis, and monitoring treatment response[61,64,65]. MiRNAs contribute to the biogenesis and progression of MM, including tumor survival, proliferation, migration, angiogenesis, and transformation of the nearby normal cells. In addition, several mechanisms of drug resistance have been identified as being controlled by upregulation or downregulation of miRNAs: regulation of drug transporters and associated proteins, of drug targets, regulation of programmed cell death and cell proliferation, of DNA damage repair, of oncogenic pathways, of tumor cell metabolism and hypoxia, and microenvironment modulation. From disease staging to drug resistance, miRNAs have been found to be predictive of patient outcomes.

The biogenesis of miRNAs is a multistep and highly coordinated process involving several molecular participants that start in the nucleus and end in the cytoplasm, where several post-transcriptional modifications occur[751-753]. The canonical biogenesis pathway begins with the transcription of miRNA-coding genes, normally by polymerase II, into transcripts with a hairpin structure called long primary miRNAs (pri-miRNAs). These pri-miRNAs are subsequently cleaved and processed into precursors miRNAs (pre-miRNAs) by the microprocessor complex, the Drosha-DiGeorge syndrome critical region 8 (DGCR8) complex, consisting of the ribonuclease (RNase) III endonuclease, Drosha, which cleaves the pri-miRNAs 5' and 3' arms, and its co-factor DGCR8, a double-stranded RNA-binding protein that recognizes the precise site of pri-miRNAs cleavage[751-755]. The pre-miRNAs, which are stem-loop RNAs, are then exported to the cytoplasm by the Ran-Guanosine-triphosphate (GTP)-dependent Exportin-5 (XPO5). In the cytoplasm, the RNase enzyme Dicer associated with TAR RNA binding protein (TRBP) cleaves the pre-miRNAs, creating duplexes with 19e25-nt known as miRNA:miRNA*. These duplexes are unwound,

resulting in two single strands. The resulting strand with lower 5' stability or 5' uracil, named the "guide strand", is generally the strand that preferably turns out to be the mature miRNA[752,754]. The miRNA-induced silencing complex (miRISC or RISC) is then assembled through the association of the guide strand with Argonaute (AGO) family proteins[751,753]. The strand that is not retained in/associated with AGO proteins, known as the "passenger strand" (or miRNA*), is normally degraded by the cells' machinery[754]. The mature miRNA, stabilized via the miRISC complex, is then guided to its target mRNAs, where it binds mainly to the 3'-untranslated region (3'UTR). However, the miRISC complex can interact with other regions of the target mRNAs, namely the 5'UTR or the coding sequences. The 2-8 nucleotides from the miRNA's 5' end, named the "seed sequence," are essential for this process of miRNA's target recognition[751-755]. Depending on the degree of complementarity between miRNAs and their target mRNAs, miRNAs can have different regulatory actions: (i) if there is full complementarity, the target mRNA is degraded/ cleaved; (ii) if there is partial complementarity, a repression of the target mRNA translation occurs. Partial complementarity is the case most observed in humans[751,752].

In addition to these canonical miRNAs, other non-canonical miRNAs have been found, e.g., mirtrons, small nucleolar RNA (snoRNA) fragments, and 5'-capped miRNA precursor-derived 3p miRNAs, resulting from non-canonical biogenesis pathways, which have been named the "Drosha—or Dicer-independent biogenesis pathways" since they do not require these proteins[751,752,754].

Functional miRNAs are important cellular players because they regulate critical cellular events, including cell cycle, proliferation, cell death, differentiation, and metabolism[61,65,756]. Their expression can be dysregulated due to genetic/cytogenetic alterations, epigenetic modifications, single nucleotide polymorphisms in miRNAs regulatory networks (miRSNPs), deficiency in the miRNA biogenesis machinery, or extracellular factors/components. The dysregulation of miRNA expression is associated with disease development and initiation, including cancer. Depending on their target mRNAs, miRNAs can act as tumor promoters (named "oncomiRs") by inhibiting the expression of tumor suppressors or as tumor suppressors (named "tumor suppressor miRs") by reducing the expression of oncogenes. Thus, oncomiRs are often upregulated, and tumor suppressor miRs are frequently downregulated in cancer. Interestingly, some miRNAs are found to act always as oncomiRs or tumor suppressor miRs in cancer, in a tumor type-independent manner, but other miRNAs present a context-dependent role, acting as oncomiRs in some tumors and as tumor suppressor miRs in others[751,753,755].

In MM, miRNAs show consistent expression patterns in PB and BM aspirates[80,757]. This makes miRNAs valuable for diagnosing MM, monitoring treatment response, and identifying relapse through less invasive peripheral blood sampling rather than relying solely on bone marrow aspirates. Notably, regarding PB, serum and plasma cannot be automatically assumed to be interchangeable concerning miRNA concentrations, as these are higher in plasma than in serum. However, plasma processing to remove subcellular/cellular components can reduce miRNA concentrations to serum ones[758]. The circulating miRNAs are stable at room temperature for 24 hours and when frozen or refrigerated for up to 72 hours, making them accepted and attractive biomarkers[758,759]. While PB is a valuable source of miRNAs, other body fluids, such as urine, are particularly interesting in MM due to their pathophysiology and clinical manifestations[760].

As discussed, several miRNAs were dysregulated in MM and its asymptomatic pre-malignant stages (Table 3). **An essential tool for differentiating MM patients from healthy controls and MGUS patients is the observation of distinctive circulating miRNA patterns in MM patients compared to MGUS patients and healthy donors.**

For instance, serum analysis revealed a combination of up-regulated miR-34a and down-regulated let-7e could distinguish MM from healthy donors with a sensitivity of 80.6% and specificity of 86.7%, and from MGUS with a sensitivity of 91.1% and specificity of 96.7%[761]. Circulating miR-720 and miR-1246 were increased in the serum of MM patients, while miR-1308 was decreased. Further research revealed that the combination of miR-720 and miR-1308 was beneficial in differentiating between pre-symptomatic stages and MM patients from healthy controls and patients with unrelated disorders. Furthermore, the expression of miR-1246 and miR-1308 may help to identify MGUS from MM patients[762]. The expression of miR-202 was significantly higher in MM patients compared to healthy controls, and this diagnosis of MM patients used miR-202 had a sensitivity and specificity of 80.0% and 60.0%, respectively[763]. In the same way, it has been reported that the level of miR-4449 present in the serum of MM patients is significantly higher than in healthy controls. This miRNA could be helpful for MM diagnosis when used along with FLC and β 2-microglobulin[764]. Indeed, various patterns have been identified that enhance the accuracy of MM diagnosis. For instance, samples obtained from patients with both MGUS and MM revealed that miR-181a/b, miR-21 and miR-106b-25 were over-expressed in MGUS cases compared to healthy controls. Further, miR-181a/b, miR-21, and miR-106b/25 were up-regulated in both MGUS and MM patients. Nonetheless, miR-32 and miR-17-92 were detected exclusively in MM patients, suggesting a potential role for these miRNAs in the progression and transformation of MGUS to MM[61,65,637,756].

Table 3. Overview of the potential role of miRNAs as biomarkers for the diagnosis of MM

microRNA	Expression changes	Sensitivity	Specificity	Ref.
miR-34a	↑ in MM vs HC	80.6%	86.7%	[761]
Let-7e	↓ in MM vs MGUS	91.1%	96.7%	[761]
miR-202	↑ in MM vs HC	80%	60%	[763]
miR-4449	↑ in MM vs HC	79%	91%	[764]
miR-125b-5p	↑ in MM vs HC	86%	96%	[64]
miR-490-3p	↑ in MM vs HC	60%	85%	[61]
miR-29a	↑ in MM vs HC	88%	70%	[64]
miR-203	↓ in MM vs HC	83%	83%	[64]
miR-30d	↓ in MM vs HC	89%	63%	[61]
miR-483-5p	↑ in MM vs HC	58%	90%	[64]

↑—increased, ↓—decreased

Several miRNAs were observed to be associated with some of the analytical parameters, clinical manifestations, and staging. A study on circulating miRNAs showed that lower levels of miR-744, let-7d, miR-130a, and let-7e were related to higher creatinine and β 2-microglobulin levels and associated with advanced ISS stage. However, no correlation was observed with the infiltration of BM by PCs in MM and MGUS[761]. Levels of miR-125b-5p were associated with extramedullary infiltration and were significantly higher in stage III patients compared to stage I/II patients[765]. Likewise, plasma miR-483-5p level in MM patients was also correlated with the ISS stage[766]. It was found that miRNAs in the miR-29 family generally decrease in the urine of kidney disease patients but increase in the serum of MM patients. These miRNAs were closely related to the number of marrow plasma cells and the level of M-protein[767,768]. Another study showed that miR-134-5p, miR-6500-5p, miR-548q, and miR-548y in urine, found to be reduced in MM patients in comparison to healthy controls, were negatively

correlated with the total urinary light chain and positively correlated with serum albumin, becoming potential noninvasive diagnostic biomarkers for MM[760].

In the same way, there seems to be a **relationship between alterations in miRNA expression patterns and specific MM cytogenetic abnormalities that impact prognosis**[769]. The overexpression of miRNAs located at 1q, including miR-1231, miR-205, miR-215, miR-19a, and miR-488, was correlated with chromosome 1q gain, which is observed in more than 50% of patients and associated with poor prognosis[770,771]. For instance, miR-215 targets MDM2, a negative regulator of p53, and IGF-1 and IGF-1[771]. Regarding chromosomal losses, low expression of miR-221 was associated with the presence of del(13q), highlighting the role of chromosome 13 deletion as a prerequisite for tumor clonal expansion. Therefore, this finding suggests a potential tumor-suppressor role for miR-221 despite its known function as an oncogene in various cancers[772]. Moreover, del(13q14) in MM correlated with lower levels of let-7e, miR-744, and miR-19a[761]. Regarding miR-19a and other members of the miR-17-92 cluster (miR-17 and miR-20a), their association with the advanced ISS stage is probably due to their chromosome location on chromosome 13q31.3, which is very close to the RB tumor suppressor gene on 13q14[770]. Also located in the same region, the miR-15a/16 cluster is reported to be downregulated in MM and contributes to MM pathogenesis by promoting cell growth and angiogenesis in the BM[773]. Curiously, for miR-744, in which the gene lies in the 17p12 region, close to the TP53 gene (17p13), there was no relationship between the levels of miR-744 and deletion of TP53, characteristically associated with very poor prognosis, even with new drugs available[761]. Conversely, miR-22, encoded at the 17p locus, is significantly less expressed in those MM patients with the 17p deletion[774]. Multiple different miRNA expression patterns have been identified concerning translocations involving the IGH gene, although they are not always consistent across different studies. A higher expression of miR-133a was seen in MM with t(14; 16), while downregulation of miR-135b and miR-146a in MM with t(4; 14), which target genes involved in the IL-1 signaling pathway[775]. Overexpression of miRNAs from the let-7e family, miR-125a-5p, and miR-99b were also associated with t(4;14) [771,772].

As presented earlier, **miRNA profiles can serve as prognostic markers for drug response or resistance** (Figure 4, Table 4). For example, a study examining the miRNA profiles of patients before and after drug resistance to bortezomib, thalidomide, and lenalidomide found that downregulation of serum exosomal miR-16-5p, miR-15a-5p, miR-20a-5p, and miR-17-5p was associated with bortezomib resistance[776]. This highlights how miRNAs can be used as biomarkers to decide on treatment and personalized approaches.

Table 4. Overview of the potential role of miRNAs in drug resistance and sensitivity in MM.

Drugs	Resistance		Sensitivity		Ref.
	Upregulated	Downregulated	Upregulated	Downregulated	
Bortezomib	miR-451, miR-22-3p, miR-155, miR-21	miR-29b, miR-155, miR-34a, miR-15a/16, miR-27a-5p, miR-137/197, miR-520g/mir-520h, miR-101-3p	miR-631, miR-497, miR26a, miR-21-5p, miR-202, miR-197-3p	-	[439,502-504,510-512,517,519,521,522,525,529,533,534,536,538,543,546,554]
Izaxomib	-	miR-29b, miR-101-3p	miR-33b	-	[96]
Carfilzomib	-	miR-29b	-	-	[510,511]
Lenalidomide IMiDs (including Lenalidomide)	miR-30e	miR-22		miR-103a-3p, miR-145-3p	[514] [515,516]
Daratumumab	-	miR-26a	-		[87]
Melphalan	miR-451	miR-221/222, miR-15a/16,	-	-	[386,524]
Dexamethasone	miR-130b, miR-221/222, miR-221, miR-21	-	miR-150	miR-142-5p, miR-181a	[502,508,518,520,523]
Multidrug	miR-19a, miR-125b, miR-34a, miR-210, miR-21	miR-489, miR-30-5p	-	-	[89,326,499,503,522,542,544,546,550,553]

Besides being a diagnostic marker or associated with specific MM characteristics associated with prognosis, such as cytogenetics, numerous miRNAs have been suggested to predict survival outcomes

(Table 5). A pioneering study by Wu et al. showed a significant association between high levels of miR-17 and miR-886-5p and decreased OS in ndMM patients[777]. This allowed the construction of an outcome classifier to divide patients into three risk groups. The first group, classified as high risk, consists of individuals with elevated expression levels of both miRs, accounting for 13.5% of the total patients with a median OS of 19.4 months. Next, the middle risk includes patients with high expression levels of either of the miRs, representing 54% of the cases, with a median OS of 40.6 months. Lastly, the third group, identified as low risk, comprises individuals with low expression levels of both miRs, making up 32.5% of the cases, with a median OS of 65.3 months. In addition, the miRNA-based classifier significantly improved the predictive power of the ISS/FISH approach and was independent of GEP-derived prognostic signatures[777]. Another study found that the ndMM patients with low expression of miR-15a had significantly shorter PFS and OS, becoming an independent prognostic factor for survival even in multivariable analysis[778]. It was observed that MM patients with elevated levels of miR-410, which regulates cell proliferation and apoptosis by targeting KLF10 via activating PTEN/PI3K/AKT pathway, in both ndMM and rrMM were associated with higher tumor burden and significantly reduced PFS and OS[779]. With respect to miR-19a, its association with poor survival prognosis MM was not influenced by genetic abnormalities, notably del(13q14) and amp 1q21. The downregulation of miR-19a is associated with shorter PFS and OS. Nevertheless, although miR-19a levels portend a poor prognosis, patients with decreased levels of miR-19a showed improved responsiveness to bortezomib treatment and a significant increase in OS after therapy. Therefore, miR-19a serves as an important prognostic indicator for identifying high-risk MM[770]. In different studies, low levels of serum miR-744 and let-7e were associated with reduced remission and shorter OS of MM patients, while high levels of plasma miR-483-5p, serum miRNA-720 and miRNA-1246 were associated with shorter progression-free survival, indicating poor prognosis,

while low miR-153, miR-296, miR-490, miR-455, miR-500, and miR-642, are associated with longer remissions and PFS[766,780-782].

Table 5. Overview of the potential role of miRNAs as biomarkers for the prognosis of MM

microRNA	Expression changes	Prognostic significance	Ref.
miR-17	↑	Poorer OS	[777]
miR-886-5p	↑	Poorer OS	[777]
miR-410	↑	Poorer PFS and OS	[779]
miR-19a	↓	Poorer OS	[770]
miR-744	↓	Poorer OS	[64]
Let-7e	↓	Poorer OS	[64]
miR-483-5p	↑	Poorer PFS	[766]
miR-720	↑	Poorer PFS	[783]
miR-1246	↑	Poorer PFS	[783]
miR-153	↓	Longer PFS	[766]
miR-296	↓	Longer PFS	[766]
miR-490	↓	Longer PFS	[766]
miR-455	↓	Longer PFS	[766]
miR-642	↓	Longer PFS	[766]
miR-30d-3p	↓	Poorer OS	[784]
miR-16-2-3p	↑	Poorer PFS and OS	[784]
miR-15a	↓	Poorer PFS and OS	[778]
miR-16	↓	Poorer PFS and OS	[778]
miR-25	↓	Poorer PFS and OS	[64]
miR-92	↑	Poorer PFS	[64]
miR-194	↑	Longer OS	[64]

↑—increased, ↓—decreased

A meta-analysis involving more than 600 MM patients identified 37 differentially expressed miRNAs and found a statistically significant correlation between low expression of miR-30d-3p and reduced OS and high expression of miR-16-2-3p and reduced OS and PFS[784]. Another one identified miR-19a, miR-221, miR-25, and miR-223, which were found to be associated with the highest number of genes and to be highly conservative in their sequences, suggesting to be indicative of their role as prognostic or diagnostic biomarkers in MM[785]. A final meta-analysis specifically designed to examine the prognostic value of miRNA expression levels in MM concluded that down-regulation of miRs-15a, 16, 25, 744, and let-7e was predictive of decreased OS and that reduced expression of miRs-15a, 16, 25, or increased expression of miR-92 was predictive of reduced PFS[786].

Considering all this, the most promising clinical application of miRNAs in MM is likely prognostication. Nonetheless, most of these data originated from basic and translational research or meta-analysis, lacking information from clinical trials. Including miRNA as prognostic biomarkers in clinical trials will be fundamental to its widespread use[787,788].

Despite these numerous studies demonstrating their value in prognosis in MM, their widespread adoption has not yet occurred. Several limitations can be found in using microRNAs. First, there is some controversy regarding the relationship of miRNA levels between serum and corresponding tissue, in which circulating miRNAs might only sometimes reflect the changes occurring in tumor tissue of interest. The origin of circulating miRNAs and their function is still unclear as they may not always be directly associated with malignant cells but may also reflect indirect effects, such as being secreted by non-malignant cells or actively taken up by malignant cells. Also, the

regulatory networks involving miRNAs are complex, and a single miRNA can target multiple genes, making it sometimes difficult to link specific miRNAs directly to cancer-related processes[789,790]. Second, the kinetics of circulating miRNAs are influenced by health conditions, age, gender, diet, and dynamic changes in different individuals. These variations can complicate the interpretation of miRNA profiles and reduce their specificity as cancer biomarkers[791]. Third, miRNAs are often present at low concentrations, making their detection challenging. This low abundance requires highly sensitive detection methods and can lead to issues with reproducibility and reliability[792]. Finally, there is a lack of standardized protocols for miRNA extraction, normalization, and quantification. Current protocols begin with the extraction and purification of circulating miRNAs from plasma or serum, followed by screening and identifying sequences of interest requiring sophisticated and sensitive techniques, such as NGS or micro-arrays, with further RT-PCR confirmation and validation. These methods can be technically challenging and expensive, limiting their widespread use, and variations can lead to inconsistent results, making it difficult to compare and value their impact across different studies[793-795].

3.2.5. Extracellular vesicles

Extracellular vesicles (EVs) are a heterogeneous group of particles that are cell-released, enclosed within a phospholipid bilayer, and that do not replicate. Importantly, they contain cargo representatives of their cell of origin, such as transmembrane proteins, enclosed cytosolic proteins, lipids, metabolites, RNAs, small non-coding RNAs (such as miRNAs and long non-coding RNAs), and even DNA fragments [121,796-798]. In addition to managing cellular waste, they play a pivotal role in mediating intercellular communication under both physiological and pathological conditions and are gaining recognition in scientific research as potential non-invasive biomarkers. Tumor-derived EVs educate recipient cells towards a tumor-promoting phenotype, playing a role in multiple steps of cancer development and contributing to all cancer hallmarks[451,798]. As discussed previously, EVs can modify the tumor microenvironment to support tumor growth and survival, induce angiogenesis and osteolysis, tumor cell invasion and migration, initiate resistance to cell death, evade the immune response, and reprogram cellular energy metabolism and drug resistance[121,796,797].

EVs can vary in size, properties, biological functions, and mechanisms of release. EVs released directly from the plasma membrane vary in size (100–1000 nm) and are known as microvesicles (MVs), ectosomes, and microparticles. On the other hand, EVs released from multi-vesicular bodies (MVB) are known as exosomes and have a size of 30–150 nm. Early endosomes will mature into MVB, fusing with the plasma membrane to release their content as exosomes. Finally, apoptotic bodies fall into the range of 500–5000 nm and are formed during cytoskeletal rearrangement, released by outward blebbing and decomposition of the cell membrane of apoptotic cells. From all EVs, apoptotic bodies are less frequently involved in intercellular communication[121,798].

Currently, a combination of ultracentrifugation with purification techniques, such as size-based filtration or chromatography, can enhance the purity and recovery of this method[799,800]. Several commercial kits based on precipitation are available with high purity, but some have low specificity, making extra purification measures necessary. Also, these kits should be cautiously approached, especially for biomarker studies, as it is nearly

impossible to exclude coprecipitated cargo[796,797]. Differential ultracentrifugation remains the most used technique to isolate EVs with intermediate specificity, but it can be time-consuming. Density gradient or size exclusion chromatography further improves the purity of EVs by removing non-EV contaminants. Combinations of different isolation techniques will increase specificity, which is necessary to detect specific EV cargo, even with lower yield and more time-consuming[799,800]. Isolated EVs must be verified by size, shape, physical features, and biochemical properties. Typically, Transition Electron Microscopy (TEM) is used to estimate the shape and size of EVs, and Nanoparticle Tracking Analysis (NTA) is also used as a rapid alternative for semi-quantification and size EVs[801,802]. Additionally, multi-parameter analysis techniques, such as nano-flow cytometry (nFCM), are becoming crucial for accurately estimating size, concentration, purity, and morphological characterization[803]. Western blotting is routinely applied to the detection of EV marker proteins[799,800]. Minimal information advocates for the minimum of 3 protein markers: one transmembrane protein (including CD63, CD9, and CD82) associated with the plasma membrane and/or endosomes, one cytosolic protein (including TSG101 and/or ALIX) or flotillins that associated with membrane microdomains, and one negative marker or other intracellular proteins such as calreticulin, calnexin, prohibitin, or GM130. HSP70, ARF6, and tubulin can serve as cytosolic markers, while syntenin is recognized as the most abundant protein in EVs[804,805].

MM, as well as other hematological malignancies, has been shown to have elevated serum EV levels compared to healthy individuals[806]. For instance, MM patients produce approximately four times more EVs than healthy individuals and MGUS and SMM patients[807,808]. Since EVs can be recovered from common body fluids, including peripheral blood and urine, and their content reflects their cell of origin, EVs can be useful as potential noninvasive biomarkers for diagnosis, bone disease, graft-versus-host disease, drug response/resistance, and survival outcomes(Table 6)[798].

When examining **plasma cell markers in EVs**, both the monoclonal immunoglobulin-free light chain and the monoclonal immunoglobulin of the B-cell receptor have been found on MM-derived EVs[823,824]. Moreover, it was demonstrated that CD138 was more specific and highly expressed in MM-EVs than in healthy individuals and was correlated to therapeutic response, tumor burden, and disease stage[825,826]. Regarding clinical presentation, patients with bone disease showed higher levels of CD138-positive EVs in the PB[827]. It was also seen that the presence of multidrug resistance protein P-glycoprotein, along with the stem cell marker CD34 on CD138-negative MM EVs in patients with MM, could be a marker of disease progression and treatment unresponsiveness in MM patients, specifically with aggressive disease. This indicates the existence of a resistant immature phenotype or could be the consequence of CD138 shedding. CD138 cannot be considered a “static” biomarker of MM disease evolution as the presence of a CD138-negative population increases in aggressive disease[822]. In addition, CD38+ EVs appear to be more present in BM plasma and serum of patients with MM compared to MGUS and SMM, being related to the clinical ISS. CD38-positive EVs were significantly higher in patients with ISS3 than those in ISS2 and ISS1[806]. Another study showed a positive trend between BM PC percentage and bone lesions and CD38-positive MM-EVs, as already seen for CD138-positive[828]. In the same study, using the MM markers CD38 and CD138, it was possible to correlate the double positive EVs from PB and the percentage of PC in the BM[828]. Adenosine is a potent immunosuppressive compound produced in the BM of MM patients from either ATP (via CD39/CD73) or

NAD⁺ (via CD38/CD203a (PC-1)/CD73). Since BM adenosine levels are elevated in MM patients compared to those with MGUS or SMM, EVs CD38, CD39, and CD73 have potential as promising biomarkers for distinguishing between MM and its precursors[162]. Other proteins, such as MHC class I, B2-microglobulin, and CD44, were also abundant in EVs derived from MM cell lines and PB of MM patients and could be potentially used as markers. In particular, CD44 was highly enriched in corticosteroid-resistant MM cell lines[808,820].

Table 6. Overview of the MM-derived EVs as potential biomarkers of disease, treatment response, and survival prognosis based on their content.

EV content	Specific EV content	EVs cell of origin	Biomarker use	Ref.
miRs	miR-129-5p	MM	Discriminate between MM (↑) and MGUS/SMM (↓) Advance bone disease	[809]
	miR-155	MM	Discriminate between MM (↑) and HC (↓) Worse prognosis	[810]
	↓ miR-let-7b	MM	High-risk disease, shorter PFS and OS	[783]
	↑ miR-10a, miR-346, miR-135b	BMSC	Discriminate between MM (↑) and HC (↓)	[811]
	↑ miR-27b-3p, miR-214-3p	BMF	Discriminate between MM (↑) and MGUS (↓) Discriminate between rrMM (↑) and ndMM (↓)	[811]
	↓ miR-18a	MM	High-risk disease, shorter PFS and OS	[783]
	↑ miR-34	MM	Shorter OS	[811]
	↑ miR-16	MM	Longer OS	[812]
	↑ miR-15a	BMSC	Drug resistance	[776]
	↑ miR-20a	MM	Drug resistance	[776]
	↑ miR-17	MM	Drug resistance	[776]
	↑ miR-16	MM	Drug resistance	[776]
lncRNAs	PRINS	MM	Discriminate between MM (↑) and MGUS/SMM (↓)	[813]
	LINC00461	SCM	Discriminate between MM (↑) and HC (↓)	[811]
	MALAT1	BMSC	Discriminate between MM (↑) and post-treatment patients and healthy controls (↓)	[814]
	↑ PCAT-1	MM	Prognosis Correlated with the β2-microglobulin concentration	[815]
	↑ FFAR1 and SP9	MM	Bortezomib sensitivity	[797]
Other snRNA	↓ HIST1H2BG and ITIH2	MM	Bortezomib sensitivity	[816]
	↑ circMyc	MM	Discriminate between MM (↑) and HC (↓) Bortezomib resistant Correlated to 17p deletion and translocation of t(4;14)	[817]
	↑ piRNA-823	MM	High-risk disease Discriminate between MM (↑) and HC (↓)	[811]
Lipids	Acid Sphingomyelinase	MM	Melphalan and bortezomib resistance	[818]
Other content	SAA1, SAA2, and SAA4		Discriminate between MM (↑) and MGUS (↓)	[819]
	MHC-1, β2-MG	MM	Discriminate between MM (↑) and MGUS (↓)	[820]
	↑ CD71		Prognosis	[450]
	↑ CD38, CD138, CD44	MM	Prognosis, Disease progression	[450]
	↑ CD46, ↑ CD26, ↓ CD31 and ↓ CD106	MM	GVHD	[450]
	CD138-P-gp+CD34+	MM	Multidrug resistance	[822]

↑—increased, ↓—decreased

Circulating-free miRNAs have been identified as noninvasive solid biomarkers in MM. However, one of the main limitations of circulating-free miRNAs as liquid biopsy is their instability and short half-lives in biological fluids. EVs appear to be a robust biomarker platform because of their high abundance and ability to protect their cargo from nuclease and proteasome degradation[783,829]. In particular, **miRNAs carried by EVs** were differentially expressed between MM and healthy individuals and can be a valuable tool to discriminate between MM and its precursor stages[783,809]. EV-miR-129 was detected in high levels in the BM and serum of MM patients and was absent in SMM. This miR targets several genes involved in osteoblast differentiation, and its overexpression is

associated with advanced bone disease. Therefore, EV-miR-129 levels could be a marker of MM bone disease, absent in MGUS and SMM[809]. EV-miR-155 levels in the serum of MM patients are significantly lower than those in other hematological malignancies, making it useful as a diagnostic biomarker[810]. Furthermore, measuring EV-miR levels serves as a valuable tool not only for monitoring MM treatment and progression but also for predicting survival outcomes. In a pilot cohort of ndMM treatment with bortezomib-based protocols, RNA sequencing using a qRT-PCR array for 22 miRNA showed that only EV-miR-let7b and EV-miR-18a were associated with disease prognosis. Patients with severe disease, mainly in ISS3, had low levels of EV-let7b and EV-miR-18a in circulation, and their serum levels were negatively correlated with PFS and OS. This prognosis was still statistically significant after adjusting for the ISS and adverse cytogenetics in the multivariate analysis, which enhanced the stratification of MM patients with high-risk factors[783]. EV-miR-16, other than its tumor suppressive role in communicating between MM-PCs and other cells of the BM microenvironment, is associated with MM prognosis in patients also receiving bortezomib-based protocols. Patients with high levels of EV-miR-16 in serum had significantly longer five-year OS than those with lower levels[812]. This was confirmed in exploring miR patterns in bortezomib-resistant patients, being found that not only EV-miR-16 but also EV-miR-15a, EV-miR-20a, and EV-miR-17 were strongly downregulated in circulation and were associated with drug resistance[776].

In addition to miRNAs, long-non-coding RNAs (**lncRNAs**) are other regulatory RNAs delivered by EVs and are released at large levels in circulation. Among 84 lncRNAs secreted, only one EV-lncRNA, PRINS (psoriasis susceptibility-related RNA gene induced by stress), was dysregulated in MM and MGUS patients compared to healthy controls. Levels of EV-PRINS positively correlated with clinical parameters such as bone marrow plasma cell infiltrate percentage, albumin, creatinine, and lactate dehydrogenase levels. This work suggested a possible diagnostic function for EV-lncRNA PRINS in patients with monoclonal gammopathies, with a specificity of 83.3 % and a sensitivity of 84.9 % to distinguish MM and MGUS participants from healthy controls[813]. Similarly, EV-lncRNA PCAT-1 expression levels were significantly correlated with the β 2-microglobulin concentration and MM subtypes, suggesting its potential use in auxiliary diagnosis[815]. Similarly to EV-miRNAs, when comparing EV-lncRNA between responders and non-responders to bortezomib-based treatment, FFAR1 and SP9 were increased, whereas HIST1H2BG and ITIH2 were decreased. Of these, FFAR2 and SP9 could be potential independent prognostic indicators of survival in patients with MM[816]. Also, EV-lncRNA MALAT1 derived from BM mononuclear cells is overexpressed in ndMM patients compared to posttreatment patients and healthy controls, possibly being used to monitor response[814].

More recently, a study showed that **circular RNAs (circMyc)** levels were elevated in the EVs of MM patients and were associated with 17p deletion and t(4;14) translocation. Additionally, circMyc levels were higher in bortezomib-resistant patients compared to responders and served as an independent predictor of poor prognosis[817].

Although circulating cell-free DNA could serve as a biomarker, double-stranded **DNA inside EVs** remains a subject of debate. This controversy is likely due to inconsistencies in the preparation methods and size of the isolated EVs[830]. Some data exist on pancreatic cancer, in which KRAS and TP53 mutations in EV-DNA were detected. Nonetheless, no significant data is known in MM[687]. The same happens with evaluating **lipids** in EVs, which is

not straightforward because of several risks, including co-isolation of lipoproteins, poor lipid extraction, exclusion of lipophages, and the complex nature of biological membranes[818]. Regarding **proteins**, a high proteomic similarity between PB and BM-matched pairs was reported, supporting the use of circulating EV as a counterpart of the BM EV proteome. High EV protein/particle ratio, or EV cargo >0.6 µg/10⁸ particles, was found to be related to poorer survival and immune dysfunction, being identified as a set of PB EV-proteins (PDIA3, C4BPA, BTN1A1, and TNFSF13) with a new biomarker potential for myeloma patient outcomes[821]. Another study showed the differences in the proteome within extracellular vesicles between precursor stages and MM. The transferrin receptor (or CD71) protein was upregulated in both populations of EVs derived from MM patients compared to MGUS patients and was of prognostic significance. Similarly, three isoforms of serum amyloid A (SAA) protein, SAA1, SAA2, and SAA4, were also highly upregulated in EVs within MM patients relative to MGUS patients[819].

Nonetheless, the heterogeneity of the EVs populations within a biological sample might complicate the biomarker analysis, particularly since a single cell can produce different subtypes of these vesicles[831]. Moreover, the EVs retrieved from liquid biopsies do not necessarily represent the most abundant cell type in a certain tissue[832]. Individual factors such as donor age, biological sex, diet, specific diseases, medications, and many other variables can influence the characteristics of circulating EVs in peripheral blood. Also, technical factors such as pre-analytical variables of the EV source, storage conditions, source material handling, and experimental conditions can all impact EV recovery[804]. EVs encapsulation of their cargo provides them greater stability, a longer half-life, higher resistance to degradation, and a greater ability to travel long distances when compared to circulating free proteins, lipids, and nucleic acids. Of note, EVs have the ability to transfer their cargo to recipient cells and organs that are protected by physiological barriers, such as the blood-brain barrier[833,834]. However, EVs selective packaging is highly heterogeneous and changeable under different physiological conditions, internal cellular processes, or external stimuli. Therefore, they provide only a snapshot of the molecular environment in their cell of origin at the time they are released from the cells, hampering a spatial and temporal evaluation[835,836].

Importantly, there are no standardized protocols for the isolation, purification, characterization of EVs and separation of EV subpopulations with high sensitivity and specificity. For instance, although there is no consensus, some studies indicate that cancer cells produce more EVs than normal cells, while others report no significant difference in plasma EV levels between cancer patients and healthy individuals[806,808,824,837]. This conflicting evidence may be due to low inter-laboratory reproducibility and the lack of standardized protocols for EV isolation and purification[796-798].

Finally, several challenges must be addressed before implementing EV-based liquid biopsies in the clinic, such as the time required for analysis, the high costs involved, the need to balance high specificity with high sensitivity, and the necessity to correlate EVs findings with clinical data and the natural history of the disease.

CHAPTER 2

AIMS

The **OVERALL AIM** of this thesis was to identify biomarkers of measurable residual disease and drug response/resistance using liquid biopsies from patients with Multiple Myeloma and contribute to guiding personalized therapeutic decisions.

For this, the following **SPECIFIC AIMS** were pursued:

1. **Detect measurable residual disease (MRD) biomarkers in Extracellular Vesicles (EVs) isolated from BM and PB samples of MM patients.** To achieve this objective, EVs were isolated from the BM and PB of MM patients at distinct disease stages (diagnosis and remission), and the levels of MRD-associated protein markers were compared between BM and PB EV samples. PB samples from healthy controls were also tested to support the feasibility of using the identified markers as possible MM biomarkers to monitor MRD in a minimally invasive way.
2. **Identify miRNAs in bone marrow (BM) and peripheral blood (PB) samples of MM patients as putative biomarkers of MM drug response/resistance to treatment.** To address this objective, next-generation sequencing (NGS) was performed in BM and PB samples of sensitive and resistant MM patients treated with the same first-line protocol. Subsequently, the most relevant results were confirmed by qT-PCR. Additionally, we used bioinformatics analysis to explore the potential signaling pathways regulated by the identified miRNAs.
3. **Explore the possibility of isolating and quantifying miRNAs in the cargo of EVs from liquid biopsies of MM patients as preliminary results with the long-term objective of identifying MM biomarkers of drug response/resistance in PB samples, which could replace the bone marrow biopsy/aspirate as a valid and accessible sample to assess drug resistance/response patterns and prognostic significance.** To accomplish this, preliminary comparisons of selected microRNA levels were made between BM versus PB at diagnosis and remission stages.
4. **Contribute to better stratification of MM patients towards a personalized medicine approach.** For this, when possible, correlations between the biological results obtained and specific clinical characteristics of MM patients (and known disease staging systems) were established, considering the patient's clinical evolution and outcome, namely drug response, progression-free survival, and overall survival.

CHAPTER 3

RESULTS & DISCUSSION

ORIGINAL ARTICLE

3.1. Detection of Measurable Residual Disease Biomarkers in Extracellular Vesicles from Liquid Biopsies of Multiple Myeloma Patients**Published**

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Article

Detection of Measurable Residual Disease Biomarkers in Extracellular Vesicles from Liquid Biopsies of Multiple Myeloma Patients—A Proof of Concept

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Abstract: Monitoring measurable residual disease (MRD) is crucial to assess treatment response in Multiple Myeloma (MM). Detection of MRD in peripheral blood (PB) by exploring Extracellular Vesicles (EVs), and their cargo, would allow frequent and minimally invasive monitoring of MM. This work aims to detect biomarkers of MRD in EVs isolated from MM patient samples at diagnosis and remission and compare the MRD-associated content between BM and PB EVs. EVs were isolated by size-exclusion chromatography, concentrated by ultrafiltration, and characterized according to their size and concentration, morphology, protein concentration, and the presence of EV-associated protein markers. EVs from healthy blood donors were used as controls. It was possible to isolate EVs from PB and BM carrying MM markers. Diagnostic samples had different levels of MM markers between PB and BM paired samples, but no differences between PB and BM were found at remission. EVs concentration was lower in the PB of healthy controls than of patients, and MM markers were mostly not detected in EVs from controls. This study pinpoints the potential of PB EVs from MM remission patients as a source of MM biomarkers and as a non-invasive approach for monitoring MRD.

Keywords: multiple myeloma; extracellular vesicles; measurable residual disease; liquid biopsy

1. Introduction

Treatment of Multiple Myeloma (MM) has improved substantially, demonstrated by the current sequential and integrative approach using drugs of different classes in combination, which has clear benefits for patients [1]. In recent years, several drugs, such as proteasome inhibitors, immunomodulators, and monoclonal antibodies, have been approved for the treatment of MM, and several others with novel mechanisms of action are currently under pre-clinical and clinical trials, namely bi-specific monoclonal antibodies and chimeric antigen receptor (CAR)-T cells. Despite the unprecedented response rates and prolonged survival achieved with these significant advances in treatment, MM remains incurable and, eventually, all patients inevitably relapse [2].

A deep and sustained complete response (CR) to treatment is the ultimate objective in any phase of MM treatment. Nonetheless, achieving CR is not enough to ensure longer survival in MM. Indeed, the persistence of very low levels of plasma cells (PCs) in patients' bone marrow (BM), as measurable residual disease (MRD) after treatment, will constitute the soil for a subsequent relapse and are a major cause of drug resistance. Quantifying MRD allows for the assessment of chemotherapy efficacy and clinical outcome, the identification of patients at high-risk of recurrence, and provides important information for therapy decisions [3,4]. MRD evaluation is most important to determine when the myeloma clone is disappearing during treatment (remission) and when the myeloma burden, even low, is reappearing after treatment (relapse) [5,6]. Thus, its routine monitoring has the potential to guide therapeutic decisions [4,7,8].

Indeed, MRD constitutes the most significant predictor of clinical outcome [9,10]. In large meta-analyses and several multivariate analyses in clinical trials, achieving negative MRD is the major and the strongest independent prognostic factor, outweighing classical favorable prognostic factors [9,11–13]. MRD is associated with unprecedentedly improved progression free-survival (PFS) and overall survival (OS), regardless of the depth of the International Myeloma Working Group (IMWG) criteria response at the time of MRD evaluation (CR versus above or very good partial response) [14,15], the cytogenetic risk (high or standard risk) [16,17], the time of MRD assessment (at induction, transplant, consolidation, before or after maintenance treatment initiation) [18,19], the status of the MM disease (newly diagnosed or relapsed/refractory disease) [20], or the fact that patients are eligible or non-eligible for transplant [10,19]. Moreover, the real clinical impact of MRD is reproducible in different centers enrolling patients in clinical trials, and also in the clinical practice [21].

MRD evaluation can be divided into two approaches: measuring intramedullary disease by multiparameter flow cytometry (MFC) immunophenotyping or by high-throughput/next-generation sequencing (NGS) molecular assessment of immunoglobulin gene rearrangements; or by quantifying extramedullary disease (EMD) using functional imaging methods [21–23]. Using MFC makes it possible to identify myelomatous PCs based on light-chain clonality of phenotypically aberrant tumor cells with a sensitivity of 10^{-6} using next-generation flow (NGF) cytometry [24–26]. Clonal immunoglobulin gene rearrangements, initially identified by the allele-specific oligonucleotide polymerase chain reaction (AS-PCR), are now detected by NGS that performs millions of reads of DNA fragments with a sensitivity of 10^{-6} . Both NGF and NGS lack standardization over different laboratories but yield similar results [23,27,28]. The 18-fluoro-2-deoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) is the optimal method to evaluate the disease outside the BM, allowing for lesions with metabolically active disease to be distinguished, which is mostly useful for patients with EMD; nonetheless, this approach has not yet been validated in randomized clinical trials and cannot be currently used to guide therapeutic decisions [29,30]. MRD determination by either NGF or NGS relies on BM samples and specific pitfalls can compromise its overall success [21]. MM is characterized by a patchy pattern of BM infiltration and different clonal PCs may reside in different areas of the BM, reflecting its spatial molecular heterogeneity [31]. When using NGF and NGS, no EMD is assessed, which may hamper an MRD result from being truly negative, mainly in relapses. Moreover, BM can be hemodiluted and, to minimize false-negative MRD results, the result should be confirmed in a second assessment [21]. These limitations allied to the painful and discomfiting collection of BM samples led to efforts to identify alternative approaches using peripheral blood (PB).

Extracellular Vesicles (EVs) are a group of lipid bi-layer circular particles with different biogenesis that are heterogenous in size and content. EVs include exosomes (with endosomal origin and sizes ranging from 30 to 100 nm), microvesicles (with plasma membrane origin and sizes ranging from 50 to 2000 nm), or even apoptotic bodies (with both cytosolic components and nuclear fragments and sizes ranging from 50 to 5000 nm) [32–34]. Initially, EVs were considered as cellular waste with no biological activity, but now are recognized as essential players in the intercellular communication mediated by their cargo [35–38]. In

fact, EVs are responsible for the horizontal transfer of phenotypes between cells since they transport cellular contents from the donor cells (e.g., proteins, microRNAs, or fragments of DNA), which may be incorporated by recipient cells [39,40]. EVs are released by all cell types, both normal and malignant, and have recently emerged as a possible source of blood biomarkers for several diseases, particularly cancer [32,41,42]. In fact, cancer cells produce and release a higher amount of EVs when compared to healthy control cells [43–46]. In recent years, several studies have shown how EVs are relevant for several hallmarks of cancer, such as cellular proliferation, immune modulation, or metastasis [35,47–50].

In MM, EVs play a crucial role in mediating the mutual crosstalk between the MM PCs and cells from the tumor microenvironment by transferring active molecules such as lipids, proteins, and regulatory RNAs, thus contributing to MM pathobiology [44,51]. Another example of the relevance of this communication is the interplay between BM mesenchymal stem cells (MSCs) and PCs, in which MM-MSCs-derived EVs are taken up by MM-PCs, causing proliferation, survival, and migration of the latter by activating oncogenic factors [52,53]. EVs secreted by MM-PCs also contribute to angiogenesis by transferring high amounts of vascular endothelial growth factor (VEGF) to endothelial cells [54]. Interestingly, drug-resistant cells appear to release higher amounts of EVs than drug-sensitive ones [55,56]. Moreover, some studies have shown the transfer of EVs from drug-resistant PCs to sensitive cells, protecting the recipient cells from apoptosis, influencing the activation of several survival pathways, and promoting drug resistance [55].

Considering the flow of communication between MM-EVs and other cells in the BM microenvironment, as well as the dynamic quantity of EVs in relation to disease severity, stage, or treatment phase [57–60], it is conceivable that MM-EVs and their cargo could be used as potential biomarkers of the disease and to monitor MRD in PB. This study aimed to implement a protocol for the isolation of EVs from the BM and PB of MM patients at distinct disease stages (diagnosis and remission) and to compare the levels of some MRD-associated protein markers between BM and PB EV samples.

2. Results and Discussion

2.1. EVs Were Isolated from the PB and BM of MM Patients

EVs from the PB and BM of MM patients and EVs from the PB of healthy controls were isolated by Size Exclusion Chromatography (SEC) followed by ultrafiltration (UF) and were characterized by Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), and Western Blot (WB) in terms of size distribution, morphology, and the presence of well-known protein EV markers to confirm the identity and purity of the isolated EVs.

Our results show the possibility of isolating EVs from the BM and PB of patients with MM. Choosing the optimal strategy to isolate EVs is a critical step in achieving enough of a yield of EVs with minimal contamination by non-vesicular proteins/particles in order to assure their accurate study [34,61]. SEC was previously described to be efficient in isolating EVs from plasma and separating them from some of the protein contaminants [62,63]. As seen in Figure 1(A2,A4,B1,B2), NTA and TEM analysis showed that the EVs of higher sizes are separated in the earlier fractions, while the EVs of smaller sizes are separated in later fractions. Particles between 50 and 1000 nm are mostly obtained on fractions 3 to 6 (Figure 1(A2,A4)). TEM analysis confirmed the NTA results, indicating that particle size decreases while the number of particles increases from fractions 3 to 7 (Figure 1A,B). The mean particle range determined by TEM was approximately between 80 and 40 nm for fractions 3 to 6, respectively (Figure 1B). Irrespective of the type of sample analyzed (PB or BM), the protein concentration rises considerably from fraction 7 onwards (Figure 1A,B), where the main protein contaminants such as albumin and apolipoprotein B are found in both PB and BM samples (Figure 1(C1,C2)). This is in agreement with other studies showing that SEC is an efficient method for isolating EVs from plasma samples and that from fractions 8 to 10 there is an increased level of contaminants [64–68]. TEM also showed the morphological heterogeneity of the isolated particles, with particles from fractions 3

to 6 being mostly round or with an irregular surface, as has been reported for EVs [57,69], contrasting with a more uniform and spherical morphology found on particles isolated from fraction 7 onwards, as expected for apolipoproteins [64].

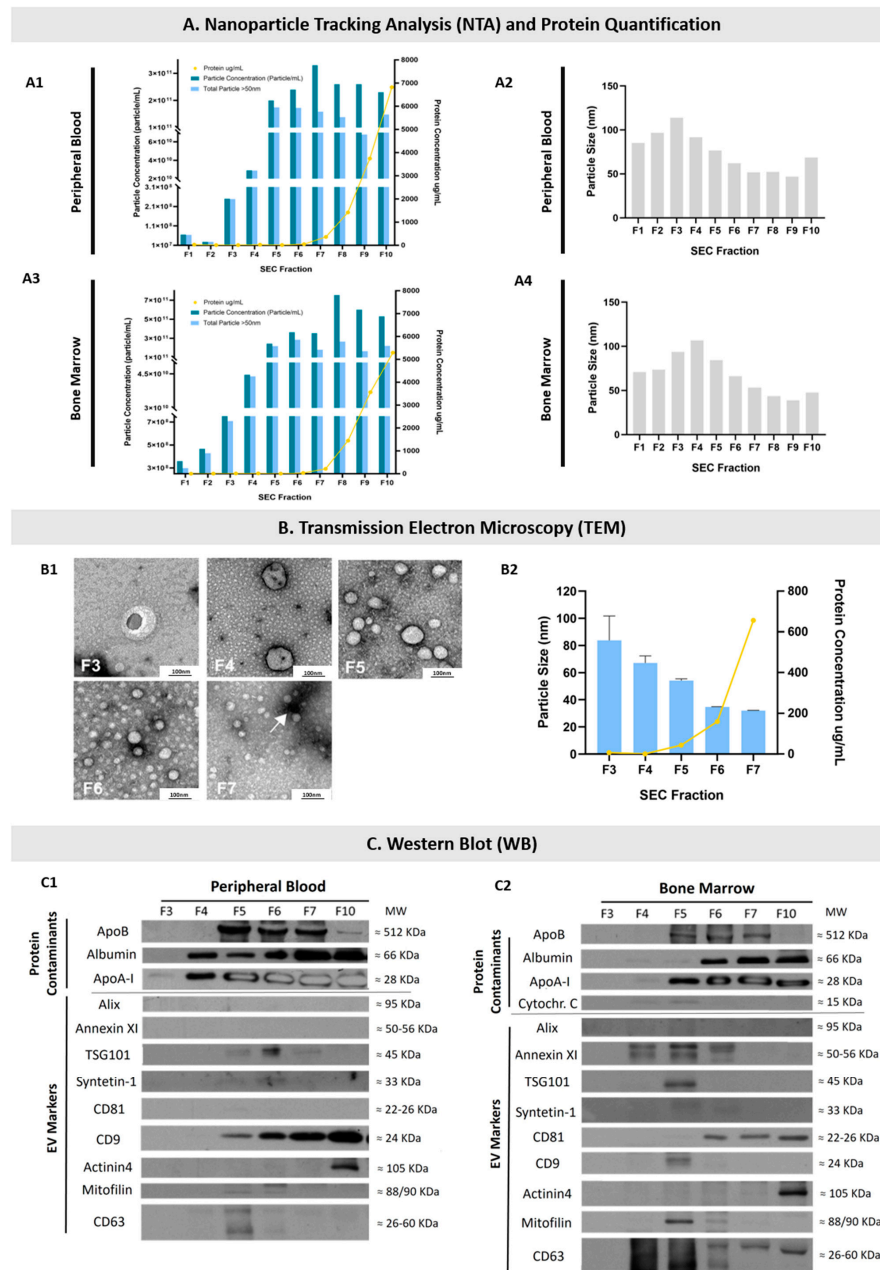


Figure 1. Characterization of the EVs isolated from peripheral blood (PB) and bone marrow (BM) samples by Size Exclusion Chromatography (SEC) and concentrated by ultrafiltration (UF). (A) NTA and protein quantification results suggest the presence of EVs from SEC fractions 3 to 6. Graphics on

the left show particle concentrations in each SEC fraction (dark blue bars; left axis), the particle concentrations above 50 nm of size (light blue bars; left axis), and the total proteins quantified (yellow dots and lines; right axis) throughout the SEC fractions from a MM patient PB (A1) and BM (A3) sample. Graphics on the right show the mean particle size distribution in each of the PB (A2) and BM (A4) eluted SEC fractions analyzed by NTA. (B) TEM results confirm the presence of EVs on SEC fractions 3 to 7 isolated from PB samples. (B1) Representative images of EVs from SEC fractions 3 to 7 obtained by TEM. The white arrow in fraction 7 indicates the presence of protein contaminants, more frequently found in the latter SEC fractions. The scale bar displayed corresponds to 100 nm. (B2) Representative results of the particle size (blue bars; left axis) analyzed by TEM, and the amount of total protein quantified (yellow line; right axis) in each SEC fraction. Results are the mean $\pm$ standard error (SE) of a minimum of 200 EVs analyzed from each SEC fraction isolated from a PB sample (with the exception of fractions 3 and 4, in which only 6 and 18 EVs were analyzed, respectively). (C) EV markers and protein contaminants in SEC fractions 3 to 10 analyzed by WB. The blot displays representative results from markers found in PB (C1) and BM (C2) samples. The molecular weight (MW) of the bands is shown on the right side of each blot in kDa. Fractions 3 to 6 were ultrafiltrated separately. NTA: Nanoparticle Tracking Analysis; MM: Multiple Myeloma; PB: peripheral blood; BM: bone marrow; TEM: Transmission Electron Microscopy; WB: Western Blot.

Finally, the presence of several EV markers (CD63, Mitofilin, CD9, CD81, Syntenin-1, TSG101, and Annexin XI) was found by WB in fractions 4 to 10 (Figure 1(C1,C2)). Most interestingly, when comparing the presence of EV markers between PB- and BM-isolated EVs, differences were found regarding Annexin XI, CD81, CD9, and CD63. Indeed, the PB EV marker found between fractions 3 and 6 was mostly CD9. However, in EVs isolated between fractions 3 and 6 from BM samples, the EV markers mostly found were Annexin XI and CD63. To our knowledge, this interesting finding had not been previously reported and suggests that even though the size of the EVs isolated (from fractions 3 to 6) is similar between PB and BM samples, those EVs have a different abundance of typical EV markers which therefore may correspond to distinct EV subsets. This difference may not reflect the protein content of the cells of origin of the isolated EVs, since selective packaging of proteins into EVs has been described [70–72]. This further enhances the relevance of comparing the cargo of MM-associated proteins in the EVs isolated from PB and BM samples.

The presence of EV markers in fraction 10, mainly Actinin4 and CD9 in PB EVs and CD81, Actinin4, and CD63 in BM EVs, might be due to the presence of these proteins in a free form, as EVs are not expected to be obtained from fractions 8 to 10, as demonstrated by the NTA and TEM results.

2.2. EVs Were Isolated from Diagnosis and Remission PB and BM Samples of MM Patients

It was possible to confirm the presence of EVs in PB and BM samples from MM patients obtained at either diagnosis or complete remission. The presence of the EV markers CD63 and CD81 was found in all samples (Figure 2A) without statistically significant differences, even though some differences in the levels of these markers were found at diagnosis and remission. Indeed, the levels of CD63 were lower at diagnosis than at the remission stage, while the levels of CD81 were higher at diagnosis than at the remission stage. This trend was observed both in the PB and BM samples. In agreement with what had been previously observed (Figure 1C), the EVs isolated from BM samples had higher levels of CD81 than the EVs isolated from PB samples, reflecting a difference in the protein cargo of EVs from both origins.

NTA results showed consistency regarding the EV sizes isolated from all samples (PB and BM at diagnosis and remission), which presented mean EV sizes between approximately 40 and 100 nm (Figure 2B), without statistically significant differences. This was confirmed by TEM (Figure 2C).

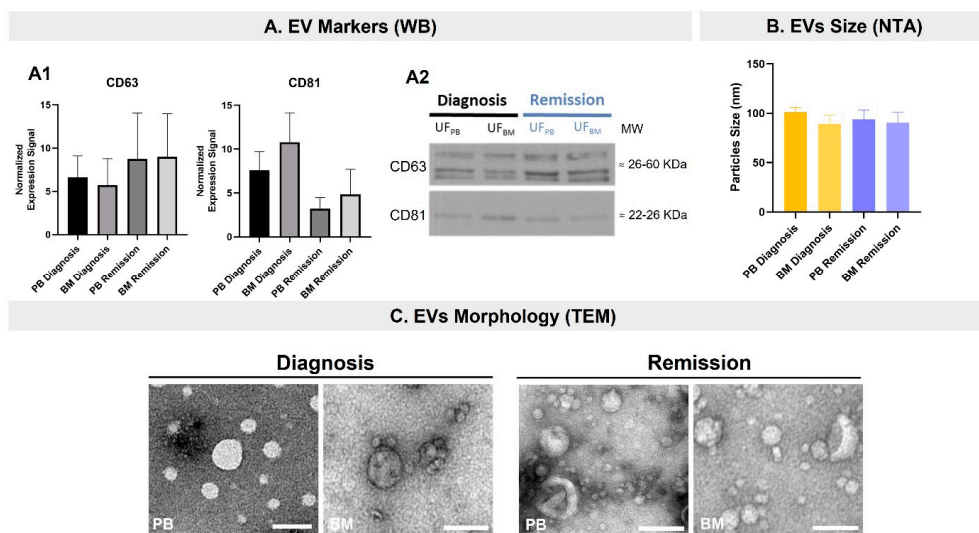


Figure 2. Characterization of the EVs isolated by Size Exclusion Chromatography (SEC) and concentrated by ultrafiltration (UF) from diagnostic and remission samples of peripheral blood (PB) and bone marrow (BM). (A) EV markers found by WB in the diagnostic and remission PB and BM EV samples. (A1) Quantification of CD63 and CD81 signals in the ultrafiltrated (UF) samples, normalized for the total protein of the lane (Ponceau staining). Results are the mean $\pm$ standard error (SE) from three MM patients. (A2) Representative blots of CD63 and CD81. (B) Average size of the isolated EVs. Results are the mean $\pm$ standard error (SE) from three MM patients. (C) Representative images of the isolated EVs. The scale bar displayed corresponds to 100 nm. PB: peripheral blood; BM: bone marrow; WB: Western Blot; UF: ultrafiltrated pool of SEC fractions 3 to 6; NTA: Nanoparticle Tracking Analysis; MM: Multiple Myeloma; TEM: Transmission Electron Microscopy.

2.2.1. EVs Isolated from Diagnostic PB and BM Samples Present MM Markers in Their Cargo

Different immunophenotypic markers of MM were analyzed by WB in the ultrafiltrated pools from SEC fractions 3 and 6 for both PB and BM patient samples at diagnosis and remission status. The MM EV markers analyzed consisted of the well-known MM markers related to specific PC lineages, such as CD38 and CD138, and others used by the Euroflow Consortium for the diagnosis and response evaluation to treatment by flow cytometry: CD45, CD56, CD19, CD81, CD117, CD27, and cytoplasmic immunoglobulin kappa and lambda light chains [73]. Results are presented in Figure 3, with the first two lanes of each blot presenting results from diagnostic samples (from PB and BM).

Levels of CD38 and CD138 were analyzed in ten patients. A higher number of CD38 positive samples were observed compared to CD138 and co-presentation of CD38/CD138. This co-presentation was particularly evident in patients 4 and 8. However, other patients, such as patient 1 and 3, did not present CD38+ in EVs at diagnosis. Recently, it was described that low levels of CD38 were found in PCs of patients with EMD, conferring poor response to anti-CD38 monoclonal antibodies. Interestingly, our patients without CD38 in EVs at diagnosis, and CD38-negative by flow cytometry (Table 1), did present EMD with low medullary plasmacytosis [74]. MM patients without CD38 are also reported to have worse prognosis while being refractory to treatment [75].

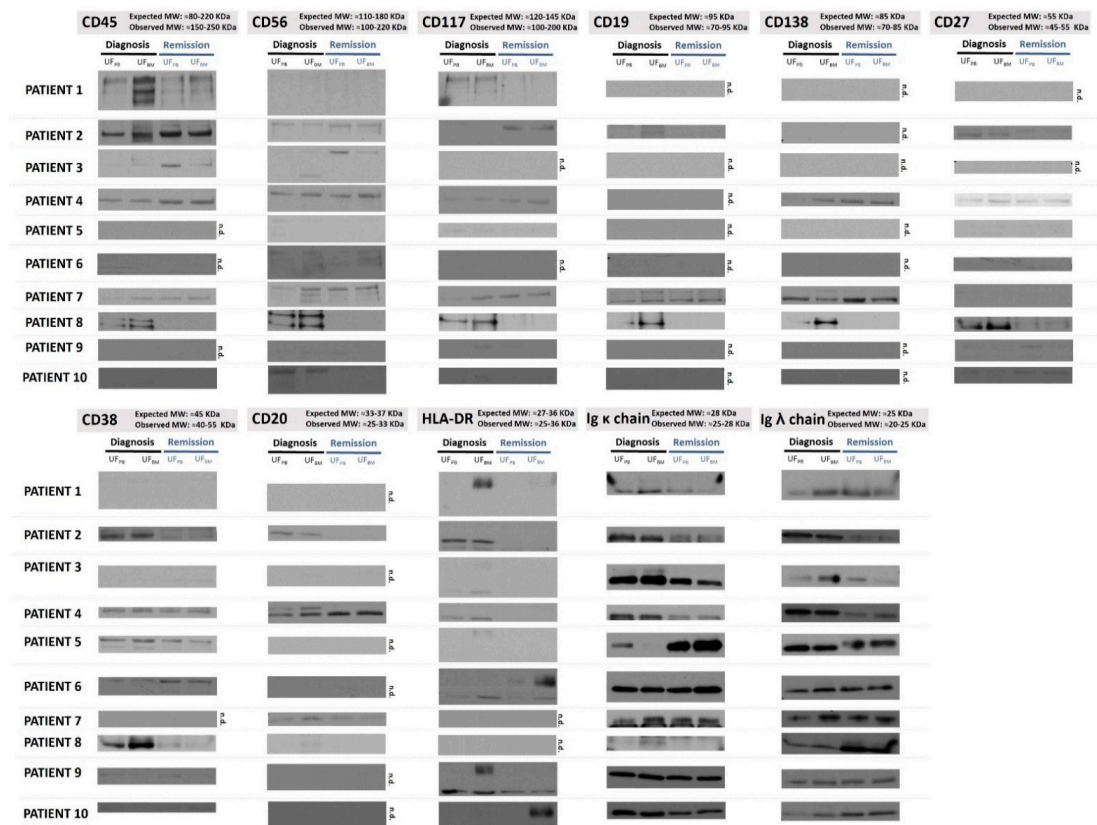


Figure 3. Detection of Multiple Myeloma (MM) and Minimal Residual Disease (MRD) Markers in EVs from diagnosis and remission samples from the peripheral blood (PB) and bone marrow (BM) of MM patients. Different markers of MM and MRD were analyzed by WB in the ultrafiltrated pool from SEC fractions 3 to 6 (UF) isolated from PB and BM samples collected at both diagnosis (first two lanes) and remission (final two lanes) from 10 MM patients (n.d.: signal not detected). The molecular weight (MW) of the bands is shown in kDa on the right side of the blots. MM: Multiple Myeloma; MRD: Minimal Residual Disease; PB: peripheral blood; BM: bone marrow; SEC: Size Exclusion Chromatography; WB: Western Blot; UF: ultrafiltrated pool from SEC fractions 3 to 6.

Table 1. Comparison of Multiple Myeloma (MM) patients' characteristics at diagnosis and at day 100 of evaluation regarding response criteria, measurable residual disease (MRD) by flow cytometry, and Extracellular Vesicle (EV) markers both in peripheral blood (PB) and bone marrow (BM).

Patient	MM Isotype	ISS			FISH		EMD		% of PCs at Diagnosis					MM Markers by WB at EVs PB/BM at Diagnosis					IMWG Response Criteria at D100					textbMRD at D100 by Flow Cytometry					% of PCs at D100					MM Markers by WB at EVs PB/BM at D100																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															

BM: bone marrow; CR: complete response; D100: day 100 after autologous stem cell transplant; EMD: extramedullary disease; EVs: Extracellular Vesicles; FISH: Fluorescence in situ hybridization; Ig: immunoglobulin; IMWG: International Myeloma Working Group; ISS: International Staging System; K: immunoglobulin kappa (κ) chain; L: immunoglobulin lambda (λ) chain; MM: Multiple Myeloma; MRD: measurable residual disease; nd: signal non-detectable in all samples in the same WB; PB: peripheral blood; PCs: plasma cells; sCR: stringent complete response; VGPR: very good partial response; WB: Western Blot; wd: signal weakly detected in WB; (−) negative in WB; (+) positive in WB; (++) intense positive in WB.

Both CD38 and CD138, as specific lineage markers, were described to be the best markers to use when identifying the source EVs derived from monoclonal PCs [24,57]. Nonetheless, it is necessary to carefully interpret results from CD38+ EVs and CD138+ EVs once they can derive from other cells rather than PCs. Indeed, CD38 is also detectable in low levels in other lymphoid cells (NK, B, and activated T cells) and myeloid cells (monocyte), while CD138 can be expressed in low levels by epithelial cells [76,77]. When possible, co-presentation of CD38/CD138 should be used to identify MM EVs [57]. In a recent study, these double positives showed that their concentration was on average two-fold greater in MM patients when compared to healthy controls, whereas single CD38+ or CD138+ positivity in EVs was lower (less than one-fold) when compared to healthy controls [57].

Expression of CD45 was found in EVs from six patients (Figure 3), and in half of them CD45 was more expressed in BM than in PB (patients 1, 2, and 8). The impact of CD45 positivity in MM at diagnosis is controversial in terms of prognostic value, despite being associated with higher proliferative cell rates [78]. It was difficult to establish a pattern between proliferation and the level of CD45 EVs in our patients.

Restriction for kappa (κ) or lambda (λ) light chains is usually used as a surrogate for clonality in MM, with abnormal ratios suggesting the presence of such a restriction [73]. Importantly, there were different levels of kappa or lambda light chains in the samples analyzed (Figure 3), with predominance of one of the light chains corresponding to the PC isotype defined by serum immunofixation (Table 1).

Other markers were reported to be increased in MM EVs, although they are not specific to MM and could be used as aberrant markers. For example, increased levels of the transmembrane glycoprotein CD147 were found in MM-derived EVs and appeared to be related to tumor cell growth and MM progression [79,80], while the adhesion molecule CD44 was increased in MM-derived EVs from patients treated with steroids and lenalidomide [81,82].

2.2.2. MM Markers Can Be Found in EVs from Both BM and PB Samples

When comparing the presence of MM markers between paired PB and BM samples, it was possible to verify that, with some minor exceptions, most of the markers present in the EVs isolated from BM were also present in EVs isolated from PB (Figure 3). However, differences were found in the levels of those markers when comparing paired PB and BM samples for CD45 (patients 1, 2, 3, 7, and 8), CD56 (patient 3, 7, 8, and 10), CD19 (patient 2 and 8), CD138 (patients 4, 7, and 8), CD27 (patient 8), CD38 (patient 8 and 9), HLA-DR (patients 1, 3, 5, 6, and 10), immunoglobulin kappa (Ig κ) (patients 1, 5, 7, and 8), and immunoglobulin lambda (Ig λ) (patients 1, 3, 7, and 10). The observed differences were found mostly in the diagnostic samples. In most cases, the differences found at diagnosis consisted of higher levels in BM than in PB samples. However, and surprisingly, in the remission samples the levels of the markers were very similar between PB and BM paired samples. More similar results between PB and BM were only obtained for CD117 and CD20 in all analyzed patients.

Even though this WB analysis is not a quantitative analysis, and the analysis of results is limited by the lack of a proper EV protein loading control, this suggests that the cargo of immunophenotypic markers on EVs from BM and PB paired samples is not the same. Therefore, the search for these biomarkers in liquid biopsies (blood samples, PB) from diagnostic samples may not provide similar results to the ones obtained from BM aspirates. Nonetheless, similar levels of immunophenotypic markers are present in EVs from BM and PB at the remission stage, suggesting that the follow up of MM patients by analysis of EVs in liquid biopsies (PB samples) may be representative of the disease markers in the BM. This finding is of major relevance regarding the potential use of EVs as biomarkers in liquid biopsies for MRD monitoring.

Detection of MRD in PB mirroring BM and/or EMD would bring obvious advantages to patients and clinicians, allowing frequent and minimally invasive real-time monitoring of the disease [83–86]. In fact, the use of a liquid biopsy has been proposed as an alternative approach to monitoring MRD in MM and mainly uses circulating tumor DNA

(ctDNA) [83,87,88], circulating tumor cells (CTCs) [89–91], serum monoclonal immunoglobulins [14], and, recently, EVs [44,92]. EVs are easy to assess and considered as possible minimally invasive biomarkers for cancer because of: (i) their capacity to carry important and relevant cargo that reflects the cell of origin; (ii) their ability to protect their cargo from external degradation; and (iii) their considerable longevity and stability in circulation [93]. These are major advantages compared to other circulating cell-free molecules such as proteins and microRNAs that are susceptible to degradation and have a short half-life [85,94]. Compared to CTCs, EV assessment requires a smaller sample volume to guarantee the likelihood of detection since they are easily found in PB [85]. The ctDNA also requires large volumes of plasma to be analyzed, having unpredictable half-lives compared to EVs [95]. Additionally, only 0.1–10% of the total circulating cell-free DNA (cfDNA) consists of ctDNA, with non-tumoral cfDNA hampering the use of ctDNA as a biomarker [85,96].

2.2.3. EVs from Diagnosis and Remission PB and BM Patient Samples Present Different Levels of MM Markers

In all the ten patients included in the analysis, there was a lack of consistency regarding the levels of most MM markers found in the cargo of EVs isolated from paired diagnosis or remission samples (Figure 3). Nonetheless, in some patients, clear changes were found according to the stage of the disease in terms of the levels of some markers, such as CD38, CD45, CD56, and HLA-DR.

Regarding the comparison of MRD status determined by flow cytometry and the EV MM markers found at remission, it is difficult to establish a definite correlation, even though some patterns can be found (Table 1). Indeed, in some patients, the disappearance at remission of specific markers in the EVs could support their possible use as MRD markers (Table 1). For example, regarding CD38 levels, results (Figure 3) show that there is a reduction in CD38 levels for patients 2 and 8 at remission when compared to diagnosis, both in PB- and BM-isolated EVs, possibly reflecting a change in the disease burden. Both patients presented a high-risk disease with International Staging System 3 (ISS 3) and *amp1q21* in Fluorescence in situ hybridization (FISH) analysis (with bad prognosis) and also had a CR after an autologous stem cell transplant (ASCT) which achieved MRD negativity when assessed by flow cytometry (Table 1). It was previously reported that in EVs derived from MM-PCs expressing CD38, their quantity was positively correlated to the clinical staging system, in which the number of CD38+ EVs was significantly higher in patients with an ISS of 3, echoing aggressive disease, when compared to those with an ISS of 2 or ISS of 1 [46].

Interestingly, patient 8 also presented a disappearance of CD138 in EVs isolated from remission (in both PB and BM samples, Figure 3). In another study, positive CD138 EVs were associated with disease stage and therapeutic response, with CD138+ circulating EVs increasing gradually in patients with relapsed MM, reflecting the disease burden and resistance to treatment [97]. Another study evaluated CD138 as a possible marker in the PB to assess response and showed that the levels of CD138+ EVs were higher in newly diagnosed MM patients when compared to patients in remission or healthy donors, thus providing support for the use of EVs as a tool for monitoring MM in PB [98]. This suggested the possibility of using CD138+ EVs as a prognostic tool and surrogate of treatment response [97,98]. In fact, a specific resistance signature of CD138+/P-glycoprotein+(P-gp+)/CD34+ EVs was found to be significantly elevated in the plasma of patients with aggressive disease and persistent residual disease, supporting its possible role as an MRD marker in EVs [99].

Strikingly, patient 8 presented a decrease in all analyzed MM markers in the remission sample, together with a decrease in kappa but an increase in lambda chains (Figure 3). The pattern observed may represent a CR to ASCT with recovery of polyclonality, restoration of the kappa/lambda ratio, and no residual disease detected in the EV cargo. This patient after ASCT reached a CR with normalization of the ratio of free light chains and negative residual disease as assessed by flow cytometry.

Interestingly, CD56 increased in remission for patient 3, while it was reduced or disappeared for patients 8 and 10 (Figure 3). The expression levels of CD56 in MM are reported to be up to 80% but its impact is controversial, being mainly associated with EMD and worse prognosis [100,101]. Patient 3 had no EMD, but had a revised-ISS of 3 and t(4;14) (Table 1), which is associated with severe prognosis; at day 100 after ASCT this patient did not achieve negative residual disease [102].

Regarding CD45, its levels decreased on the remission EV samples from patient 1 and disappeared on the remission EV samples from patient 8. However, its levels increased in the remission samples of patients 2, 3, and 4 (Figure 3). It is accepted that, during normal PC development and differentiation, CD45 levels progressively reduce, but its role in MM is controversial. Its persistence appears to reflect more immature PCs and an aggressive phenotype [78]. As with patients 2 and 3 with high ISS or revised-ISS (R-ISS) stages and high-risk features in cytogenetics harboring bad prognosis, patient 4 also had bad prognosis with an R-ISS of 3 and double the amount of high-risk features in the cytogenetics analysis, specifically t(4;14) and del17p (Table 1). The increased levels of CD45 in MM EVs may reflect drug resistance and may be implicated in a compromised depth of response, with both patients 3 and 4 being positive in the flow cytometry MRD assessment at day 100 after ASCT (Table 1).

HLA-DR also presented different levels at diagnosis and remission, being reduced at remission in most patients but intensively increased in the BM EVs from patients 6 and 10 (Figure 3). The HLA-DR antigen most frequently appears on the cytomembrane of macrophages and B lymphocytes and may aid the host immune system in identifying and attacking tumor cells. Its relation to MM is less explored but its persistence is associated with shorter survival [103]. Nonetheless, it was not possible to establish a pattern between these bad prognoses and our patients. Patient 6 had no cytogenetic high-risk features while patient 10 had an R-ISS of 3 and t(4;14) (Table 1).

2.3. EVs from Healthy Controls Do Not Present Most of the MM Markers

The same markers of MM and MRD were analyzed in the EVs isolated from the PB of ten healthy controls by WB of the ultrafiltrated pool of SEC fractions 3 to 6 (UF). Results are presented in Figures 4 and 5. EVs concentration was lower in the PB of healthy controls than in the PB or BM of patients with MM (even though only the comparison between PB of healthy controls and PB of patients at remission was considered to be statistically significant) (Figure 4B), while the average size of the isolated EVs was very similar (Figure 4A). Overall, the levels of EVs in cancer, including hematological malignancies, are reported to be considerably higher than in healthy controls [46], highlighting their potential as cancer biomarkers [43].

As expected, IgG kappa and lambda were detected in EVs isolated from healthy donors (Figure 5). However, HLA-DR is also detected in healthy controls, indicating that this is not a suitable biomarker to be used for MM diagnosis or monitoring of MRD. However, some MM markers (CD56, CD117, CD27) were not identified in healthy controls, with the exception of control 8 for CD56. In addition, CD38 was barely detected in healthy controls (Figure 5). This agrees with other studies, which found CD38+ EVs in MM serum but not in healthy controls [46,98]. A pivotal study previously demonstrated that serum samples from patients with PC diseases contained higher levels of Hsp70, Annexin IV, and c-Src-positive EVs derived from monoclonal PCs than healthy controls [104]. Additionally, the quantity of serum EVs expressing CD38 [46,98] or CD138 [97,99,105] was found to be significantly elevated in MM patients compared to healthy controls and related to disease severity [46], stage, and response/resistance to treatment [97,99,105].

The absence of MM markers in EVs from healthy controls further suggests that markers such as CD38, CD56, CD117, and CD27 are suitable for use as biomarkers of MM or in response monitoring of MRD.

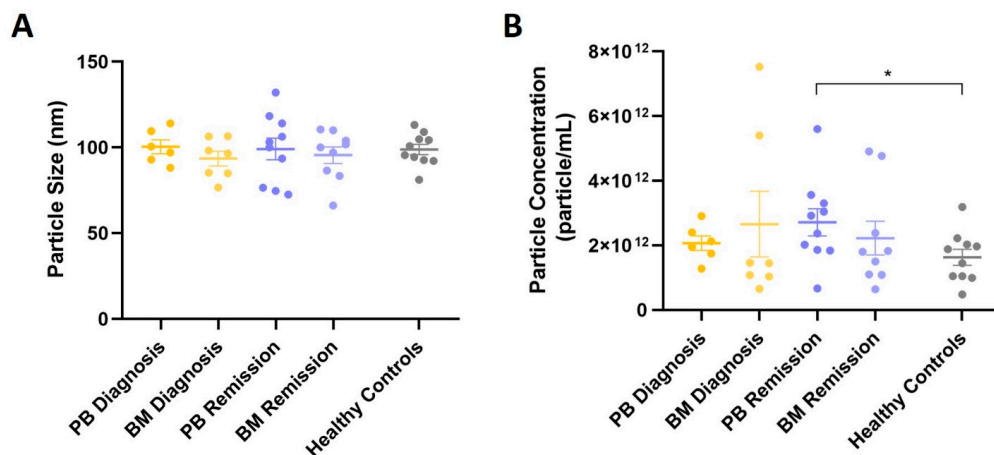


Figure 4. Comparison of the size and concentration of the EVs isolated by Size Exclusion Chromatography (SEC) and concentrated by ultrafiltration (UF) from Multiple Myeloma (MM) patients and healthy controls. **(A)** Average size of the isolated EVs from PB and BM samples of both diagnosis and remission MM patients and from healthy controls. Results are the mean $\pm$ standard error (SE) of the particle size obtained by NTA from at least six samples. **(B)** Particle concentration of the isolated EVs from PB and BM samples from both diagnosis and remission MM patients and from healthy controls. Results are the mean $\pm$ standard error (SE) of particle concentration obtained by NTA from at least six samples. * $p < 0.05$. PB: peripheral blood; BM: bone marrow; SEC: Size Exclusion Chromatography; UF: ultrafiltrated pool of SEC fractions 3 to 6; NTA: Nanoparticle Tracking Analysis; MM: Multiple Myeloma.

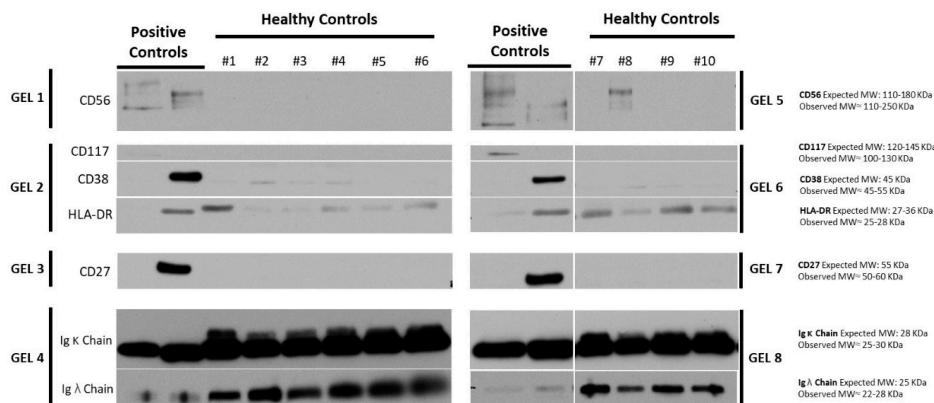


Figure 5. Analysis of Multiple Myeloma (MM) and Minimal Residual Disease (MRD) markers in EVs from the peripheral blood (PB) of healthy donors. Different markers of MM and MRD were analyzed by WB in the ultrafiltrated pool of SEC fractions 3 to 6 (UF) from the PB of 10 healthy donors. The UF from two MM patients was used as positive controls (left side of the blots). The molecular weight (MW) of the bands is shown in kDa on the right side of the blots. MM: Multiple Myeloma; MRD: Minimal Residual Disease; PB: peripheral blood; BM: bone marrow; SEC: Size Exclusion Chromatography; WB: Western Blot; UF: ultrafiltrated pool of SEC fractions 3 to 6.

3. Material and Methods

3.1. Patients and Samples

Patient peripheral blood (PB) and bone marrow (BM) samples were collected from Multiple Myeloma (MM) patients at diagnosis and at remission on the 100th day after autologous stem cell transplant (ASCT) at the Clinical Hematology Department from Centro Hospitalar Universitário São João (CHUSJ, Porto, Portugal). All patients were newly diagnosed with MM and treated upfront with the triplet combination of bortezomib, thalidomide, and dexamethasone. Treatment response on the 100th day after ASCT was assessed according to the International Multiple Myeloma Group recommendations [13,14,24,106].

3.2. Minimal Residual Disease (MRD) Determination by Flow Cytometry

All Minimal Residual Disease (MRD) assays were performed on the BM aspirates within 24 h of collection according to the Eight Color EuroFlow panel for MM, combining surface antigens for the identification of phenotypically abnormal clonal plasma cells (PCs)—CD38, CD138, CD45, CD19, CD56, CD27, CD81, and CD117—and cytoplasmic lambda and/or kappa light chains to confirm clonality. MRD was considered negative with a cut-off level of less than 20 clonal PCs in 2 million nucleated cells (minimal sensitivity of 10^{-5}) and the limit of detection (LOD) in each assay was determined according to the formula $(20/\text{nucleated cells}) \times 100$. Any patient was considered MRD negative if there was an absence of clonal PCs or if they were present below the LOD achieved in the corresponding sample [24,107,108].

3.3. Platelet-Poor Plasma (PPP) Isolation

Samples were collected in tubes buffered with 3.8% of sodium citrate (Vacutest® KIMA, Arzergrande, Italy). The PB and BM samples were transferred to centrifuge tubes using an aseptic technique, and an equal amount of phosphate-buffered saline 1× (PBS 1×) was added to each. The mix was gently transferred to tubes containing Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany) and centrifuged for 30 min at room temperature (RT) at $400 \times g$. The Platelet-Rich Plasma (PRP) was centrifuged (at $2500 \times g$, at RT, for 15 min), resulting in Platelet-Poor Plasma (PPP). The PPP was divided into aliquots and immediately stored at -80°C until further use.

3.4. Extracellular Vesicles (EVs) Isolation from Platelet-Poor Plasma (PPP)

3.4.1. Size Exclusion Chromatography (SEC)

The isolation of Extracellular Vesicles (EVs) was performed using a previously described method with some alterations [62]. After being washed with filtered 0.32% (*w/v*) trisodium citrate dihydrate (Merck Life Science, Darmstadt, Germany) in PBS (PBS-citrate, pH 7.4), Sepharose cross-linked 2B (CL2B300, Merck Life Science, Darmstadt, Germany) was placed in a 10 mL syringe (10 mL SOFT-JECT®, Henke Sass Wolf, Tuttlingen, Germany) with its tip filled with a piece of nylon stocking (20 denier). When the compacted Sepharose reached the 11 mL mark, a 3 MM paper filter (3 MM CHR; Cytiva, Marlborough, MA, USA) was added to the top. The column was kept in 20% ethanol and stored at 4°C until further use.

For EVs isolation, the 20% ethanol solution was removed by loading PBS-citrate into the SEC column. The PPP (1 mL) was loaded with the continuous addition of PBS-citrate. Ten sequential 1 mL fractions were collected. Fractions were either used immediately for EVs characterization or stored at -80°C until further use.

3.4.2. Ultrafiltration (UF)

To concentrate the EVs, around 4 mL from pools of EV-rich and protein-low fractions (i.e., SEC fractions 3 to 6) was filtered with a 100 kDa cut-off membrane (Amicon® Ultra-4 Centrifugal Filters Ultracel®—100 K, Milipore, Merck Life Science, Darmstadt, Germany) and centrifuged at 4°C at $3200 \times g$. The ultrafiltrated (UF) sample was collected, quantified (as described below), and stored at -80°C until downstream analysis.

3.5. Protein Quantification

The protein amount of each SEC fraction (either from the EV cargo or protein contaminants) was determined using the Lowry protein assay (DC™ Protein Assay kit, Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The absorbance was analyzed in a microplate reader (Synergy™ Mx, BioTek Instruments Inc., Winooski, VT, USA) with a 488 nm excitation wavelength and read at the 655 nm emission.

3.6. EVs Characterization

3.6.1. Nanoparticle Tracking Analysis (NTA)

Particle concentration and size distribution were obtained by Nanoparticle Tracking Analysis (NTA). The SEC fractions and the UF sample were pre-diluted (1:10 to 1:10,000) in 0.22 µm of filtered PBS-citrate (to reach the optimal concentration read-out range of 10^7 to 10^9 particles/mL). The samples were loaded at a constant rate in a NanoSight NS300 (Malvern Instruments Ltd., Malvern, UK) with a 1 mL syringe (Omnifix® 100 Solo, B1 BRAUM, Melsungen, Germany) using a Nanosight syringe pump (Malvern Instruments Ltd., Malvern, UK) at RT. Three separate 30 s videos were recorded with the following specifications: camera type, sCMOS; laser type, Blue488; camera level, 15/16; slider shutter, 1206/1300; slider gain, 366/512; FPS, 25.0; temperature, 21.1–25.5 °C; viscosity, 0.878–0.974 cP; syringe pump speed, 40. Particles were detected by video analysis using NanoSight NTA Software (NTA version 3.2, Dev Build 3.2.16) with the following settings: detection threshold, 5; blur size, auto; max jump distance, auto (8.3–16.2 pixels). The mean, mode, and median vesicle size (nm) and estimation of the particle concentration (particles/mL) were determined. The data obtained by NTA were then analyzed using GraphPad (Prism 8).

3.6.2. Transmission Electron Microscopy (TEM)

EVs size, morphology, and integrity were visualized by Transmission Electron Microscopy (TEM) using negative staining. Each sample was resuspended in PBS-citrate or in a 1:2 solution of PBS-citrate and HEPES 20 mM (Merck Life Science, Darmstadt, Germany) +4% (*w/v*) sucrose (Merck Life Science, Darmstadt, Germany), mounted in Formvar-carbon-coated electron microscopy grids (Electron Microscopy Sciences, Hatfield, PA, USA) for 2 min in the dark at RT, and dried with a filter paper. TEM grids were then counterstained with 5% uranyl acetate and visualized under the transmission electron microscope (Jeol JEM 1400, JEOL, Tokyo, Japan) with an acceleration voltage of 80 kV. The data were obtained by the Histology and Electron Microscopy Service, i3S, Porto, Portugal. Representative TEM photographs were acquired and the EV size for each SEC fraction was measured by image analysis using ImageJ software.

3.6.3. Western Blot (WB)

The SEC fractions and the UF sample were denatured in loading buffer (Tris-HCl 1 M pH 6.8, 10% SDS, 85% glycerol, β-mercaptoethanol, 1% bromophenol blue) and boiled at 95 °C for 5 min. A total of 15 µg of protein from each fraction was separated using SDS-PAGE (Mini-PROTEAN® Tetra Vertical Electrophoresis Cell, Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane (GE Healthcare Life Science, Chalfont St Giles, UK) using a Mini Trans-Blot® cell system (Bio-Rad, Hercules, CA, USA). After the transfer, the proteins were stained with Ponceau S Solution (PanReac AppliChem, Barcelona, Spain) and images were acquired with Chemidoc XRS+ System equipment (Bio-Rad, Hercules, CA, USA). The membranes were blocked for at least 2 h at RT in a blocking solution consisting of 5% (*w/v*) non-fat dry milk (Molico, Nestlé, Vevey, Switzerland) in TBS-T (Tris-buffered saline solution pH 7.4 with 0.1% Tween-20, [Promega Corporation, Madison, WI, USA]). After blocking, membranes were incubated with primary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA; GeneTex, Irvine, CA, USA) diluted in the blocking solution in accordance with the manufacturer's instructions (Supplementary Table S1) and stirred for 90 min at RT. After being washed in TBS-T, membranes were incubated with the secondary antibodies (GE Healthcare Life Science, Chalfont St Giles, UK; Santa Cruz Biotechnology,

Dallas, TX, USA) (Supplementary Table S2) for 1 h with agitation at RT. The signal of the membranes was then detected using the enhanced chemiluminescence (ECL) Western Blotting Detection Reagent (GE Healthcare Life Science, Chalfont St Giles, UK), Amersham Hyperfilm ECL (GE Healthcare Life Science, Chalfont St Giles, UK), and a Fuji Medical Film Processor (FPM-100A Model, Fuji Photo, Tokyo, Japan). The molecular weight of protein bands was estimated by comparison with an established protein marker (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, ThermoFisher Scientific, Waltham, MA, USA). Band quantification was carried out using Image Lab™ Software version 6.0.1 (Bio-Rad, Hercules, CA, USA). Signal quantification of the bands was normalized to the total protein of the lane, which was obtained by Ponceau staining.

3.7. Multiple Myeloma (MM) Marker Analysis by Western Blot (WB)

A total of 15 µg of the UF samples from the PB and BM patient samples and from the PB of healthy donors was denatured and separated by SDS-PAGE followed by WB using the same protocol and systems as described above. Antibodies (GE Healthcare Life Science, Chalfont St Giles, UK; Santa Cruz Biotechnology, Dallas, TX, USA; Abcam, Cambridge, MA, USA) for MM-specific markers (such as CD38 and CD138) were used, as well as other antibodies relative to the antigens used in the EuroFlow panel, such as CD19, CD27, CD45, CD81, CD56, CD117, and cytoplasmic lambda and/or kappa light chains [13,24] (Supplementary Tables S2–S4, and Figure S1).

3.8. Statistical Analysis

The statistical analysis was performed using the two-tailed unpaired *t*-test which was obtained in GraphPad Prism 8.0 software. Statistical significance was considered whenever $p < 0.05$.

4. Conclusions

In the present study, we demonstrated the possibility of isolating EVs from the BM and PB of MM patients at distinct disease stages (diagnosis and remission) and compared the MRD-associated markers between paired BM and PB EVs at diagnosis and remission stages. Firstly, our results demonstrated that EVs from BM and PB samples, both from diagnosis and remission, were successfully isolated using the SEC method, concentrated by UF, and characterized by NTA, TEM, and WB in terms of size distribution, morphology, and the presence of well-known EV protein markers. Secondly, EVs isolated from PB or BM at diagnosis present specific MM markers, such as CD38 and CD138, confirming the potential of EVs as MM biomarkers. Thirdly, by analyzing alterations in the levels of MM markers in EVs isolated from paired diagnosis and remission samples, it was possible to verify differences between those markers, enhancing the possibility of using EVs to monitor MRD. Fourthly, even though in the diagnostic samples the levels of MM markers were different in several PB and BM samples, in the remission samples those levels were very similar, thus suggesting that EVs have the potential to monitor MRD in liquid biopsies from remission samples. Finally, the much lower levels of MM markers in the EVs isolated from the PB of healthy controls supports the possibility of using those markers as specific MM biomarkers to non-invasively monitor MRD.

Nonetheless, some caveats are exposed in our study. It was not powered to provide definite clinical advice; thus, further studies should be attempted to extend this study to a larger sample of MM patients and integrate this approach with NGS or NGF at different disease timepoints. Moreover, even if the SEC method proved to be efficient in isolating EVs and the protocols to characterize those EVs were well described, overall, the methodology is time consuming and needs to be further optimized to become reproducible and easier to use in clinical practice. Furthermore, there is no standardization in the interpretation of results, which relies on sample-by-sample analysis by experienced operators.

Overall, this study suggests the possibility of isolating EVs from PB samples of MM remission patients for use in the monitoring of MRD. Our results warrant a prospective and

larger study with more samples from MM patients to confirm and reproduce the herein presented data, to optimize the methodology in order for it to become easier to use in clinical practice, and to determine its sensitivity regarding MRD detection so that it can possibly assume its role as a method for analyzing non-invasive liquid biopsies of MM.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms232213686/s1>.

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Informed Consent Statement: All samples were collected after obtaining informed consent from all individual participants in accordance with the Declaration of Helsinki.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request, provided no ethical, legal, or privacy issues are raised.

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Abbreviations

AS-PCR	allele-specific oligonucleotide polymerase chain reaction
ASCT	autologous stem cell transplant
BM	bone marrow
CAR-T cells	chimeric antigen receptor-T cells
CHUSJ	Centro Hospitalar Universitário São João
cfDNA	cell-free DNA
CTCs	circulating tumor cells
ctDNA	circulating tumor DNA
CR	complete response
D100	day 100 after ASCT
ECL	enhanced chemiluminescence
EMD	extramedullary disease
EVs	Extracellular Vesicles
FDG-PET/CT	18-fluoro-2-deoxyglucos positron emission tomography/computed tomography
FISH	Fluorescence in situ hybridization
FPM	Fuji Medical Film Processor
Ig	immunoglobulin

IMWG	International Myeloma Working Group
ISS	International Staging System
K	immunoglobulin kappa (κ) chain
L	immunoglobulin lambda (λ) chain
LOD	limit of detection
MFC	multiparameter flow cytometry
MM	Multiple Myeloma
MRD	measurable residual disease
MSCs	BM mesenchymal stem cells
MW	molecular weight
NGF	next-generation flow
NGS	next-generation sequencing
NTA	Nanoparticle Tracking Analysis
OS	overall survival
PB	peripheral blood
PBS	phosphate-buffered saline
PC	plasma cells
PFS	progression free-survival
PPP	Platelet-Poor Plasma
PRP	Platelet-Rich Plasma
R-ISS	revised-ISS
RT	room temperature
sCR	stringent complete response
SEC	Size Exclusion Chromatography
TBS-T	Tris-buffered saline solution with 0.1% Tween-20
TEM	Transmission Electron Microscopy
UF	ultrafiltration
UF	ultrafiltrated pool from SEC fractions 3 to 6
VEGF	vascular endothelial growth factor
VGPR	very good partial response
WB	Western Blot

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Supplementary Data

Detection of measurable residual disease biomarkers in extracellular vesicles from liquid biopsies of Multiple Myeloma patients – a proof of concept

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Supplementary tables

Supplementary Table S1. Analyzed EV markers and protein contaminants and their respective antibody dilutions.

Protein	Reference	Dilution factor	Expected Molecular Weight (KDa)
Actinin 4	GTX113116 (GeneTex)	1:500	105
Albumin	sc-271605 (Santa Cruz)	1:100	66
Alix	sc-49268 (Santa Cruz)	1:400	95
Annexin XI	sc-46686 (Santa Cruz)	1:100	50-56
ApoA-I	sc-376818 (Santa Cruz)	1:100	28
ApoB	sc-393636 (Santa Cruz)	1:100	512
CD63	sc-5275 (Santa Cruz)	1:200	26-60
CD81	sc-7637 (Santa Cruz)	1:200	22-26
CD9	sc-13118 (Santa Cruz)	1:200	24
Cytochrome C	sc-13560 (Santa Cruz)	1:200	15

HSP70	sc-66048 (Santa Cruz)	1:200	70
Mitofilin	sc-390707 (Santa Cruz)	1:100	88/90
Syntenin-1	sc-100336 (Santa Cruz)	1:200	33
TSG101	sc-136111 (Santa Cruz)	1:200	45

Supplementary Table S2: Secondary Antibodies and their respective dilution factors.

Antibody	Reference	Dilution Factor
Anti-mouse	NA931V (GE Healthcare)	1:2000
Anti-goat	sc-2354 (Santa Cruz)	1:2000
Anti-rabbit	NA934V (GE Healthcare)	1:2000

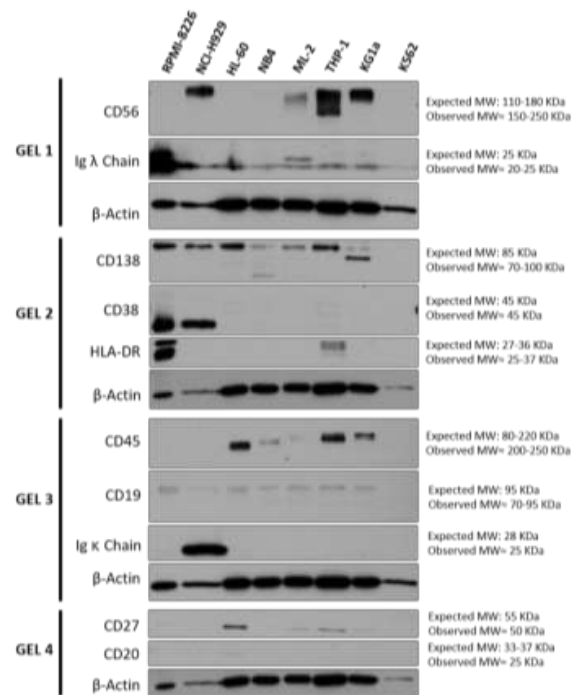
Supplementary Table S3: Analyzed Multiple Myeloma Markers and their respective antibody dilutions.

Protein	Reference	Dilution factor	Expected Molecular Weight (KDa)
CD56 (NCAM)	sc-7326 (Santa Cruz)	1:200	110-180
CD79B	Sc-18902 (Santa Cruz)	1:200	39
CD117 (c-kit)	sc-17806 (Santa Cruz)	1:100	120/145
CD138 (Syndecan-1)	sc-12765 (Santa Cruz)	1:200	85
CD38	sc-374650 (Santa Cruz)	1:100	45
HLA-DR	sc-53319 (Santa Cruz)	1:200	27/36
CD45	sc-1178 (Santa Cruz)	1:200	180-220
CD19	sc-373897 (Santa Cruz)	1:100	95
CD28	sc-70612 (Santa Cruz)	1:200	44
Ig λ chain [EPR5367-62]	ab124719 (abcam)	1:20000	25
Ig κ chain	sc-59265 (Santa Cruz)	1:200	28
CD44 (HCAM)	sc-9960 (Santa Cruz)	1:200	90-95
CD20	sc-393894 (Santa Cruz)	1:100	33-37
CD27	sc-25289 (Santa Cruz)	1:150	55

Supplementary Table S4: Expected and observed Molecular weights for the bands of the Multiple Myeloma and Minimal Residual Disease protein Markers, analyzed in both human samples and in cell lines of hematological malignancies. n.d. – not detected

Protein	Expected Weight (KDa)	Observed Molecular Weight (KDa)	
		Human Samples	Cell Lines
CD56	110-180	100-220	150-250+
CD45	80-220	150-250	200-250
CD117	120-145	100-200	n.d.
CD19	95	70-95	70-95
CD138	85	70-85	70-100
CD27	55	45-55	50
CD38	45	40-55	45
CD20	33-37	25-33	25
HLA-DR	27-36	25-36	25-37
Ig κ Chain	28	25-28	25
Ig λ Chain	25	20-25	20-25

Supplementary Figures



Supplementary Figure S1: Analysis of Multiple Myeloma (MM) and Minimal Residual Disease (MRD) protein Markers in cell lines of hematological malignancies. Different markers of MM and MRD were analyzed by WB in cell lysates of several cell lines of hematological malignancies. Actin staining was used as loading control. The molecular weight (MW) of the bands is shown in KDa on the right side of the blots.

ORIGINAL ARTICLE

3.2. microRNA-665 as a possible biomarker of drug response in Multiple Myeloma patients**Submitted for publication**

Bergantim, Rui; Peixoto da Silva, Sara; Pinto, Vanessa; Pereira, Joana; Sousa, Diana; Trigo, Fernanda; Matthiesen, Rune, Guimarães, José E.; Vasconcelos, M. Helena. "microRNA-665 as a possible biomarker of drug response in Multiple Myeloma patients: preliminary study".

microRNA-665 as a possible biomarker of drug response in Multiple Myeloma patients: preliminary study

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ABSTRACT

Background: Multiple myeloma (MM) is a complex hematological malignancy with heterogeneous clinical and pathophysiological backgrounds that influence treatment responses and outcomes. Identifying biomarkers to predict drug response and guide treatment decisions, particularly regarding drug combinations, is essential to improve therapeutic efficacy and patient outcomes. This study explores the role of microRNAs (miRNAs/miRs) derived from bone marrow (BM) and peripheral blood (PB) to serve as biomarkers to predict responses to treatment in newly diagnosed MM (ndMM) patients.

Methods: This study included patients with ndMM undergoing first-line treatment with bortezomib, thalidomide, and dexamethasone. miRNAs were isolated from BM and PB, and their profiles were analyzed using Next-Generation Sequencing (NGS), with differentially expressed miRNAs validated by quantitative real-time PCR (qPCR). Clinical and response data were collected to assess correlations between miRNA levels, clinical characteristics, and patient outcomes. In silico analysis for target-prediction and gene ontology (GO) enrichment was performed to explore the potential biological and functional role of the identified miRNAs.

Results: NGS profiling revealed several miRNAs differently expressed between treatment-refractory and sensitive patients, as well as between PB and BM. Among these, miR-665, miR-483-5p, miR-143-3p, and miR-145-5p were selected for further validation through qPCR. It was observed that miR-665 was significantly elevated in treatment-refractory patients compared to treatment-sensitive patients.

Additionally, miR-665 levels were higher in PB than in BM. Elevated miR-665 levels were associated with more aggressive disease characteristics and poorer clinical outcomes, including reduced overall survival.

Discussion: Our findings suggest that miR-665 could potentially serve as a non-invasive biomarker for predicting drug resistance and guiding treatment decisions in MM. These findings highlight the potential utility of miRNAs in liquid biopsies as predictive biomarkers of drug response in MM and could pave the way for personalized treatment strategies, improving patient outcomes. Future research is needed to validate these results in larger cohorts and explore the underlying mechanisms of miR-665 in MM pathogenesis and drug resistance.

KEYWORDS

miRNAs, multiple myeloma, biomarkers, drug resistance, drug response.

INTRODUCTION

Multiple myeloma (MM) is a highly clinical, phenotypical, and molecular heterogeneous hematological neoplasia, making the perfect treatment approach challenging to establish. Over the last few years, the MM treatment landscape has significantly changed with new drugs such as proteasome inhibitors (PIs), immunomodulators (IMiDs), and several monoclonal antibodies approved and broadly used to treat MM. Additionally, other therapies with new mechanisms of action, such as conjugated and bi-specific monoclonal antibodies and Chimeric antigen receptor (CAR) T-cells, have been integrated into clinical practice, allowing new treatment perspectives (1, 2). The impact of these treatments, both at diagnosis and relapses, has been substantial, enabling MM patients to achieve better responses, longer progression-free survival (PFS), and ultimately better overall survival (OS) (3). This is particularly evident in the first line of treatment, which usually includes a PI, an IMiD, and, recently, an anti-CD38 monoclonal antibody. These improvements have led to higher rates of complete response and undetectable measurable residual disease (MRD) in patients either eligible or non-eligible for transplant (4, 5).

Nonetheless, a small percentage of patients fail to respond to initial therapy and are classified as primary refractory MM patients (6-8). It has been reported that around 10-20% of MM patients are refractory to first-line treatments, including PIs and IMiDs, with around 5% being refractory to recent anti-CD38⁺ monoclonal antibodies-based protocols (6, 9). There is no consensus on the management of these patients, making them a particularly complex group to treat, exhibiting very aggressive disease characteristics and markedly shorter survival outcomes (7, 8).

The current treatment algorithm includes first-line therapy with multiple drug combinations, intensified or not with autologous stem cell transplant. Subsequent lines of treatment are determined according to the mechanisms of action, the response achieved, and the toxicity of previous therapies (10). At each step of the treatment, patient characteristics (such as performance status) and disease characteristics (including cytogenetic abnormalities) are evaluated to contribute to informed therapeutic decisions (11-13). However, these approaches fail to identify primary resistant patients and different outcomes are achieved by patients treated with the same therapeutic protocol (10, 14). Thus, biomarkers to predict

drug response and guide treatment decisions, particularly in selecting optimal drug combinations, are essential to improve therapeutic efficacy and outcomes in MM.

MicroRNAs (miRNAs/miRs) are highly conserved, small non-coding RNAs (approximately 19 to 25 nucleotides long) that modulate gene expression at both transcriptional and post-transcriptional levels (15). miRNAs specifically bind (fully or partially) to complementary regions within target mRNAs to promote their degradation and/or inhibit their translation, depending on the degree of complementarity (16). By degrading or blocking the translation of mRNA targets, miRNAs regulate gene expression in cancer pathophysiology, such as genes involved in several cellular signaling pathways, apoptosis, cell growth, proliferation, invasion, and metastasis (17-19). By targeting oncogenes and tumor suppressor genes, miRNAs are major players in the hallmarks of cancer and drug response (20-22). In MM, aberrant miRNA expression has been reported, with numerous upregulated or downregulated miRNAs identified across different processes of tumorigenesis (23, 24). For instance, several studies demonstrated that specific miRNAs may negatively influence the drug sensitivity of MM cells at different stages of the disease, emphasizing their possible role in developing drug resistance (25-30).

Considering the biological and clinical heterogeneity observed in MM and the knowledge that miRNAs are regulators of various pathways contributing to the hallmarks of cancer, they have recently emerged as potential key biomarkers for diagnosing this disease (20, 31, 32). Since miRNAs may be found in biological fluids such as blood (within circulating cells, associated with extracellular vesicles, or free) and can be easily detected, they represent promising candidates as non-invasive biomarkers in MM (33-37). Identifying and quantifying free miRNAs circulating in the peripheral blood of MM patients may contribute to understanding drug resistance mechanisms and improve the prediction of treatment responses (38-41). In this study, we explore the role of miRNAs isolated from the bone marrow (BM) and peripheral blood (PB) of MM patients undergoing treatment with bortezomib, thalidomide, and dexamethasone to assess their potential as biomarkers of treatment response.

MATERIAL & METHODS

Patients with MM and treatments

This study included patients with newly diagnosed MM (ndMM), proposed for the first line of treatment with bortezomib, thalidomide, and dexamethasone at Centro Hospitalar and Universitário São João (CHUSJ) - Porto, Portugal. None of the patients had undergone any prior therapy before collecting PB and BM samples. Patients were classified according to their response to the treatment regimen into two groups: treatment-sensitive or treatment-primary refractory. Sensitive patients had a complete response (CR) after the second and fourth cycles of treatment that persisted with negative MRD at day 100 following autologous stem cell transplant. Primary refractory patients had no response after two cycles of the first-line treatment. For these primary refractory patients, additional therapeutic regimens were explored. The criteria for diagnosis, clinical staging, risk stratification, and treatment response were assessed according to the International Multiple Myeloma Group (IMWG) recommendations (10, 42-44). This study was approved by the Ethical Committee for Health at CHUSJ, and written informed consent was obtained from all participants in compliance with the Declaration of Helsinki.

Collection and preparation of PB and BM samples

PB and BM samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes simultaneously before any treatment and processed within 1 to 4 hours after collection. All samples were collected in the morning while patients were fasting to avoid possible physiologic confounding factors. The samples were mixed with an equal volume of sterile phosphate saline buffer (PBS). Then, 4 mL of the patient samples (PB or BM) in PBS were carefully loaded over the top of Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany) and placed in a 15 mL centrifuge tube. Then, the samples were centrifuged at $400\times g$ (Centrifuge 5810R, Eppendorf), without brake, for 30 minutes at room temperature (RT). From this centrifugation, four layers resulted: a bottom layer containing erythrocytes and granulocytes, a second layer corresponding to the Histopaque, a third layer of mononuclear cells, and an upper layer corresponding to the cell-free plasma. This latter layer was then collected and aliquoted in several vials, immediately stored at -80°C to preserve RNA for further processing and analysis.

miRNA extraction for library preparation

Volumes of approximately 1mL of PB plasma and 1.5mL of BM plasma were used to extract miRNAs. Before the extraction, and to increase the extraction yield, samples were treated with proteinase K ($3\text{ }\mu\text{g}/\mu\text{L}$) at 37°C for 10 minutes. Then, miRNA extraction was performed using the miRCURY™ RNA Isolation Kit (Exiqon, Foster City, CA, USA), following the manufacturer's instructions. Before starting the lysis step, $10\mu\text{L}$ of β -mercaptoethanol and $1\mu\text{g}$ of MS2 RNA (RNA from bacteriophage MS2, Roche, Germany) were added to the lysis solution. The remaining protocol was performed as per the manufacturer's instructions. To elute the RNA, $30\mu\text{L}$ of RNase-free water was used. The RNA concentration of the samples was measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All the purified RNA samples were stored at -80°C .

Library preparation and miRNA profiling by Next Generation Sequencing (NGS)

miRNA cDNA libraries were constructed following the Ion Total RNA-Seq kit v2 protocol (Life Technologies, Carlsbad, CA, USA). After RNA quality control of samples, using the small RNA assay kit in the Bioanalyzer (Agilent 2100, Santa Clara, CA, USA), $3.5\text{ }\mu\text{L}$ of total RNA from each PB and BM sample was incubated for 16 hours to facilitate adaptors hybridization and ligation. After incubation, reverse transcription was performed, and cDNA was purified and size-selected by magnetic beads. Afterward, cDNA was amplified with a specific primer with unique barcodes to identify and track each sample. Due to the presence of an adapter dimer in some samples, an adapted protocol was developed where final libraries run in agarose gel and bands were excised, avoiding the adapter dimer band. Then, the pooled libraries were processed using the Ion Chef™ System (Life Technologies, Carlsbad, CA, USA), and the resulting 550™ chips were sequenced on the Ion S5™XL System (45).

Bioinformatics

A total of 35 FASTQ files from the Ion Torrent sequencer were analyzed. The files underwent trim processing using CutAdapt with the following settings: a minimum fragment length of 10 base pairs and a minimum quality score of 20 were specified (46). Following the trimming process using CutAdapt, the quality of the resulting reads was evaluated with FASTQC to ensure acceptable quality standards (FastQC. Available online: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>, accessed on 20 December 2019). Reads were then aligned by Bowtie1 to human genome assembly (hg19), allowing for one mismatch. Prebuild indexes were downloaded from the Bowtie webpage. The

length of seed substrings in the bowtie analysis was set to 10, and the additional parameters—best —nomaqround were also specified. The read count was calculated using FeatureCounts (47) software implemented in miARma-Seq (48) using a minimum quality of 10. The genome annotation files were miRBase_Annotation_20_for_hsa_mature_miRNA.gtf and/Homo_sapiens.GRCh37.75.gtf for miRNAs and all RNAs, respectively. Significant differential expression was calculated using the EdgeR package (49), with a minimum count per million cut-off of 2. biomaRt (50) was used to retrieve transcript annotation. To generate visualizations for our data analysis, we utilized the ggplot2 R package to create Venn diagrams, Pie charts, and Volcano plots.

Candidate miRNA confirmation by qPCR

miRNAs found differentially expressed between PB and BM samples and differentially expressed between samples from refractory and sensitive patients were selected for further validation of the NGS results by quantitative real-time PCR (qPCR). RNA from those samples was extracted using miRNeasy Serum/Plasma Advanced Kit (217204, Qiagen, Germany), following the manufacturer's instructions. To increase the recovery, 1 µg of MS2 RNA (RNA from bacteriophage MS2, Roche, Germany) was added to the RPL Buffer on each sample, as recommended by the manufacturer. For each sample, 400 µL were used as input, which resulted in a 30 µL final elution volume. Total RNA concentration and purity were measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA (10 ng) served as a template for cDNA synthesis using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol and using the T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The miRNAs expression levels were assessed using 2X TaqMan™Fast Advanced Master Mix (Applied Biosystems, Foster City, CA, USA) and 20X TaqMan™ Advanced miRNA Assays probes (hsa-miR-145-5p, 477916_mir; hsa-miR-143-3p, 477912_mir; hsa-miR-483-5p, 478432_mir; hsa-miR-665, 479150_mir – Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions (in a final volume of 20 µL per well). The qPCR reactions were performed in a 7500 Fast Real-Time PCR device (Applied Biosystems, Foster City, CA, USA). For each sample, triplicates were performed, and negative controls lacking cDNA template (no template control) or lacking reverse transcription enzyme (no reverse transcription) were included in duplicate in the qPCR plates. Three miRNAs found by NGS to be abundantly expressed in all samples were tested as endogenous controls (hsa-miR-26a-5p, 477995_mir; hsa-miR-17-5p, 478447_mir; hsa-miR-103a-3p, 478253_mir – Applied Biosystems, Foster City, CA, USA). The miR-26a-5p was then selected for expression normalization since its expression was the most stable in all samples (when compared to the other two candidates by using the BestKeeper software (51) and the analysis of cycle threshold (Ct) values among the samples - Supplementary Figure 1).

The efficiency of all the qPCR assays was between 90% and 110%, as recommended by the manufacturer. The Ct values for all miRNAs in each sample were acquired with the 7500 Software v2.06 (Applied Biosystems, Foster City, CA, USA). The Livak Method ($2^{-\Delta\Delta C_t}$), along with the Student's t-test (when data followed normality) or with the Mann-Whitney U test (when data did not follow normality), were used to determine statistical differences in the normalized relative expression of miRNAs among the different groups.

Statistical Analysis

The clinical characteristics of primary refractory patients to treatment were compared with those sensitive to treatment, along with the differential levels of the miRNAs of interest. Nominal variables

were expressed as percentages and analyzed using the Chi-square test, with appropriate corrections applied when needed, such as the Yates correction for continuity or Fisher's exact test. For continuous variables, differences between the two groups were evaluated using a two-sided independent Student's t-test when the data were normally distributed and with the Mann-Whitney U test when the assumption of normality was not met or the variable was ordinal. The prognostic potential of the identified miRNAs was evaluated using receiver operating characteristics (ROC) and area under the curve (AUC) analysis. For the cutoff of the identified miRNAs, the mean ΔCT values were used once it averaged across multiple replicates to reduce variability. Youden's index was calculated to identify the best cut-off, sensitivity, and specificity for each identified miRNA. Analyses of OS and progression-free survival (PFS) were conducted. Kaplan–Meier curves for OS and PFS were constructed, and differences were assessed using the Mantel-Cox (log-rank) test. Univariate and multivariate Cox regression analyses were performed with 95% confidence intervals (CIs) of each hazard ratio (HR) to identify independent predictors of outcomes after adjusting for confounders such as the Revised International Staging System (R-ISS) and cytogenetics. All statistical analyses were conducted using IBM SPSS Statistics for Windows (Version 27.0, IBM Corp., Armonk, NY, USA). Results were considered statistically significant when p-values were ≤ 0.05 .

In silico gene ontology analysis

miR-665 gene targets were predicted using an ensemble approach composed of three different target prediction bioinformatic tools (miRDB, TargetScanHuman 8.0, and DIANA TOOLS—microT-CDS) (52-54). To minimize prediction errors, only gene targets with a miRDB Target Score ≥ 80 , TargetScan cumulative weighted context score ≤ -0.3 , and DIANA micro-T-CDS prediction score ≥ 0.8 were selected. Genes identified by at least two different prediction tools were used for enrichment analysis using Enrichr (55). All Gene Ontology (GO) terms with a q-value ≤ 0.05 and a combined enrichment score over 10 were considered. GOnet was used to visualize relationships between miR-665 target genes and statistically significant GO terms (56).

RESULTS

Baseline clinical characteristics of MM patients

A cohort of twenty newly diagnosed patients with MM was included in the study. The median age at diagnosis was 58 years old (ranging from 44 to 67), with 60% of male patients. At diagnosis, the predominant clinical feature was anemia (80%), followed by lytic lesions (60%), renal failure (30%) and hypercalcemia (25%). Additionally, 15% of patients presented extramedullary disease, primarily non-paraskeletal. Regarding the R-ISS, three patients (15%) were R-ISS stage I, eleven (55%) were R-ISS stage II and six (30%) were R-ISS stage III. Cytogenetics by Fluorescence In Situ Hybridization (FISH) revealed high-risk cytogenetics abnormalities in 20% of the patients, with two of them exhibiting double-hit features, including the deletion 17p and t(4;14)(p16;q32). In terms of treatment response, eight patients achieved a CR with the induction treatment and maintained CR after autologous stem cell transplant, while twelve were classified as primary refractory. Of note, abnormal FISH results ($p=0.017$) and high lactate dehydrogenase (LDH) levels ($p=0.035$) were statistically significantly different between sensitive and primary refractory patients. Additionally, there was a trend suggesting R-ISS stage III to be more related to primary refractory patients ($p=0.053$). A detailed summary of the baseline clinical characteristics of the MM patients is provided in Table 1.

Table 1. Baseline and clinical characteristics of Multiple Myeloma (MM) patients according to their treatment response

Baseline and Clinical Characteristics	Total (n=20)	Refractory (n=12)	Sensitive (n=8)	p-value
Age				
Median (range) - yr	58 (44-67)	56 (47-63)	63 (44-67)	0.163
Sex - no. (%)				
Male	12 (60%)	9 (75%)	3 (37.5%)	0.094
Female	8 (40%)	3 (25%)	5 (62.5%)	
Myeloma isotype - no (%)				
Light Chain Kappa	4 (20%)	2 (16.7%)	2 (25%)	0.247
IgG/k	6 (30%)	2 (16.7%)	4 (50%)	
IgG/L	5 (25%)	5 (41.7%)	0 (0%)	
IgA/k	2 (10%)	2 (16.7%)	1 (12.5%)	
IgA/L	3 (15%)	1 (8.3%)	1 (12.5%)	
ISS stage - no (%)				
I	3 (15%)	1 (8.3%)	2 (25%)	0.300
II	8 (40%)	4 (33.3%)	4 (50%)	
III	9 (45%)	7 (58.3%)	2 (25%)	
R-ISS stage - no (%)				
I	3 (15%)	1 (8.3%)	2 (25%)	0.053*
II	11 (55%)	5 (41.7%)	6 (75)	
III	6 (30%)	6 (50%)	0 (0%)	
FISH				
Normal	11 (55%)	4 (33.3%)	7 (87.5%)	0.017*
Abnormal	9 (45%)	8 (66.7%)	1 (12.5%)	
High-risk cytogenetic profile				
No	5 (25%)	4 (33.3%)	1 (12.5%)	0.134
Yes	4 (20%)	4 (33.3%)	0 (0%)	
High-risk cytogenetic profile				
del(17p)	2 (10%)	2 (22.2%)	0 (0%)	0.571
t(4;14)	2 (10%)	2 (22.2%)	0 (0%)	0.572
t(14;16)	1 (5%)	1 (8.3%)	0 (0%)	0.708
t(11;14)	3 (15%)	2 (16.7%)	1 (12.5%)	0.134
amp1q21	3 (15%)	3 (15%)	0 (0%)	0.453
Double-Hit (del17p and t(4;14))	2 (10%)	2 (16.7%)	0 (0%)	0.571
Anemia				
<10g/dL or <1g/dL of normal Hb	16 (80%)	11 (91.6%)	5 (62.5%)	0.110
Creatinine				
<2mg/dl	14 (70%)	7 (58.3%)	7 (87.5%)	0.163
≥2mg/dL	6 (30%)	5 (41.7%)	1 (12.5%)	
Hipercalcemia				
yes	5 (25%)	4 (33.3%)	1 (12.5%)	0.292
Lytic lesions				
yes	12 (60%)	6 (50%)	6 (75)	0.264
B2-microglobulin				
Increased	9 (45%)	7 (58.3%)	2 (25%)	0.142
Normal	11 (55%)	5 (41.7%)	6 (75)	
LDH				
Increased	5 (25%)	5 (41.7%)	0 (0%)	0.035*
Normal	15 (75%)	7 (58.3%)	8 (100%)	
Extramedullary plasmacytoma				
Yes	3 (15%)	3 (25%)	0 (0%)	0.125
No	17 (85%)	9 (75%)	8 (100%)	
Clonal plasma cell in BM (median, range)	20.9 (5-72%)	26.5 (5-72%)	16.4 (12-64%)	0.702
Clonal plasma cell in PB (median, range)	0 (0.35-4%)	0.58 (0.35-4%)	0 (0-0%)	0.089

BM: bone marrow; FISH: Fluorescence in situ hybridization; ISS: International Staging System; LDH: Lactate dehydrogenase; PB: peripheral blood; R-ISS: Revised International Staging System; * $p \leq 0.05$.

miRNA profiling by NGS

To characterize the small RNAs, sequencing was performed on samples from both BM and PB of the twenty patients included in this study. The distribution patterns of the mapped reads revealed distinct RNA compositions between sensitive and primary refractory patients, as illustrated in the pie chart in Figure 1A. In sensitive patients, a significant majority (55%) of mappable RNAs were miRNAs, whereas in primary refractory patients, miRNAs represented only 15% of the reads. The remaining RNA types in both groups were attributed to small nuclear or nucleolar RNA, long intergenic noncoding RNA, and miscellaneous small RNA. Notably, in primary refractory patients, most mapped reads corresponded to processed mRNA (60%).

Venn diagrams (Figure 1B) were used to compare the differences between sensitive and primary refractory patients regarding the expression of miRNAs in PB and BM samples. This analysis identified 653 miRNAs in BM, of which 600 were in primary refractory patients and 283 in sensitive patients, and 1063 miRNAs in PB, of which 996 were in primary refractory patients and 563 were in sensitive patients. Furthermore, this analysis showed an overlap of 248 miRNAs in the BM and 496 miRNAs in the PB when comparing the expression profiles of primary refractory versus sensitive patients.

To identify differentially expressed miRNAs with statistical significance, volcano plots (Figure 1C and 1D) were generated, applying filtering criteria for both PB and BM samples, as well as distinguishing between sensitive and refractory patients. The applied threshold used to screen up- or downregulated miRNAs was a log₂ fold change ≥ 1.5 and p-value ≤ 0.05 . In the plots, the red points represent the differentially expressed miRNAs with statistical significance. The top ten upregulated and top ten downregulated miRNAs in refractory patients compared to sensitive patients are summarized in Table 2. Additionally, the top upregulated and downregulated miRNAs in peripheral blood compared to bone marrow are shown in Table 2.

For validation purposes, specific miRNAs were selected based on their differential expression. miR-483-5p and miR-665, both upregulated in primary refractory patients compared to the sensitive ones, were chosen for further analysis. Additionally, miR-143-3p and miR-145-5p, both upregulated in BM compared to PB samples, were also selected for subsequent validation studies. The selection for miRNAs further analysis was based on several criteria: the higher log Fold Change (logFC) reflecting the most significant expression level variations, the smallest p-values suggesting that the observed difference in expression is unlikely to have occurred by chance, and those with the lowest False Discovery Rate (FDR) indicating a higher confidence in the result being a true positive rather than a false discovery.

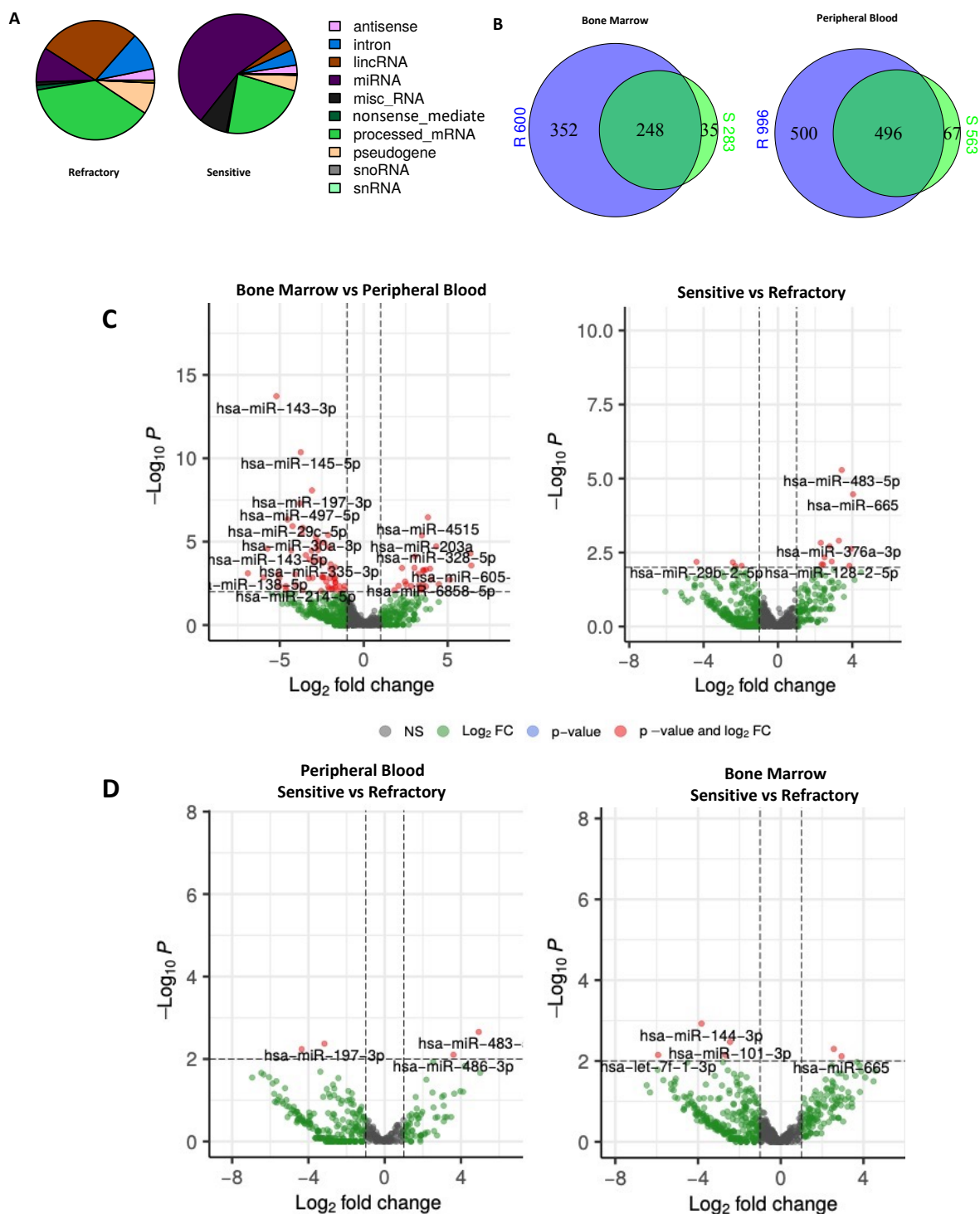


Figure 1. Characteristics and expression of circulating miRNAs in bone marrow (BM) and peripheral blood (PB) of primary refractory and sensitive Multiple Myeloma (MM) patients. A) Composition of the mapped reads of circulating RNAs in primary refractory and responsive MM patients combining BM and PB. B) Venn diagram of significantly differentially expressed miRNAs according to response status (refractory vs sensitive) and type of sample (BM vs PB); C) Volcano plots of the results of the differential miRNAs expression analyses according to response status (refractory vs sensitive) and type of sample (BM vs PB). The miRNAs with a $|\log_2$ fold change| ≥ 1 and p-value < 0.05 are labelled in red in the plots. miRNA, microRNA; lincRNA, long intergenic noncoding RNA; misc_RNA, miscellaneous other RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA.

Table 2. Upregulated and downregulated miRNAs according treatment outcome and type of sample

	miRNA	logFC	logCPM	<i>p-value</i>	FDR
UPREGULATED IN REFRACTORY PATIENTS	hsa-miR-187-5p	4,467481157	4,999912736	0,014689868	0,495159246
	hsa-miR-5001-3p	4,11926043	5,037955191	0,026838139	0,512046727
	hsa-miR-665	4,039370272	5,932134772	3,42067E-05	0,012981428
	hsa-miR-4667-5p	3,933875948	5,304446229	0,002388779	0,302180498
	hsa-miR-3176	3,841951429	5,468643693	0,008870548	0,460440365
	hsa-miR-520g-3p	3,52583699	4,858377697	0,044649489	0,560622822
	hsa-miR-483-5p	3,430800011	7,839680282	5,18076E-06	0,003932195
	hsa-miR-376a-3p	3,272721022	6,099817551	0,001257833	0,283355694
	hsa-miR-873-3p	3,252375639	4,839430741	0,032849171	0,553122668
	hsa-miR-4722-5p	3,217018137	4,872146192	0,047432659	0,560622822
DOWNREGULATED IN REFRACTORY PATIENTS	hsa-miR-877-3p	-3,306627765	5,898643163	0,034312164	0,553122668
	hsa-miR-361-3p	-3,465796319	5,992564948	0,02388589	0,503594174
	hsa-miR-95-3p	-3,910144848	6,013312209	0,02079349	0,503594174
	hsa-miR-616-5p	-4,134781364	5,353101873	0,039685056	0,55493667
	hsa-miR-29b-2-5p	-4,379510925	6,573692081	0,006573483	0,460440365
	hsa-miR-1246	-4,430617075	6,174884833	0,023838552	0,503594174
	hsa-miR-338-3p	-4,479214903	6,276779402	0,038914405	0,55493667
	hsa-miR-330-5p	-4,484324636	6,227873871	0,041484991	0,55493667
	hsa-miR-33a-3p	-4,487403017	5,565536783	0,048197106	0,560622822
	hsa-let-7f-1-3p	-4,848958739	5,735267299	0,023131113	0,503594174
UPREGULATED IN PERIPHERAL BLOOD	hsa-miR-605-5p	6,407563131	6,004300064	0,000267493	0,007097483
	hsa-miR-4269	6,354529751	6,110361793	4,96048E-05	0,001794793
	hsa-miR-586	5,146168885	5,39453953	0,001897352	0,027459854
	hsa-miR-4505	4,470723829	5,332474608	0,003524915	0,046763876
	hsa-miR-583	4,462684862	4,89663433	0,041073489	0,256811436
	hsa-miR-328-5p	4,314346645	6,469368173	1,92026E-05	0,000955328
	hsa-miR-6858-5p	4,011935719	5,530086494	0,001843167	0,027169646
	hsa-miR-4669	3,949008303	6,415231537	0,000417561	0,009775833
	hsa-miR-4515	3,821164973	7,354851532	3,4694E-07	5,52328E-05
	hsa-miR-1304-3p	3,707346522	5,731713067	0,004909534	0,055042094
DOWNREGULATED IN PERIPHERAL BLOOD	hsa-miR-138-5p	-6,901637807	7,171435152	0,000785347	0,016029124
	hsa-miR-5571-3p	-5,955884531	6,457745261	0,001385308	0,023686303
	hsa-miR-143-5p	-5,734877574	7,014021433	2,59283E-05	0,001146606
	hsa-miR-1275	-5,47465861	6,156047374	0,013981566	0,123659182
	hsa-miR-143-3p	-5,201131981	12,24935304	1,89177E-14	1,50585E-11
	hsa-miR-218-5p	-5,093952615	7,252905501	0,0008404	0,016677647
	hsa-miR-642a-3p	-5,032561789	5,897212976	0,001532273	0,023878673
	hsa-miR-511-5p	-5,012709257	5,857941789	0,004520526	0,05363306
	hsa-miR-582-5p	-4,924638241	5,825759013	0,016247235	0,136134725
	hsa-miR-145-5p	-3,752119667	12,73229925	4,32236E-11	1,7203E-08

FDR: False Discovery Rate; logCPM: log count-per-million; LogFC: log Fold Change; miRNA: microRNA.

Candidate miRNA confirmation by qPCR

miR-665 and miR-483-5p levels are higher in primary refractory patients compared to sensitive patients. It was observed that miR-665 was significantly more abundant in the PB than in the BM, with a fold change ($2^{-\Delta\Delta C_t}$) of 5.91 ($p=0.015$) (Figure 2A). The same trend was observed for miR-483-5p, with a fold change ($2^{-\Delta\Delta C_t}$) of 2.95 ($p=0.022$) (Figure 2B). Additionally, a statistically significant increase was observed in the levels of miR-665 in primary refractory patients when compared to sensitive patients, with a fold change ($2^{-\Delta\Delta C_t}$) of 5.78 ($p=0.021$) (Figure 2C). There was also an increase in the levels of miR-483-5p in primary refractory patients compared to sensitive patients, although without statistical significance, with a fold change ($2^{-\Delta\Delta C_t}$) of 1.23 ($p=0.487$) (Figure 2D).

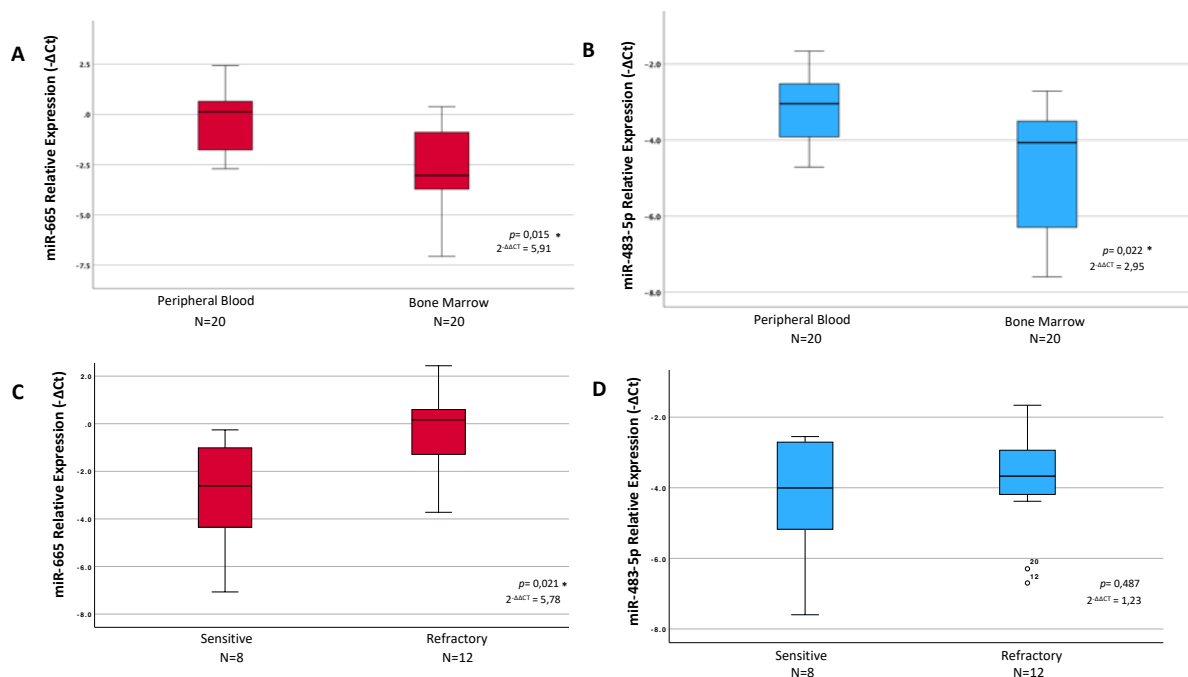


Figure 2. Relative expression of miR-665 and miR-483-5p in different samples [peripheral blood (PB) vs. bone marrow (BM)] (A and B) and regarding response to treatment (sensitive vs. refractory) in MM patients (C and D). Results are presented in boxplots that present the median with interquartile range. The circles outside the boxes indicate outliers. * $p \leq 0.05$.

miR-143-3p and miR-145-5p levels are higher in BM when compared to PB

A statistically significant increase was observed in miR-143-3p levels in the BM compared to the PB samples, with a fold change ($2^{-\Delta\Delta C_t}$) of 7.17 ($p=0.003$) (Figure 3A). Regarding miR-145-5p, there was a trend toward increased levels in BM compared to those of PB with fold change ($2^{-\Delta\Delta C_t}$) of 1.99, albeit not reaching statistical significance ($p=0.112$) (Figure 3B). Neither miR-143-3p nor miR-145-5p showed statistically significant differences concerning treatment outcomes (Figure 3C and Figure 3D).

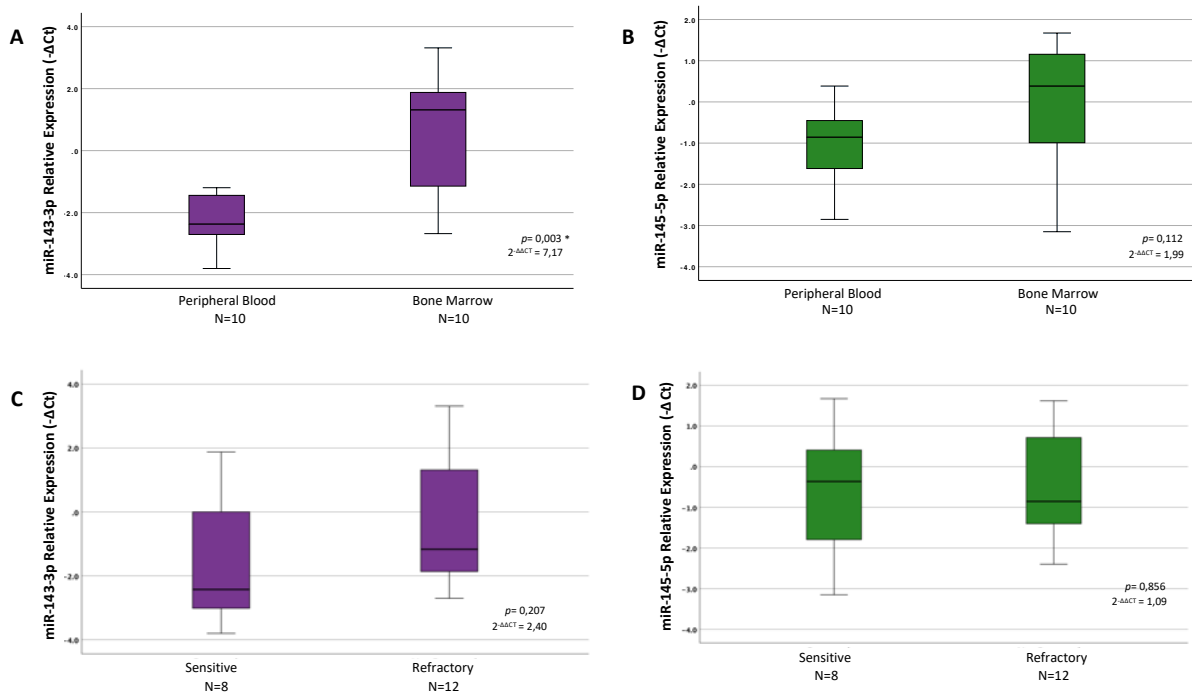


Figure 3. Relative expression of miR-143-3p and miR-145-5p in different samples [peripheral blood (PB) vs. bone marrow (BM)] (A and B) and regarding response to treatment (sensitive vs. refractory) in MM patients (C and D). Results are presented in boxplots that present the median with interquartile range. The circles outside the boxes indicate outliers. * $p \leq 0.05$.

Correlation of miRNAs expression with clinical characteristics and survival outcomes

miR-665 is associated with aggressive clinical characteristics

All the samples were collected before treatment, underscoring the possibility of finding differences that could serve as biomarkers. Given its higher expression in primary refractory patients and detectability in both BM and PB, miR-665 was selected to be evaluated as a potential biomarker for assessing treatment response. Notably, elevated levels of miR-665 were associated with advanced stages of the R-ISS and aggressive clinical features (Supplementary Table 1 and Figure 4). In more detail, a statistically significance was found between increased expression of miR-665 and elevated creatinine ($p=0.010$) (Figure 4A), elevated b2-microglobulin ($p=0.028$) (Figure 4B), elevated LDH ($p=0.035$) (Figure 4C), the presence of extramedullary disease ($p=0.021$) (Figure 4D), and the stage III R-ISS ($p=0.031$) (Figure 4E). However, high-risk cytogenetics features were not associated with elevated miR-665 levels ($p=0.343$) (Figure 4F).

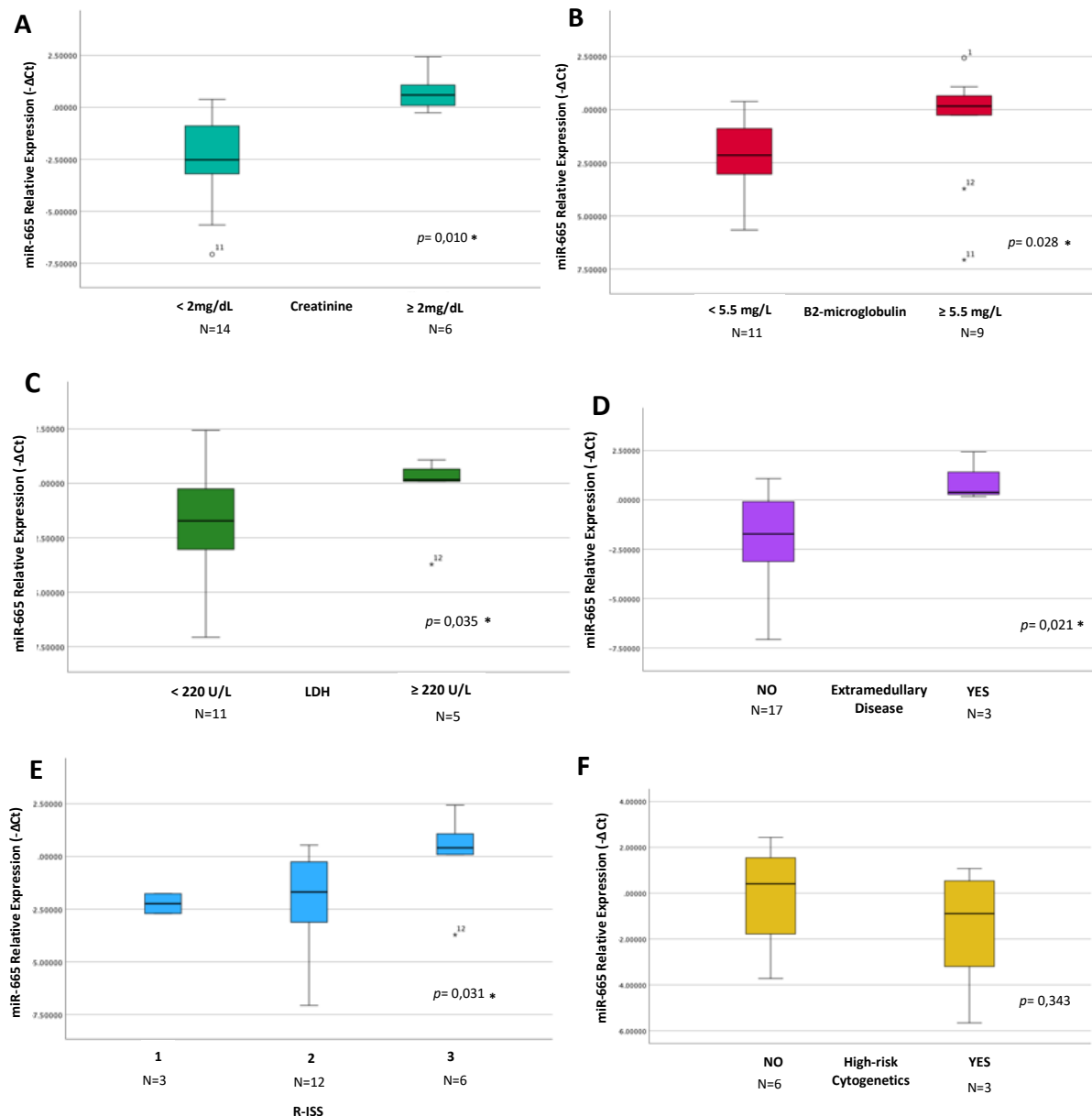


Figure 4. Circulating miR-665 is associated with aggressive disease phenotype. Boxplots presenting the correlation of plasma miR-665 levels with (A) creatinine levels, (B) B2-microglobulin levels, (C) lactic dehydrogenase (LDH) levels, (D) presence of extramedullary disease, (E) Revised-International Staging System (R-ISS); and (F) high-risk cytogenetics. Results are presented in boxplots that present the median with interquartile range. Statistical significance was calculated by Chi-Square Test A and Mann–Whitney U test. The circles outside the boxes indicate outliers. * $p \leq 0.05$

miR-665 is associated with poor survival outcomes

To further assess the prognostic significance of miR-665, survival analysis was performed using the Kaplan-Meier curves and Cox regression analysis. The prognostic potential of miR-665 was evaluated through ROC curve analysis, revealing an area under the curve of 0.83, with a 95% confidence interval ranging from 0.651 to 1.000 and a p-value of 0.014 (Supplementary Figure 2). Youden's index was calculated to determine the optimal cut-off for miR-665, identified as ΔCt 0.2206, yielding a sensitivity of 100% and a specificity of 33%. This finding supported the hypothesis that miR-665 might be a potential biomarker for treatment response, prompting subsequent survival analysis.

Kaplan-Meier survival analysis showed shorter OS in patients with higher levels of miR-665 ($\Delta\text{CT} \geq 0.2206$) in the PB, compared to those with lower levels ($\Delta\text{CT} < 0.2206$), with a median OS of 32 months for the first group and not reached for the second one (log-rank p-value < 0.001) (Figure 5A). Univariate Cox regression analysis demonstrated that MM patients with lower levels of miR-665 have significantly longer OS (HR 0.498, $p=0.002$), as well as R-ISS less than 2 (HR 4.624, $p=0.021$), increased LDH (HR 2.826, $p=0.047$) and male gender (HR 0.171, $p=0.026$). Nonetheless, multivariate Cox regression analysis did not show independent significance (Supplementary Table 2). Regarding PFS, the Kaplan-Meier survival analysis showed a shorter PFS in patients with higher levels of miR-665 compared to those with lower levels in the PB, with median PFS of 2 months and 50 months, respectively (log-rank p-value < 0.034) (Figure 5B). However, no variable showed independent significance in the multivariate Cox regression analysis (Supplementary Table 2).

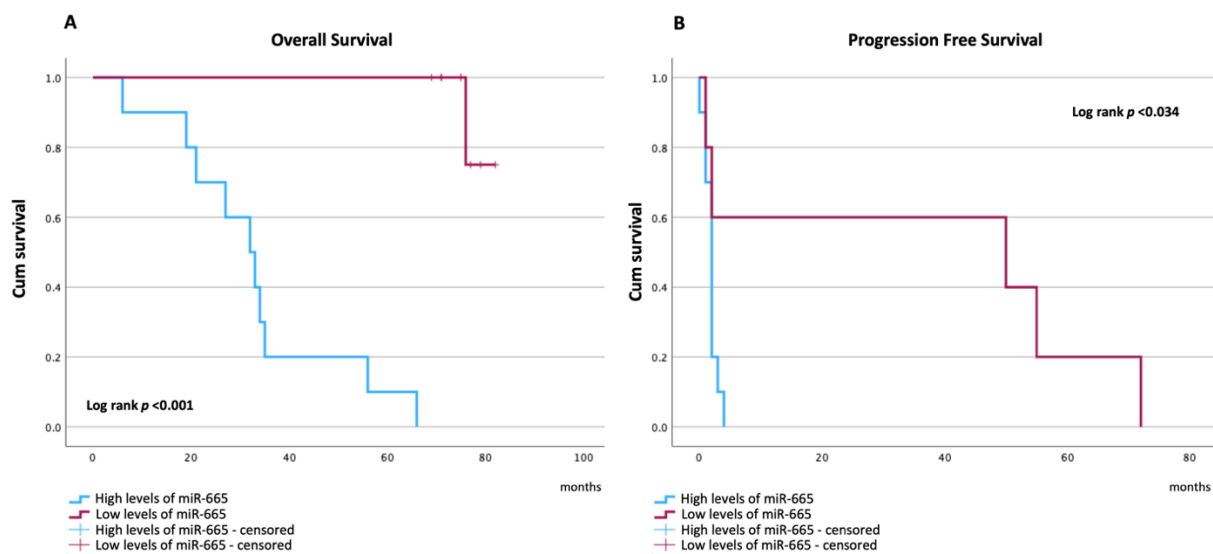


Figure 5. Kaplan-Meier curves illustrate the potential of miR-665 expression as a biomarker in peripheral blood (PB) for analyzing patient survival outcomes. The miRNA-665 was dichotomized based on low ($\Delta\text{CT} < 0.2206$) versus high expression ($\Delta\text{CT} \geq 0.2206$). A) Overall Survival (OS). B) Progression Free Survival (PFS).

In silico functional miRNA analysis

To elucidate the possible biological and functional role of miR-665, an *in silico* analysis for target prediction and GO enrichment was performed. The potential target genes of miR-665 were assessed using miRDB, TargetScanHuman 8.9, and DIANA TOOLS-microT-CDS (52-54). This comprehensive approach revealed 36 common genes across the databases and the main biological processes involved (Supplementary Figure 3A and Supplementary Figure 3B). Subsequent GO enrichment analysis showed that these genes are involved mainly in biological processes focusing on the regulation of DNA-templated transcription, regulation of transcription of RNA polymerase I and II, response to vascular endothelial growth factor (VEFG), cell migration and communication, and regulation of histone deacetylation (Supplementary Figure 3C). To further comprehend the functional roles of miR-665 and its expression, GO enrichment analysis was used to explore the correlation between the identified target genes and the statistically significant biological processes previously identified (Figure 6) (55, 56).

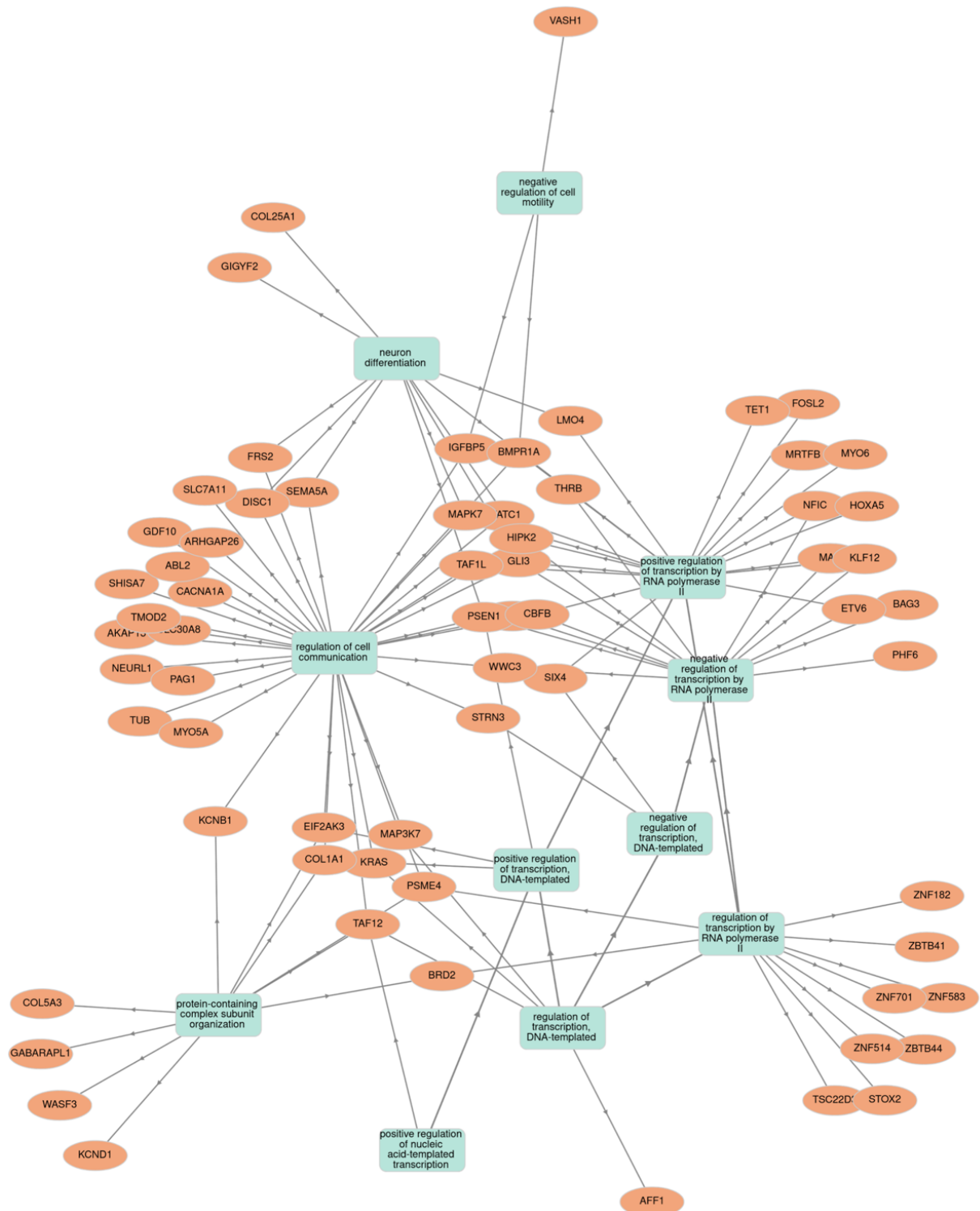


Figure 6. Association between miR-665 target genes and statistically significant biological processes terms using the Gene Ontology (GO) enrichment tool.

DISCUSSION

Treatment of newly diagnosed MM has changed substantially over the last years, with the incorporation of drugs from different classes featuring novel mechanisms of action. The current treatment algorithm includes first-line therapy with multiple drug combinations and several intensification and maintenance strategies based on the presence of high-risk cytogenetic abnormalities. This approach allows more intensive treatment of patients with these abnormalities, while those without such characteristics receive less intensive treatment. Nonetheless, there is a clear need for reliable biomarkers to guide therapeutic decisions more effectively. In this context, the study of biomarkers, such as miRNAs, for predicting treatment response is of foremost interest to enable a more personalized approach to MM treatment. The present study provides evidence that specific miRNAs are differentially expressed in newly diagnosed MM patients, their expression correlates with treatment response, and they can be assessed in BM and PB samples.

Using NGS, it was observed that miR-665 and miR-483-5p levels are elevated in primary refractory patients compared to sensitive patients. Additionally, despite having higher levels in BM samples, they were also detectable and quantified in PB samples. This variation in the expression levels of some miRNAs between groups of patients with different drug responses suggests their potential to be used as treatment biomarkers. Therefore, these miRNAs were selected for further investigation in the current study. Several miRNAs are associated with drug resistance, such as miR-29b, associated with PI3 resistance; miR-155, associated with daratumumab resistance; and miR-22, associated with IMiDs resistance (57-59). The two differentially expressed miRNAs identified in our primary refractory patients, miR-665 and miR-483-5p, have been previously described to be involved in several biological processes and signaling pathways in hematological malignancies (60-63). However, there is a lack of evidence specifically addressing their role in MM drug resistance.

Previous studies have shown that miR-483-5p is located at the 11p15.5 locus of insulin-like growth factor (IGF2) gene and has been implicated in promoting cancer cell differentiation, invasion, metastasis, escape from apoptosis, drug resistance, and decreasing radiosensitivity in several solid cancers (64-68). In MM, it was observed that plasmatic miR-483-5p was upregulated in MM patients compared to healthy controls and was associated with shorter median PFS, suggesting that miR-483-5p may serve as both a diagnostic and prognostic biomarker in MM (69). More recently, miR-483-5p has been identified as an oncomiR, promoting cell proliferation and inhibiting apoptosis in cell lines (60). Moreover, it was found that miR-483-5p is upregulated in MM-mesenchymal stem cells (MM-MSCs) and could be transferred from these cells to MM cells via Extracellular Vesicles (EVs), favoring MM progression and related bone disease by targeting tissue inhibitor of metalloproteinase 2 (TIMP2) (60).

miR-665, whose gene is located at the 14q32.2 locus next to the immunoglobulin heavy locus (IGH) gene, essential to MM pathogenesis, has a complex and context-dependent function, once it plays diverse roles in different cancer types and can act either as an oncogene or as a tumor suppressor (61). For instance, it has been reported to be downregulated in osteosarcoma, retinoblastoma, and ovarian cancer, but upregulated in non-small cell lung cancer and breast cancer (61, 70). In hematological malignancies, miR-665 appears to play a significant role in diffuse large B-cell lymphoma (DLBCL), acute lymphoblastic leukemia (ALL), and chronic myeloid leukemia (CML) (61). In DLBCL, expression of miR-665 can suppress tumor progression by targeting and inhibiting LIM and SH3 domain protein 1 (LASP1) and MYC, both essential for invasion, migration, and cell proliferation (63, 71). In ALL, increased expression of miR-665 inhibits cell growth and promotes apoptosis through targeting exotoxin glycosyltransferase 1 (EXT1) and the extracellular signal-regulated kinase 1/2

(ERK1/2) signaling pathways, translating into better survivals. miR-665 also targets the transforming growth factor beta receptor 1 (TGFBR1) and the multidrug resistance-associated protein 2 (ABCC2), increasing drug efflux, reducing apoptosis, and promoting cycle arrest, proliferation, and drug resistance (62). In CML cells, the long non-coding RNA ADORA2A-AS1 upregulates TGFBR1 and ABCC2 expression by adsorbing miR-665, further promoting drug resistance (61, 72). Regarding MM, the specific role and function of miR-665 remain unknown at the moment.

Although both miR-483-5p and miR-665 were found to be upregulated in primary refractory MM patients through NGS, further qPCR analysis showed that only the miR-665 retained statistical significance. This finding prompted us to explore its clinical implications in the analyzed patients. In addition to its association with drug response, our study highlights the potential of miR-665 as a prognostic tool in MM. Higher levels of miR-665 were associated with poorer OS, although it did not stand out as an independent factor in multivariate analysis. The same trend was observed for PFS, as expected once it had been observed that miR-665 was associated with a poor response to first-line treatment.

Furthermore, miR-665 was associated with an aggressive MM phenotype characterized by a higher disease burden, as evidenced by higher LDH, b2-microglobulin, and creatinine, as well as a higher incidence of extramedullary disease. Other studies associated higher levels of miRNAs with high-risk features, mainly extramedullary disease (73-75). For instance, miR-130a was found to be lower in the PB but higher in plasma cells from extramedullary tumors in comparison with BM plasma cells, suggesting that miR-130 could be taken up from circulation by the extramedullary tumor, thereby supporting angiogenesis and promoting cell growth and proliferation (75). However, in the present study, no samples were obtained from the extramedullary sites to confirm this association. When comparing with established prognostic models, higher levels of miR-665 were associated with a stage III of the R-ISS, indicating the potential utility of this miRNA as a biomarker or as an additional component to this prognostic staging tool, particularly to discriminate patients that are stage II. Curiously, the miR-665 gene is located next to the IGH gene in chromosome 14, which is essential in MM pathogenesis. Although higher levels of miR-665 were more common in patients with high cytogenetic risk, this difference did not reach statistical significance in the present study. Previous studies showed that, for example, that miR-133a was overexpressed in cases with t(14;16), a high-risk feature, showing a conserved inverse relation between several miRNAs deregulated in MM and cyclin D2 (CCND2) expression levels (76). Similarly, upregulated miR-99b or downregulated miR-744 have been associated with t(14;14), a major marker of poor prognosis in MM patients (77, 78). Although miR-665 was not identified as an independent factor, it may reflect the complex MM landscape in these patients and could serve as a non-invasive prognostic tool for forecasting drug resistance and patient outcomes and refining the classical models currently available.

Concerning the differences between BM and PB samples, both miR-665 and miR-483-5p presented higher levels in PB compared to BM. Even though BM studies, including cytogenetics, remain the gold standard for diagnosis and monitoring, the potential to analyze circulating miRNAs in PB as liquid biopsies can represent a non-invasive tool. This could complement other biomarkers to follow-up treatment outcomes or be used alongside MRD.

Functional *in silico* analysis for miR-665 revealed that several target genes play essential roles in various biological processes and signaling pathways related to MM. Notably, genes such as KRAS, BAG3, AFF1, CL13, MAP3K7, WWC3, and HOXA5 are critical in regulating cell proliferation and apoptosis, and TET1 plays a crucial role in epigenetic regulation (79-88). In the set of microenvironment

modulation, BAG3, TAF12, and MYO6 are important for facilitating the complex intercellular communication network (81, 89, 90). Moreover, some of these genes, including BAG3, BRD2, EIF2AK3, GABARAPL1, KRAS, PSME4, and SLC7A11, have been implicated in modulating responses to drugs such as PIs and IMiDs (90-96).

Mutations in KRAS are the most common gene mutations found in MM patients, accounting for more than 20% of all the mutations identified in the context of MM, demonstrating their fundamental role as modulators of MM pathogenesis (79, 97, 98). These mutations are frequently associated with advanced-stage disease, high-risk cytogenetics, and poorer prognosis (98, 99). Although our patients match this description, mutation analyses were not performed in our study to confirm the presence of KRAS mutations. Because KRAS is a crucial player in the MAPK pathway, patients harboring RAS mutations could benefit from inhibitors of MAPK, either directly on the specific mutation or through downstream targeting of MEK1/2 or ERK1/2, probably combining those inhibitors with IMiDs rather than PIs (79, 98). Indeed, activation of the MAPK via mutated KRAS appears to negatively impact regimens containing bortezomib by downregulating the expression of the endoplasmic reticulum stress proteins and reducing unfolded protein response activation (80, 100).

In the GO analysis performed in our study, transforming growth factor- β activated kinase1 (MAP3K7) was identified as one of the targets of miR-665, playing an essential role in the development of chemoresistance (101). MAP3K7 induces expression of survival factors through nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and mitogen-activated protein kinases (MAPK), both regulating a wide range of oncological survival signals. These pathways are involved in cellular responses to oxidative and mechanical stresses, the regulation of apoptosis, and angiogenesis (85, 101, 102). Moreover, constitutive NF- κ B activation is crucial for the MM oncogenesis and plays a pivotal role in drug resistance (103). BAG3, another miR-665 target, is known to sustain cell survival following treatment by suppressing apoptosis and autophagy (81, 91). BAG3 protects IKK γ , a subunit of the NF- κ B-activating IKK complex, from HSP70-mediated delivery to the proteasome, protects the degradation of Bcl-2 family proteins (such as Mcl-1, Bcl-x, and Bcl-2) and enhances the activation of the NF- κ B, that ultimately will induce the expression of several antiapoptotic genes (91). Additionally, BAG3 has been shown to promote MM cell proliferation through the FOXM1/ Rb/E2F axis (81). BRD2, also targeted by miR-665, appears to regulate cell cycle, proliferation, and metastasis through chromatin remodeling machines and activation of c-MYC (104). Importantly, c-MYC is fundamental for MM cell proliferation associated with shorter PFS and OS, as well as resistance to IMiDs and pathological osteoclastic activation in MM (92, 105).

This functional analysis and the potential mechanisms mediated by miR-665 regulation highlight the heterogeneity of MM and the challenge in predicting treatment outcomes. Identifying specific miRNAs associated with treatment resistance or sensitivity would represent a significant step in the personalized approach of MM patients, addressing one of the most pressing unmet needs in the field. In this study, miR-665 appears to be a promising candidate for further investigation, given its association with higher-risk features typically related to treatment resistance, increased disease burden, and poorer treatment outcomes. However, a comprehensive approach to validate the direct interaction between miR-665 and the several potential targets is essential to truly understand the role of this miRNA in plasma cell proliferation and drug resistance mechanisms. This validation could be achieved by manipulating miRNA levels using synthetic miRNA mimics or miRNA inhibitors in cells, followed by functional assays to assess the effects on cellular behavior..

Furthermore, there are challenges and limitations associated with translating this findings into clinical practice. Firstly, there is a need for standardized protocols for miRNA detection and quantification, as variability in methods can lead to inconsistent results. Secondly, the functional relevance of miRNAs remains complex to determine, as they often have multiple targets, and their targets can be influenced by other miRNAs. Thus, further research is needed to clarify and consolidate the roles of miRNAs in MM. Thirdly, the small sample size of our study limits the generalization of the results and increases the influence of confounding factors such as environmental factors, concomitant treatments, genetic background, cytogenetic abnormalities, and mutations that modulate signaling pathways relevant to MM. More controlled and extensive studies are needed to validate miR-665 as an independent biomarker of drug resistance. These studies should include larger cohorts of patients, more comprehensive multivariate analyses, experimental validation of miRNA-target interactions, improved stratification of patient subgroups based on genetic risk, clinical profiles, and different treatment protocols. For instance, miRNA analysis should be included in prospective clinical trials as possible biomarkers for future use. Of particular interest would be the integration of miRNA profiling with other biomarkers and clinical features to enhance the current predictive tools available for precision medicine.

CONCLUSIONS

The present study demonstrated that specific miRNAs are differently expressed between MM primary refractory and sensitive patients to first-line treatment, and these miRNAs can be detected in both PB and BM samples. Notably, elevated levels of miR-665 were associated with treatment-refractory disease and correlated with poor survival outcomes and aggressive MM characteristics. These results highlight the potential role of non-invasive detection of miRNAs as biomarkers of prognosis and to predict the treatment response in MM patients, paving the way for precision medicine. Furthermore, *in silico* analysis suggested that miR-665 target genes regulate cell proliferation and drug resistance, suggesting its possible role in MM heterogeneity and treatment resistance.

CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Conceptualization: R.B., J.E.G., and M.H.V.; methodology: R.B., S.P.d.S., V.P., J.P., D.S.; formal analysis: R.B., S.P.d.S., R.M., J.E.G., and M.H.V.; writing—original draft preparation: R.B., S.P.d.S., R.M., and M.H.V.; writing—review and editing: all authors.; supervision: J.E.G. and M.H.V.; funding acquisition: R.B., F.T., J.E.G., and M.H.V. All authors have read and agreed to the published version of the manuscript.

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ABBREVIATIONS

AUC: Area Under the Curve; BM: Bone Marrow; CML: Chronic Myeloid Leukemia; Ct: Cycle Threshold; cDNA: Complementary DNA; DLBCL: Diffuse Large B-Cell Lymphoma; EDTA: Ethylenediaminetetraacetic Acid; ERK1/2: Extracellular Signal-Regulated Kinases 1/2; EXT1: Exotoxin Glycosyltransferase 1; EVs: Extracellular Vesicles; FASTQ: Format for Storing Sequence Data; FDR: False Discovery Rate; GTF: Gene Transfer Format; IGH: Immunoglobulin Heavy Chain; IMWG: International Myeloma Working Group; ISS: International Staging System; KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog; LDH: Lactate Dehydrogenase; LIM: Lin-11, Isl-1 & Mec-3 Domain Proteins; MAPK: Mitogen-Activated Protein Kinase; miRNA: MicroRNA; MS2 RNA: RNA from Bacteriophage MS2; MM: Multiple Myeloma; MRD: Measurable Residual Disease; MS2: Bacteriophage MS2 RNA; NGS: Next-Generation Sequencing; NRAS: Neuroblastoma RAS Viral Oncogene Homolog; PBS: Phosphate Buffered Saline; PFS: Progression-Free Survival; PI: Proteasome Inhibitor; qPCR: Quantitative Polymerase Chain Reaction; ROC: Receiver Operating Characteristic; RT: Room Temperature; R-ISS: Revised International Staging System; SPSS: Statistical Package for the Social Sciences; TGFBR1: Transforming Growth Factor Beta Receptor 1; TIMP2: Tissue Inhibitor of Metalloproteinases 2; WB: Western Blot.

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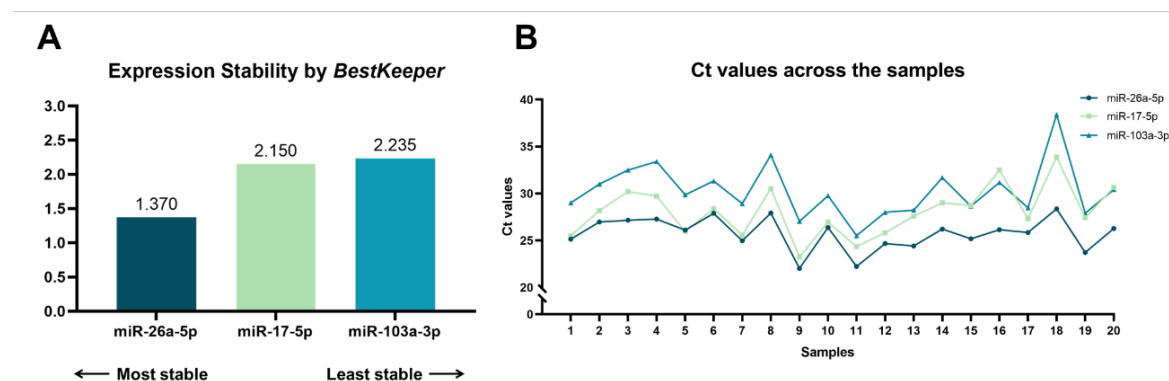
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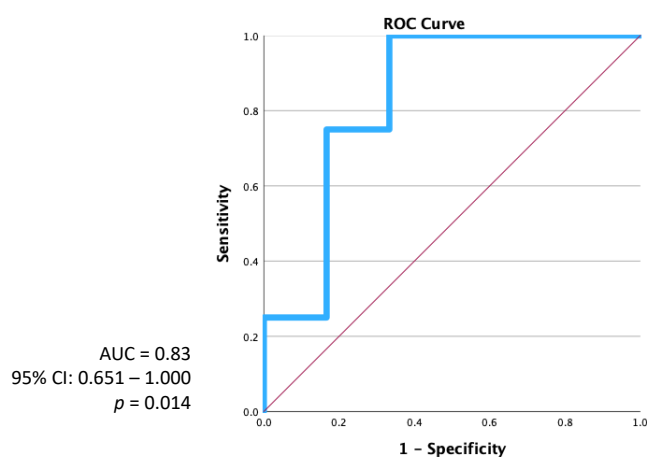
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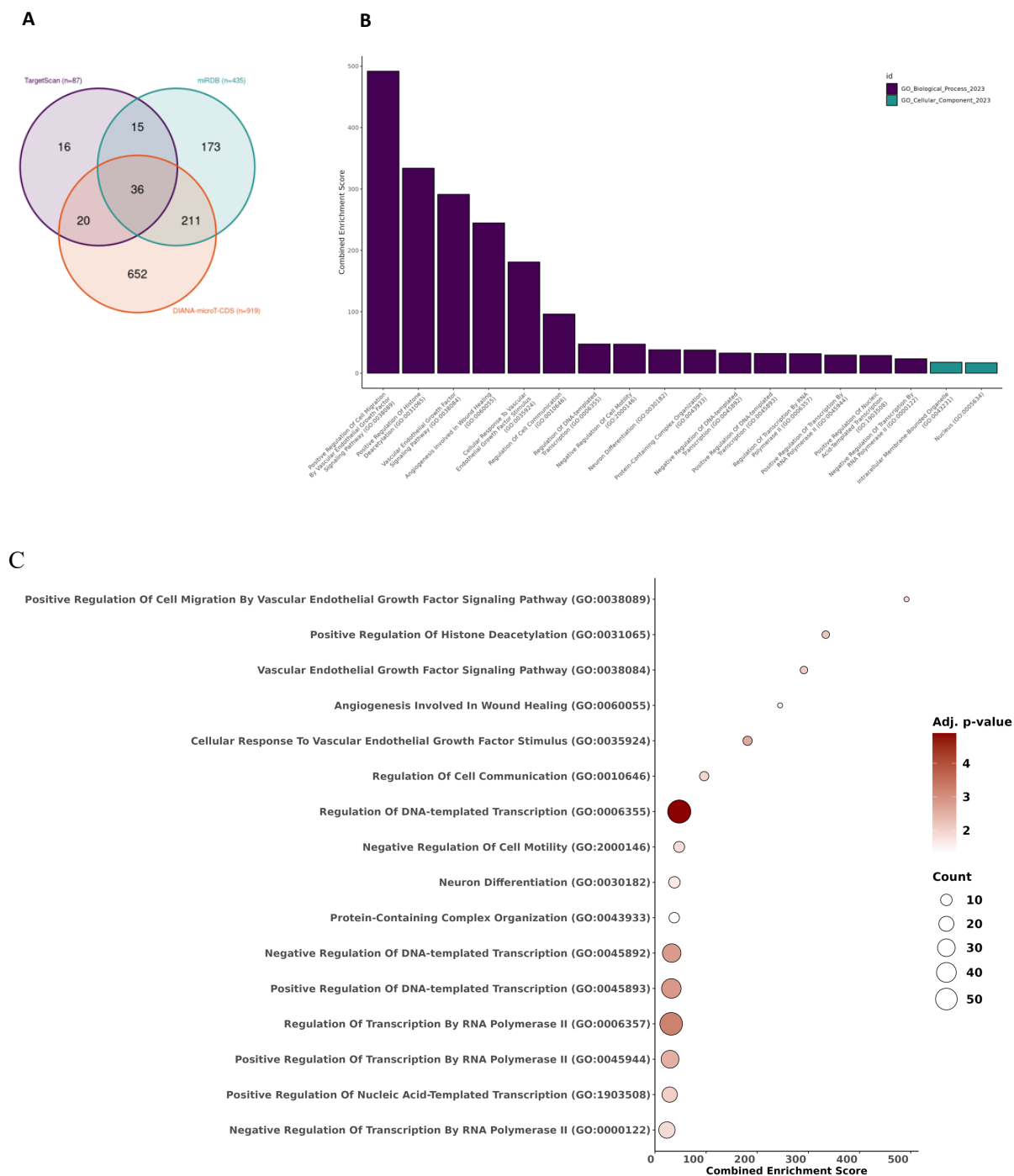
Supplementary Material



Supplementary Figure 1. Evaluation of the candidates to use as endogenous normalizer. A) Stability analyses of the expression of miRNAs normalizer candidates by *BestKeeper* software. This analysis indicates that miR-26a-5p is the most stable among all samples from the different groups. B) Cycle threshold (Ct) values across all samples of the different groups for the three candidate endogenous normalizers. The miR-26a-5p presents the lower Ct values and the most stable expression in all the samples from the different groups compared to the other two candidates.



Supplementary Figure 2. ROC curve analysis of miR-665 levels for the discrimination of high and low expression in MM samples from refractory and sensitive patients. AUC: area under the curve, 95% CI: 95% confidence intervals. p value calculated by the Hanley and McNeil method.



Supplementary Figure 3. Gene Ontology (GO) enrichment analysis of miR-665. A) Venn diagram shows the number of significant GO terms according to miRDB, TargetScanHuman 8.0, and DIANA TOOLS-microT- CDS target prediction tools. B) Significant GO terms associated with miR-665 target genes: the vertical axis indicates the combined enrichment score, while the horizontal axis categorizes the GO terms. C) Dot plot showing enrichment of GO biological processes. The color of each dot corresponds to the p-value, and the size indicates the number of miR-665 target genes associated with each biological process term.

Supplementary Table 1. Baseline and clinical characteristics of Multiple Myeloma (MM) patients according to their expression of miR-665.

Baseline and Clinical Characteristics	Total (n=20)	High miR-665 expression	Low miR-665 expression low	p-value
Age				
Median (range) - years	58 (44-67)	56 (48-63)	60 (44-67%)	0.589
Sex - no. (%)				
Male	12 (60%)	7 (35%)	5 (25%)	0.040*
Female	8 (40%)	1 (5%)	7 (35%)	
Myeloma isotype - no (%)				
Light Chain Kappa	4 (20%)	2 (10%)	2 (10%)	0.645
IgG/k	6 (30%)	1 (5%)	5 (25%)	
IgG/L	5 (25%)	2 (10%)	3 (15%)	
IgA/k	3 (15%)	2 (10%)	1 (5%)	
IgA/L	2 (10%)	1 (5%)	1 (5%)	
ISS stage - no (%)				
I	3 (15%)	1 (5%)	2 (10%)	0.073
II	8 (40%)	1 (5%)	7 (35%)	
III	9 (45%)	6 (30%)	3 (15%)	
R-ISS stage - no (%)				
I	3 (15%)	1 (5%)	2 (10%)	0.031*
II	11 (55%)	2 (10%)	9 (45%)	
III	6 (30%)	5 (25%)	1 (5%)	
FISH				
Normal	11 (55%)	3 (15%)	8 (40%)	0.199
Abnormal	9 (45%)	5 (25%)	4 (20%)	
High-risk cytogenetic profile				
del(17p)	2 (22.2%)	2 (22.2%)	0 (0%)	0.151
t(4;14)	2 (22.2%)	2 (22.2%)	0 (0%)	0.151
t(14;16)	1 (11.1%)	0 (0%)	1 (11.1%)	0.236
t(11;14)	3 (33.3%)	1 (11.1%)	2 (22.2%)	0.343
amp1q21	3 (33.3%)	2 (22.2%)	1 (11.1%)	0.635
Double-Hit (del17p and t(4;14))	2 (10%)	2 (10%)	0 (0%)	0.151
Anemia				
<10g/dL or <1g/dL of normal Hb	16 (80%)	7 (35%)	9 (45%)	0.494
Creatinine				
<2mg/dl	14 (70%)	3 (15%)	11 (55%)	0.010*
>=2mg/dL	6 (30%)	5 (25%)	1 (5%)	
B2-microglobulin				
Increased	9 (45%)	6 (30%)	3 (15%)	0.028*
Normal	11 (55%)	2 (10%)	9 (45%)	
LDH				
Increased	5 (25%)	4 (20%)	1 (5%)	0.035*
Normal	15 (75%)	4 (20%)	11 (55%)	
Extramedullary plasmacytoma				
Yes	3 (15%)	3 (15%)	0 (0%)	0.021*
No	17 (85%)	5 (25%)	12 (60%)	

Clonal plasma cell in BM (median, range)	20.9 (5-72%)	26.5 (5-66%)	16.4 (5-72%)	0.787
Clonal plasma cell in PB (median, range)	0 (0.35-4%)	0 (0-1%)	0 (0-4%)	0.166

BM: bone marrow; FISH: Fluorescence in situ hybridization; ISS: International Staging System; LDH: Lactate dehydrogenase; PB: peripheral blood; R-ISS: Revised International Staging System; * $p \leq 0.05$.

Supplementary Table 2. Univariate and multivariate Cox regression analyses regarding overall survival and progression-free survival of MM patients.

OVERALL SURVIVAL							PROGRESSION FREE SURVIVAL					
<i>Univariate analysis OS</i>				<i>Multivariate analysis OS</i>			<i>Univariate analysis PFS</i>			<i>Multivariate analysis PFS</i>		
Covariant	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Circulating miR-665												
High levels	1						1					
Low levels	0.498	0.317-0.782	0.002*	0.000	0.000-1.907E+135	0.928	0.262	0.055-1.256	0.094		n.d.	
R-ISS Stage												
R-ISS I / II							1					
R-ISS III	4.624	1.256-17.019	0.021*	0.486	0.051-4.651	0.531	1.376	0.440-4.302	0.583		n.d.	
High-risk Cytogenetics												
Yes	1				n.d.		1					
No	0.547	0.118-2.533	0.441				0.776	0.193-3.121	0.721		n.d.	
LDH												
≤ 220 U/L	1						1					
≥ 220 U/L	3.592	1.017-12.685	0.047*	0.389	0.033-1.453	0.455	1.311	0.415-4.143	0.645		n.d.	
B2-microglobulin												
≤ 5.5 mg/L	1						1					
≥ 5.5 mg/L	2.826	0.815-9.808	0.102		n.d.		1.179	0.380-3.664	0.766		n.d.	

Creatinine										
≤ 2 mg/dL	1					n.d.	1			
≥ 2 mg/dL	2.522	0.725-8.773	0.146				1.595	0.483-5.265	0.443	n.d.
EMD										
Yes	1						1			
No	0.228	0.05-1.37	0.056			n.d.	0.743	0.201-2.750	0.656	n.d.
Anemia										
Yes	1						1			
No	0.401	0.051-3.173	0.387			n.d.	1	0.000-2584	1	n.d.
Gender										
Female	1						1			
Male	0.171	0.036-0.812	0.026*	0.921	0.132-6.425	0.921	0.445	0.120-1.651	0.226	
Age										
	0.979	0.901-1.063	0.607			n.d.	0.979	0.901-1.063	0.607	
										n.d.

EMD: Extramedullary disease; FISH: Fluorescence in situ hybridization; HR: hazard-ratio; ISS: International Staging System; LDH: Lactate dehydrogenase; miR: microRNA; OS: overall survival; PFS: progression-free survival; R-ISS: Revised International Staging System; 95% CI: 95% confidence interval of the estimated HR; n.d. not done, once no statistical significance was found on univariate analysis; * $p \leq 0.05$.

ORIGINAL ARTICLE

3.3. Isolating and quantifying microRNAs from extracellular vesicles from bone marrow and peripheral blood of multiple myeloma patients**Part of this work was published as an abstract**

Bergantim, Rui; Peixoto da Silva, Sara; Polónia, Bárbara; Barbosa, Mélanie A. G.; Caires, Hugo R.; Guimarães, José E.; Vasconcelos, M. Helena. "Studying microRNAs from extracellular vesicles as potential biomarkers in liquid biopsies of multiple myeloma patients". *Clinical Lymphoma, Myeloma, and Leukemia*. 2022. Volume 22, S66-67. DOI: [https://doi.org/10.1016/s2152-2650\(22\)00387-1](https://doi.org/10.1016/s2152-2650(22)00387-1)

Isolating and quantifying microRNAs from extracellular vesicles from bone marrow and peripheral blood of multiple myeloma patients: preliminary results

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Part of this WORK was published as an ABSTRACT.

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ABSTRACT

Extracellular Vesicles (EVs) contain cargo from their cell of origin, including microRNAs (miRNAs or miRs). Therefore, the content of miRNAs packaged into EVs (EV-miRNAs) has been studied in recent years as possible biomarkers of drug response or measurable residual disease (MRD) in cancer, including in multiple myeloma (MM). The present work aimed to verify if it was possible to quantify and compare miRNA expression levels from the cargo of EVs isolated from the PB and BM of MM patients at diagnosis and remission to verify the long-term potential of analyzing EV-miRNAs as biomarkers in liquid biopsies of MM patients. Our findings demonstrate successful isolation and quantification of miRNAs from the cargo of EVs isolated from the PB and BM of MM patients at both diagnosis and remission status. Notably, miRNA levels were higher in BM than in PB at diagnosis, but this difference was not observed at remission. These preliminary results highlight the importance of sample sources for analyses of EV-miRNAs as potential biomarkers in MM and pave the way for future projects to evaluate further the potential of EV-miRNAs as biomarkers in MM.

KEYWORDS:

Extracellular Vesicles (EVs), MicroRNAs (miRNAs), Multiple Myeloma (MM), Liquid Biopsy

INTRODUCTION

miRNAs, strongly associated with cancer progression, aggressiveness, and treatment responses, are easily and readily detected in biofluids (such as PB and BM) as free circulating miRNAs or packaged into EVs (EV-miRNAs), which are attractive options for liquid biopsies[1,2]. One of the main limitations of circulating-free miRNAs as liquid biopsy is their instability and short half-lives in biological fluids[2]. Additionally, the origin of circulating-free miRNAs and their function is still unclear. They may be secreted not only by malignant cells but also by non-malignant cells. Moreover, two theories have been suggested regarding the export and function of circulating miRNA. The first one is that these miRNAs are merely byproducts of routine microvesicle secretion and cell death, and the second one is that miRNAs are specifically secreted and function in intercellular communication [3,4].

Extracellular vesicles (EVs) are a heterogeneous group of cell-released particles, enclosed within a phospholipid bilayer, and that do not replicate. Importantly, they contain cargo from their cell of origin, such as miRNAs[4,5]. EVs appear to be a robust biomarker platform because of their high abundance and ability to protect their cargo from nuclease and proteasome degradation. In fact, due to their lipid bilayer, EVs are a more stable and reliable source of miRNAs, protecting them from degradation [5-7].

Previous studies showed that EV-miRNAs were differentially expressed between MM and healthy individuals and could be a valuable tool to discriminate between MM and its precursor stage[8,9]. Moreover, several EV-miRNAs have been associated with cancer drug resistance or have been shown to be potential modulators of drug response in hematological malignancies [10-12].

This suggests that measuring EV-miRNAs levels may be a valuable tool for monitoring MM treatment response/progression and for predicting drug resistance/response and survival outcomes [7,11,12]. Therefore, this work aimed to obtain preliminary results on the possibility of quantifying EV-miRNAs isolated from bone marrow (BM) and peripheral blood (PB) of MM patients.

MATERIALS AND METHODS

Patients and samples

PB and BM samples were collected from two MM patients at diagnosis and remission at the Clinical Hematology Department from Centro Hospitalar Universitário São João (CHUSJ, Porto, Portugal). Both patients were newly diagnosed with MM and treated upfront with the triplet combination of bortezomib, thalidomide, and dexamethasone.

Isolation and concentration of EV

EV was isolated from platelet-poor plasma using a previously described method by our group, which involved size exclusion chromatography (SEC) followed by ultrafiltration (UF)[13].

Characterization of EV

EVs characterization was performed using a method previously described by our group[13]. Protein quantification of each SEC eluent was determined with the Lowry protein assay (DCTM Protein Assay kit, Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Particle concentration and size distribution were determined by Nanoparticle Tracking Analysis (NTA). EVs size, morphology, and integrity were visualized by Transmission Electron Microscopy (TEM) using negative staining. Representative TEM photographs were acquired, and the EV size for each SEC fraction was measured by image analysis using ImageJ software. EVs characterization was completed with Western Blot (WB) analysis of known protein markers.

EVs-derived microRNAs (miRNAs) Isolation and Analysis

The miRNeasy Serum/Plasma Advanced Kit (217204, Qiagen, Germany) was used for miRNA isolation from EVs previously isolated from the patient's BM or PB samples, using the manufacturer's instructions. A pre-treatment of the EVs with proteinase K (Proteinase K, recombinant, PCR grade, EO0491, Thermo Scientific, USA, 2 µg/µL of EV sample) was performed. Following the manufacturer's recommendations, to increase the miRNA isolation yield, 1 µg of MS2 RNA (RNA from bacteriophage MS2, Roche, Germany) was added to the RPL Buffer on each sample. The EVs-derived miRNAs were extracted from the BM and PB of 3 patients at both diagnosis and remission. Then, total RNA concentration and purity were quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA).

A total of 10ng RNA was used as the template for cDNA synthesis, using the TaqManTM Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, USA) according to the manufacturer protocol. The miRNAs expression levels were then assessed by quantitative Chain Reaction (qPCR) using 2X TaqManTM Fast Advanced Master mix (Applied Biosystems, USA) and 20X TaqManTM Advanced miRNA Assays probes (hsa-miR-145-5p 477916_mir; hsa-miR-143-3p 477912_mir; Applied Biosystems, USA), accordingly to the manufacturer's instructions (in a final volume of 10µL *per* well). The qPCR reactions were performed in a 7500 Fast Real-Time PCR device (Applied Biosystems, USA). For each sample, triplicates were performed, and negative controls lacking cDNA or reverse transcription enzymes were included in duplicates in the qPCR plates. Two miRNAs were tested as endogenous controls (hsa-miR-17-5p 478447_mir; and has-miR-185-5p 477939_miR; Applied Biosystems, USA). miR-185-5p was chosen as the endogenous control for normalization since its expression was more stable in all samples compared to miR-17-5p. The efficiency for all the assays was between 95 and 110%, as recommended. The threshold cycle (Ct) values for all the miRNAs in each sample were acquired from the 7500 Software v2.0.6 (Applied Biosystems, USA).

Statistical analysis was performed using the IBM SPSS Statistics for Windows (Version 27.0). The Livak Method ($2^{-\Delta\Delta C_t}$) and the Mann-Whitney U test were used to determine statistical differences in the normalized relative expression of miR-143-3p and miR.145-5p among different types of samples and disease stages. Results were considered statistically significant when p values were ≤ 0.05 .

RESULTS AND DISCUSSION

The miRNA concentrations in each sample were normalized for the initial volume of plasma used, and the results of this normalization are presented in Figure 1. Our preliminary results (from 2 patients only) indicate that BM samples present more miRNAs per mL of plasma (i.e., have a higher concentration of miRNAs) when compared to PB samples. Interestingly, we found that approximately 20% of the miRNAs found in the BM plasma are EV-miRNAs. However, in PB plasma, the results were inconsistent, with this percentage being the same or higher in one of the patients. Strikingly, even though the concentration of free-miRs was much higher in BM than in the PB, the amount of EV-miRs was similar between BM and PB samples (Figure 1).

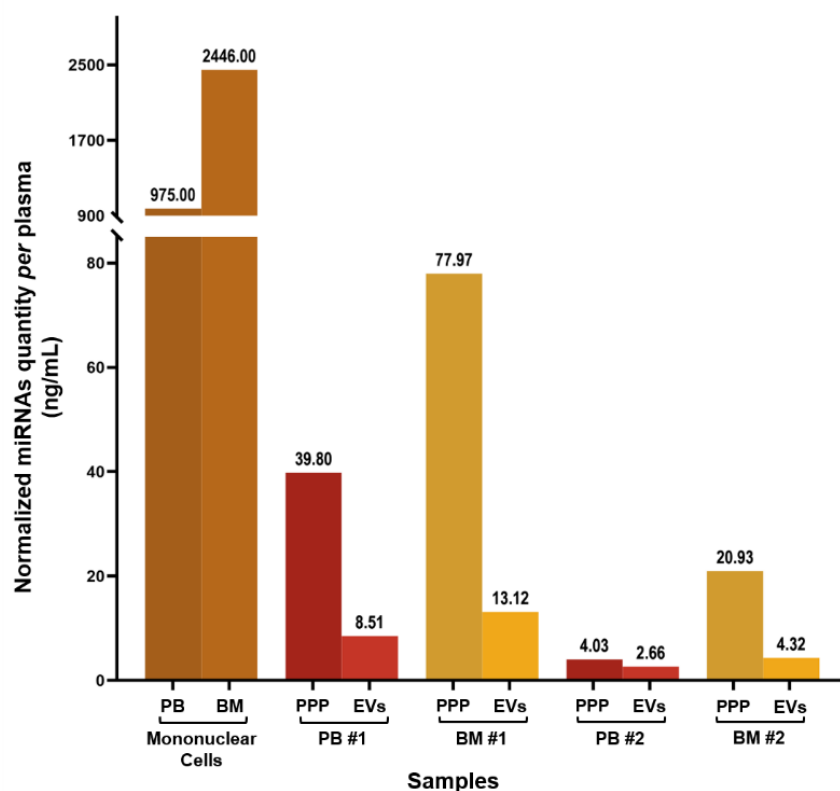


Figure 1. Normalized miRNAs quantity per ml of plasma initially used (ng/mL) of bone marrow (BM) and peripheral blood (PB) samples from multiple myeloma (MM) patients. The number of miRNAs isolated from the PPP (free miRNAs) and from the EVs (EV-miRs) of the same patient is presented side by side, allowing a comparison of the results from the samples of two different patients (#1 and #2). Also, the miRNA quantification of mononuclear cells is presented and used as a positive control. PB: Peripheral Blood; BM: Bone Marrow; PPP: Platelet Poor Plasma; EVs: Extracellular Vesicles.

The levels of two miRNAs were determined in the cargo of EVs isolated from PB and BM samples, both at the diagnosis and remission stages. This was performed for two MM patients by qPCR. The miRNAs analyzed were miR-145-5p and miR-143-3p since our team found these free miRNAs-EVs to be differentially expressed in the PB and BM of MM patients (submitted for publication). The results of the expression levels of miR-145-5p and miR-143-3p in the isolated EVs are shown in **Figure 2**.

Results indicate that it is possible to isolate miRNAs from EV samples and quantify their expression levels using qPCR. Interestingly, for both miRNAs analyzed, a statistically significant difference in their levels was observed between PB and BM at diagnosis, with higher levels being detected in the BM than in the PB, being 3-fold higher for miR-145-5p ($p=0.05$) and 15-fold higher for miR-143-3p ($p=0.05$). However, at remission, no statistically significant difference in the levels of these EV-miRNAs was found in the PB and BM patient samples ($p=0.827$ and $p=0.275$, respectively).

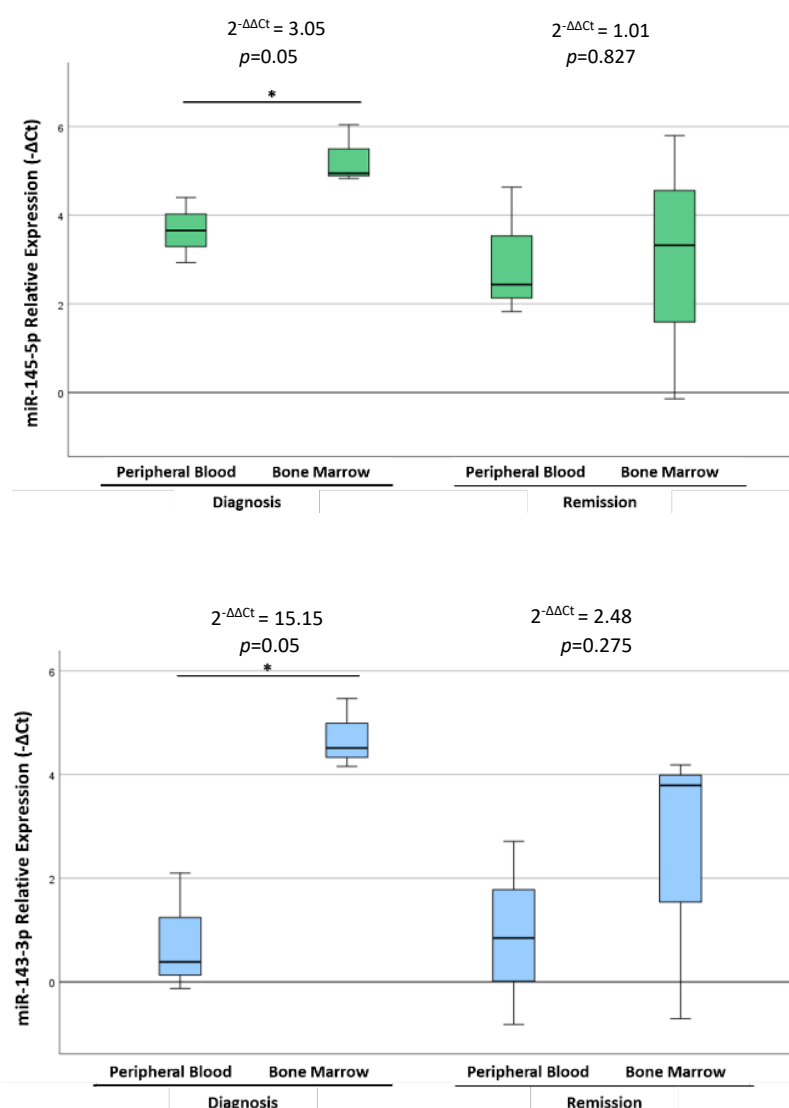


Figure 2. Relative expression of miR-145-5p and miR-143-3p in EVs isolated from the peripheral blood (PB) and bone marrow (BM) of multiple myeloma (MM) patients at diagnosis and remission stages. These data represent the mean expression levels from three different patients. Results were obtained following normalization for miR-185-5p. *indicates $p \leq 0.05$, analyzed by the Mann-Whitney U test.

DISCUSSION

Based on these preliminary results, it is possible to conclude that the SEC method used for the isolation of EVs and the miRCURY™ RNA Isolation Kit—Biofluids used for miRNA extraction are suitable methods for isolating and quantifying EV-miRNAs in MM patients. In several reports, SEC has emerged as a practical, efficient, and consistent EV isolation process that maintains the major EV characteristics, including vesicular structure and content, guaranteeing future uses [14,15].

Our results also demonstrated that it was possible to successfully isolate and quantify miRNAs in the EVs from the PB and BM samples of MM patients at both diagnosis and remission stages. The expression levels of free-miRNAs were higher in the BM than in the PB. This may suggest that BM samples may better reflect the tumor miRNAs content than PB samples, or, alternatively, simply that BM has more microRNAs than PB. Similar results were found with free-miRNA analysis in these samples, indicating that EV-miRNAs might reproduce the results obtained from free-miRNA analysis (submitted for publication). Regarding the EV cargo, it was already observed that EVs from MM patients have distinct protein cargo and different miRNAs compared to healthy donors and even other plasma cell diseases[16,17]. To confirm these hypotheses, it would be interesting to verify the levels of the identified free-miRNA-EV in BM and PB of samples from donors without MM.

However, the levels of EV-miRNAs were more similar between BM and PB samples (than the levels of free-miRNAs). The reason for this may be related to the fact that PB samples have EVs from various body sources and are not as concentrated in tumor-derived EVs as the BM samples. Some reports suggest that EVs contain approximately half of all circulating RNAs found in the PB[16,18]. These results indicate that caution should be taken when analyzing free or EV-miRNAs from PB liquid biopsies.

Nevertheless, at remission, no statistically significant difference in the levels of these EV-miRNAs was found between the PB and BM patient samples, probably reflecting the effect of drug treatment and suggesting that remission (but not diagnostic) PB samples might reproduce BM samples regarding their microRNA content. A similar suggestion was made in another published study, which examined BM and PB EVs as a source of measurable residual disease (MRD) biomarkers[13]. Specifically, in MM, distinct miRNA profiles in PB can act as prognostic markers for drug response or resistance, showing varying patterns between diagnosis and end of treatment[12]. Similarly, in other hematological malignancies, such as acute lymphoblastic leukemia, the reduction of microRNAs levels in PB was observed following chemotherapy, underscoring their utility as biomarkers in response to treatment and MRD negativity[19,20].

In MM, miR-145-5p acts as a tumor suppressor associated with better outcomes. A synthetic miR-145 mimics reduced MM progression by controlling cell proliferation, targeting *IGF1R* and *NRAS*[21,22]. Also, in MM, miR-143-3 has been associated with osteolytic bone disease, reflecting a more aggressive MM stage[23-25]. Nevertheless, in the present study, no clinical associations were attempted, considering the very preliminary nature of this work.

Finally, the number of EVs obtained from the EV isolation protocol used in this work was minimal, making it difficult to proceed with a larger sample to high-throughput proteomic and next-generation sequencing analysis and further validation by Western Blot and qPCR, respectively. For such high-throughput studies, further optimization of EV isolation protocols should be attempted to obtain faster isolation of EVs with a higher yield while maintaining their purity.

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CONFLICTS OF INTEREST

The authors acknowledge Celgene/BMS for funding this work (Project Looker—Grant_138800). The authors declare no other conflict of interest.

ABBREVIATIONS

BM: Bone Marrow; cDNA: Complementary DNA; Ct: Cycle Threshold; EV: Extracellular Vesicles; IGF1R: Insulin-like Growth Factor 1 Receptor; MS2 RNA: RNA from bacteriophage MS2; MM: Multiple Myeloma; miRNAs or miRs: microRNAs; MRD: Measurable Residual Disease; NGS: Next-Generation Sequencing; NRAS: Neuroblastoma RAS Viral Oncogene Homolog; NTA: Nanoparticle Tracking Analysis; PB: Peripheral Blood; PBS: Phosphate Buffered Saline; PPP: Platelet Poor Plasma; qPCR: Quantitative Real-Time PCR; RNA: Ribonucleic Acid; SEC: size exclusion chromatography; SPSS: Statistical Package for the Social Sciences; TEM: Transmission Electron Microscopy; UF: Ultrafiltration; WB: Western Blot.

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CHAPTER 4

GENERAL DISCUSSION & CONCLUSION

MM is a clinical, phenotypical and molecular heterogeneous hematological neoplasia, for which the perfect treatment approach has yet to be established even if it has improved substantially over the last years, including nowadays a sequential and integrative approach using drugs of different classes in combination, with a clear benefit for patients. Indeed, new drugs such as proteasome inhibitors (PIs), IMiDs, and monoclonal antibodies have been approved and widely used to treat MM. Several other therapies with new mechanisms of action, mainly based on immunotherapy, are already being used in clinical practice, especially conjugated and bi-specific monoclonal antibodies and chimeric antigen receptor (CAR) T-cells. The impact of all these treatments at both diagnosis and relapses is evident, allowing MM patients to achieve better responses, longer periods free of disease progression, and ultimately better overall survival[202].

Nonetheless, the spectrum of treatment response is widely large, ranging from primary refractory disease to negative measurable residual disease (MRD), representing the extremes of survival outcomes of MM patients. Drug sensitivity/resistance significantly contributes to this heterogeneity of results. The reasons why some patients are refractory or relapse and how drug-resistant MM clones alter their dominance and persist after therapies are not fully understood. Due to the heterogeneous nature of MM, many causes are reported to be involved in MM drug resistance, and innovative strategies should be pursued to counteract it[332].

A small percentage of patients fail to respond to initial therapy and are considered primary refractory MM patients. It has been reported that around 10-20% of MM patients are refractory to first-line treatments, including IMiDs and PIs, and that around 5% are refractory to recent anti-CD38+ monoclonal antibodies-based protocols[559,838]. There is no consensus on the management of these patients, which is becoming a complex group to treat, portraying very aggressive disease and shorter survival of less than 18 months in most reports[838,839]. A deep and sustained response to treatment is the ultimate objective in any phase of MM treatment. Nonetheless, achieving a CR is insufficient to ensure MM's long-term survival. Indeed, the persistence of low levels of plasma cells in a patient's bone marrow (BM), as a MRD after treatment, will constitute the seed for a subsequent relapse and a significant cause of drug resistance. Quantifying MRD allows the assessment of chemotherapy efficacy and clinical outcome, identifying patients at high risk of recurrence and providing important information for therapy decisions. MRD evaluation is most important to determine when and how the myeloma clone disappears during treatment and when the myeloma burden, even low, reappears during or after treatment[576].

Thus, biomarkers that predict drug response and guide treatment decisions, particularly on drug combinations, are essential to improve therapeutic efficacy and the outcome of MM treatment. Likewise, biomarkers used in routine monitoring of treatment response are required to contribute to guiding therapeutic decisions. Most current biomarkers result from the MM manifestations and intricate relations based on their pathophysiology or event genetics background, failing to represent direct plasma cells or have the clinical impact that MRD evaluated in the bone marrow has achieved. The ideal biomarker should rely on being minimally invasive and represent all the MM burden. In this context, using miRNAs and EVs (or both) as analytes in liquid biopsies provides an excellent opportunity to improve treatment choices and dynamically monitor MM patients in a noninvasive and reproducible manner[636,638,639,840].

Driven by the will to add knowledge to this area, **we aimed to identify biomarkers of MRD and drug response/resistance using liquid biopsies from patients with Multiple Myeloma and contribute to guiding personalized therapeutic decisions.** To accomplish that, we proposed to: i) detect MRD biomarkers in EVs isolated from BM and PB samples of MM patients after autologous stem cell transplant; ii) identify miRNAs in the BM and PB samples of MM patients as putative biomarkers of MM drug response to treatment during the frontline therapy; and iii) quantify miRNAs from the cargo of EVs isolated from PB or BM of MM patients at both diagnosis and remission stages.

In Chapter 3.1., we demonstrated the possibility of isolating EVs from the BM and PB of MM patients at distinct disease stages (diagnosis and remission) and compared the MRD-associated markers between paired BM and PB EVs at the diagnosis and remission stages. Our results demonstrated that EVs from BM and PB samples, both from diagnosis and remission, and EVs from the PB of healthy controls were successfully isolated using the SEC method followed by concentration by UF, and characterized by NTA, TEM, and WB in terms of size distribution, morphology and presence of well-known EV protein markers respectively. It was also possible to demonstrate that EVs isolated from PB or BM at diagnosis present specific MM markers such as CD38 and CD138, confirming the potential of analysing the cargo of EVs as MM biomarkers. Interestingly, our patients without CD38 in EVs at diagnosis and CD38 negative by flow cytometry presented more extramedullary disease with low medullary plasmacytosis, associated with treatment refractoriness and worse prognosis. CD38 and CD138, as specific lineage markers, were described as the best markers to identify the source of EVs derived from monoclonal plasma cells[582,828]. Nonetheless, it is necessary to carefully interpret results from CD38+ EVs and CD138+ EVs once they can be derived from other cells rather than PCs. Indeed, CD38 is also detectable in low levels in other lymphoid cells (NK, B, and activated T cells) and myeloid cells (monocytes), while CD138 can be expressed in low levels by epithelial cells[841,842]. When possible, co-presentation of CD38/CD138 should be used to identify MM EVs[828]. When comparing the presence of MM markers between paired PB and BM samples, it was possible to verify that most of the markers present in the EVs isolated from BM were also present in EVs isolated from PB. Detection of MRD in PB mirroring BM and/or EMD would bring apparent advantages to patients and clinicians, allowing frequent and minimally invasive real-time disease monitoring. Likewise, differences between MM markers on EVs isolated from paired diagnosis and remission samples were verified by analyzing alterations in the levels of MM markers on EVs isolated from paired diagnosis and remission samples. Comparing MRD status and the EVs MM markers found at remission is challenging, mainly regarding CD38 and CD138, even though some patterns could be found. A specific resistance signature of CD138+ P-gp+ CD34+ EVs was significantly elevated in the plasma of patients with aggressive disease and persistent residual disease, supporting its possible role as a MRD marker in EVs [822,843,844]. Our results showed that even though the levels of MM markers in the diagnostic samples differed in several PB and BM samples, those levels were very similar in the remission samples, thus suggesting that EVs have the potential to monitor MRD in liquid biopsies from remission samples. Additionally, with this approach, it was possible to demonstrate the absence of MM markers, such as CD38, CD56, CD117, and CD27, in EVs from healthy controls, further suggesting that these markers are attractive to be used as biomarkers of MM to monitor MRD non-invasively.

In Chapter 3.2., it was demonstrated that specific miRNAs are differently expressed between MM refractory and sensitive patients to first-line treatment, which can be detected in PB and BM samples. NGS profiling revealed several miRNAs expressed differently between treatment refractory and sensitive patients and between PB and BM. Among the differentially expressed miRNAs, miR-665, miR-483-5p, miR-143-3p and miR-145-5p were selected for further validation using qPCR. Although both miR-483-5p and miR-665 were found by NGS to be upregulated in the refractory MM patients analyzed, further qPCR analysis showed that only the miR-665 retained statistical significance. The difference in the expression levels of some miRNAs between groups of patients with different types of drug responses suggests their potential to be used as treatment biomarkers. Indeed, it was possible to verify that miR-665 levels were higher in refractory patients than in sensitive patients. This microRNA has an unclear function once it plays diverse roles in other cancer types and can act either as an oncomiR or a tumor suppressor miRNA [845]. This prompted us to correlate the miR-665 levels with clinical outcomes in the patients analyzed, such as clinical characteristics and survival outcomes. Our results showed that higher levels of miR-665 were associated with poor overall survival outcomes and aggressive MM characteristics. Therefore, the present study's findings underscore the potential miR-665 to be used as a predictive tool for refractoriness and poor overall survival in MM. Interestingly, this miRNA was associated with an aggressive MM phenotype having a higher burden of disease, reflected by higher LDH, higher b2-microglobulin, higher creatinine, and more frequent extramedullary disease. When comparing with established prognostic models, higher levels of miR-665 were associated with a stage III of the R-ISS, reflecting the potential use of this miRNA as a biomarker or a possible addition to this prognostic staging tool, mainly to discriminate patients that are stage II. Concerning the differences between BM and PB samples, both miR-665 and miR-483-5p presented higher levels in the PB than in the BM. Even though BM studies, namely cytogenetics, remain the gold standard, the possibility of analyzing PB circulating miRNAs as analytes in liquid biopsies can represent a non-invasive approach, together with other biomarkers or alongside MRD, to follow-up treatment outcomes. Functional in silico analysis for miR-665 revealed that several target genes play essential roles in various biological processes and signaling pathways related to MM and are associated with drug resistance, namely to PI and IMiDs[761,846,847].

Lastly, in Chapter 3.3., we showed that it was possible to successfully isolate and quantify miRNAs in the EVs previously isolated from the PB and BM samples of MM patients at both diagnosis and remission stages. The levels miR-145-5p and miR-143-3p, previously found to be differentially expressed in the free form, were determined in the cargo of EVs isolated from PB and BM samples, both at diagnosis and remission stages, using q-PCR. Interestingly, for both miRNAs analyzed, a statistically significant difference in their levels was observed between PB and BM at diagnosis, with higher levels detected in the BM than in the PB. These results indicate that PB EV-miRNAs may provide different results from BM EV-miRNAs and that caution should be taken when analyzing EV-miRNAs on PB liquid biopsies. However, at remission, no statistically significant difference in the levels of these EV-miRNAs was found between PB and BM patient samples. This is particularly interesting, once due to their lipid bilayer, EVs are a more stable and reliable source of miRNAs, protecting them from degradation compared to the free circulating miRNAs. This suggests that remission (but not diagnostic) PB samples might

reproduce BM samples regarding their microRNA content. A similar suggestion was made in Chapter 3.1, when studying BM and PB EVs as a source of MRD biomarkers.

The data gathered across this thesis provides significant insights into the potential of EVs and/or miRs as biomarkers in MM and how they can be used as analytes in liquid biopsies. **Integrating EVs and miRNAs analysis in liquid biopsies into the clinical practice could represent a significant step in MM management and treatment algorithm decisions.**

BM aspirate/biopsy constitutes the primary method for studying MM in order to identify the disease, perform a prognosis, or define a genetic classification/prognosis. However, they consist of a painful, uncomfortable, and invasive procedure, limiting the possibility of serial MM evaluation over the treatment course. This is a problem, mainly in high-risk MM patients who need regular monitoring to track disease progression and treatment response. Additionally, single-site bone marrow aspirates do not address the spatial heterogeneity of MM, marked by a patchy infiltration in the bone marrow and extramedullary sites, in which cytogenetic alterations and mutations can occur differently. Therefore, liquid biopsy, as a flexible tool for characterization and follow-up, emerges as an attractive tool in MM management to overcome challenges regarding its spatial and temporal heterogeneity. Monitoring biomarkers from the PB, such as EVs and miRNAs (as has been done in the current thesis), which can mirror the BM and extramedullary disease, would allow a **minimally invasive approach** that would facilitate and improve the management of MM.

With the emergence of novel treatment options and strategies in MM, there is an increasing need for reliable, practical, and sensitive approaches for better longitudinal monitoring of biomarkers in liquid biopsies. These approaches are especially helpful in **detecting earlier molecular relapse and clonal evolution and monitoring MRD**. In a MM phase II clinical trial, PB plasma-derived ctDNA and paired BM MM cell DNA obtained from MM patients treated with carfilzomib, thalidomide, and dexamethasone was analysed and it was possible to confirm that sequential evaluation of ctDNA predicted relapse in which at least one mutation at relapse/pre-relapse was already present at the start of therapy[848]. Negative MRD is the most important surrogate marker for survival outcomes, mainly if persistent over time, and most modern clinical trials are already designed to address MRD-driven options. However, current NGF and NGS techniques for assessing MRD only focus on bone marrow, potentially missing residual disease elsewhere[613]. The need for sequential BM aspirates/biopsies also limits the dynamic approach of MDR monitoring in MM. These methods can be complemented by imaging techniques such as PET-CT and MRI, but both failed to reflect the complex molecular landscape of MM[612,614]. PB approaches are essential to facilitate longitudinal monitoring and reflect the spectrum of MM heterogeneity. EVs, which contain cargo from their cell of origin, appear extremely interesting when evaluating MRD once, in addition to managing cellular waste, they play a pivotal role in mediating intercellular communication under physiological and pathological conditions[797,798]. Considering this and the changes in EV quantity concerning disease severity, stage, or treatment phase, it is conceivable that MM-EVs and their cargo could potentially be used as biomarkers of this disease and to monitor MRD in PB. In the present thesis, this potential future clinical application was explored. We demonstrated the possibility of isolating EVs from the BM and PB of MM patients at distinct disease

stages and further compared the MRD-associated markers between paired BM and PB EVs at the diagnosis and remission stages.

Another significant advantage of circulating biomarkers in MM relies on their prognostic potential to predict survival outcomes and identify treatment failure (due to drug resistance) and disease progression, as we demonstrated for miR-665 in ndMM treated equally with the same drug combination. Identifying specific biomarkers (such as miRNAs) associated with treatment resistance or sensitivity would represent a major step in the personalized approach to these patient's treatment and corresponds to one of the biggest unmet needs in the MM. Their use could contribute to selecting and adapting therapeutic strategies, including detecting targetable abnormalities towards **personalized treatment using liquid biopsies**. This would help to speed up the transition to more effective therapies, prevent unnecessary side effects of ineffective treatments, and optimize dosage adjustments, potentially improving patient outcomes.

The presence of the translocation t(11;14) is, so far, the unique biomarker for personalized treatment of MM using BCL-2 inhibitors, such as venetoclax[400]. Nonetheless, its identification relies on invasive BM aspiration/biopsy or extramedullary biopsy. Other attempts towards precision medicine in MM have been discussed. Recently, studies reported that small-molecule inhibitors targeting KRAS have been developed for treating RAS-mutated MM patients. Additionally, combining BRAF/MEK inhibitors in BRAF-mutated MM offers promising results[849-851]. A prospective clinical trial evaluated cfDNA sequencing in 16 patients with ndMM and linked these results to high-throughput screening of drugs to predict response to these treatments. Mutations in DIS3, FGFR3, KMT2C, MAML2, and ZFX4 predicted resistance to specific treatments, although no association was statistically significant after multiple-testing correction[852]. Again, in all these studies, identifying these biomarkers was made possible through cell lines, plasmacytomas, and BM studies. Currently, two registered clinical trials used miRNAs on PB to identify new prognostic and predictive biomarkers that could be used as therapeutic targets to improve personalized clinical therapies and highlighted the possible clinical application of liquid biopsies in MM[853,854].

Despite increasing evidence supporting the effectiveness of liquid biopsies and the results obtained in this thesis, translating this data into clinical practice presents challenges that must be addressed in future research.

The present EVs and miRNAs studies were not designed to provide definite clinical advice; thus, further studies should be attempted to extend this study to a larger sample of MM patients and integrate this approach with NGS/NGF and imaging studies at different disease time points and prospectively. An essential application of liquid biopsies could be correlating the identification of newer biomarkers with the molecular characterization of extramedullary disease. Also, miRNA and/or EVs cargo evaluation should be included in prospective clinical trials focusing on translation into the clinical setting. An exciting approach should investigate diverse biomarkers in liquid biopsies at the same time to obtain higher sensitivity and specificity. Of particular interest would be the integration, for example, of miRNA profiling and/or EVs cargo with already known biomarkers and clinical features to enhance the accuracy of predictive tools and improve patient stratification, moving towards precision medicine.

Technological limitations are also responsible for the controversial reliability of these approaches. In the present thesis, even though the SEC method proved to be efficient for isolating EVs and the protocols to characterize those EVs were well described, overall, the methodology is time-consuming and needs to be further optimized to become consistent, reproducible and more accessible to use in the clinical practice. Furthermore, there is no standardization in the interpretation of results, which relies on sample-by-sample analysis by experienced operators. Likewise, there is a need for standardized protocols for miRNA detection, quantification, and understanding of its complex network. The functional relevance of miRNAs is still being determined once they can have several targets, and those targets can be targeted by other miRNAs. Thus, further research is needed to elucidate and consolidate their roles. This research could be done by manipulating miRNA levels using synthetic miRNA mimics or miRNA inhibitors in cells, followed by functional assays.

Understanding the mechanisms by which specific miRNAs influence MM pathogenesis may also lead to developing novel targeted therapies, potentially overcoming current treatment resistance. Preclinical studies investigated the potential synergistic effect of miRNA inhibition/replacement with anti-MM drugs. For instance, inhibition of miR-221/222 via LNA-i-miR-221 restored drug sensitivity in melphalan-refractory MM cells, triggering cell apoptosis in vitro and in vivo, and replacement of miR-29-b prevented the activation of the pro-survival autophagic pathway, enhancing the anti-MM effect of bortezomib[510,855]. Using nanocarriers or EVs for miRNA delivery in combined regimens needs further investigation, although some exciting proposals are being investigated. MRX34, a liposomal nanoparticle mimetizing miR-34a, is the first human miRNA cancer therapy in a phase I clinical trial, resulting in significant down-regulation of known target genes (NOTCH1, HEY1, HES6 and HES1). Despite the off-target toxicity observed in this trial, the dose-dependent modulation of relevant target genes provided proof of concept for miRNA-based cancer therapy and prompted new research in this field[856].

Despite these challenges, **significant contributions to the management of MM were made and described in this thesis.** First, it was possible to explore reliable methods for isolating and characterizing EVs from MM patients and detecting MRD biomarkers in the BM and PB. Second, it was possible to identify miRNAs in the BM and PB that were differentially expressed in drug-resistant or sensitive patients, identifying miRNAs such as miR-665 that correlate with treatment resistance and poor clinical outcomes. Third, by comparing paired BM and PB throughout these studies, it was possible to explore the potential future clinical utility of liquid biopsies in MM follow-up by demonstrating the feasibility and benefits of using EVs and/or miRNAs as minimally invasive biomarkers.

In conclusion, this thesis underscores the promising potential of liquid biopsies in MM, mainly using EVs and/or miRNAs as analytes. Their application as a minimally invasive approach could lead to improved longitudinal monitoring of MM patients, detecting early signs of relapse/progression and identifying more profound and persistent responses to treatment. Additionally, it could be essential to the early detection of drug resistance and the selection of more personalized treatment strategies, ultimately enhancing patient outcomes and the management of this challenging hematologic neoplasia.

CHAPTER 5

FUTURE DIRECTIONS

The work presented here represents the beginning of a research trajectory, setting the way for other novel and original projects, some of which are already planned.

First, validation in larger cohorts will be essential to confirm the utility of miRs and/or EVs as biomarkers of disease monitoring and predictive treatment response. This will be possible by continuing to perform this work (isolation and analysis of EVs and/or miRs) on samples already collected and readily available. Additional samples will be collected from patients treated with new strategies, mainly immunotherapy, such as T cell engagers and CART T cells. This will establish the robustness and generalizability of these biomarkers across different patient populations.

At the same time, other molecular biomarkers used as analytes (such as proteins) in liquid biopsies and clinical data will be integrated to accelerate the translation of basic research results into better MM patient stratification and precision medicine. A step towards developing bioinformatics tools with clinical application is also an aim for future research in this field.

Finally, mechanistic studies on miRNAs will be carried out to understand further the underlying mechanism by which these miRNAs influence MM drug resistance. This may contribute to overcoming drug resistance and developing targeted therapies.

We look forward to the following challenges resulting from this PhD project, which will provide new perspectives on improving the management of MM patients, leading to future collaborative research efforts and promoting translational research towards precision medicine.

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