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CARLO BO

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PD-1 and PD-L1 expression in Multiple Myeloma patients

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Advisor:

Prof. Stefano Papa

Co-Advisor:

Dr. Massimo Valentini

Ph.D. student:

Dr. Andrea Vagnini

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ABBREVIATIONS

Abbreviation	Description
Ab	Antibody
AP-1	Activator protein 1
ADCC	Antibody-dependent cellular cytotoxicity
ADO	Adenosine
AKT	Protein kinase B
ALL	Acute lymphoblastic leukemia
APC	Antigen-presenting cell
APRIL	Proliferation-inducing ligand
ASCT	Autologous stem cell transplant
ATP	Adenosine triphosphate
BCMA	B-cell maturation antigen
BM	Bone marrow
BMSC	Bone marrow stromal cell
C	Cyclophosphamide
Ca	Calcium
CAM-DR	Cell adhesion mediated drug resistance
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CLL	Chronic lymphocytic leukemia
CRAB	Calcium, renal failure, anaemia, bone lesions
CTL	Cytotoxic T lymphocyte
D	Dexamethasone
DARA	Daratumumab
DC	Dendritic cell
EC	Endothelial cell
ECAR	Extracellular acidification rate
ECM	Extracellular matrix
EMD	Extramedullary disease
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
FDA	Food and drug administration
FGFR3	Fibroblast growth factor receptor
FISH	Fluorescence in situ hybridisation
FITC	Fluorescein isothiocyanate
FLC	Free light-chain
FSC	Forward scatter
Hb	Hemoglobin
HD	Healthy donor
HDACi	Histone deacetylase inhibitor
HIF-1	Hypoxia-inducible transcription factor 1
HMCL	Human myeloma cell line
HNSCC	Head and neck squamous cell carcinoma
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IFN-γ	Interferon-γ
IL4	Interleukin-4
IL6	Interleukin-6
IL8	Interleukin-8

iMID	Immunomodulatory drug
IMWG	International Myeloma Working Group
ISS	International Staging System
ITIM	Immune-receptor tyrosine-based inhibitory motif
ITSM	Immune-receptor tyrosine-based switch motif
JAK	Janus kinase
LDH	Lactate dehydrogenase
M	Melphalan
mAB	Monoclonal antibody
MDSC	Myeloid-derived suppressor cell
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MM	Multiple myeloma
MMSET	Multiple myeloma SET domain containing protein
MRD	Minimal residual disease
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
mRNA	Messenger RNA
MSC	Mesenchymal stromal cell
mTOR	Mechanistic target of rapamycin
MV	Microvesicle
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NDMM	Newly diagnosed multiple myeloma
NICE	National Institute for Health and Care Excellence
NK	Natural killer cell
NSCLC	Non-small-cell-lung cancer
OB	Osteoblast
OCR	Oxygen consumption rate
OC	Osteoclast
ORR	Overall response rate
OS	Overall survival
P	Pomalidomide
PC	Plasmacell
PFS	Progression free survival
PMT	Photomultiplier tubes
PBS	Phosphate buffered saline
PC	Plasma cell
PCK-θ	Protein kinasi theta
PCL	Plasma cell leukaemia
PD-1	Programmed death 1
PD-L1/L2	Programmed death ligand 1/2
PE	Phycoerythrin
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinase
pDC	Plasmacytoid dendritic cell
QIF	Quantitative immunofluorescence
R	Lenalidomide
R-ISS	Revised International Staging System
RR-MM	Relapsed/refractory multiple myeloma
RMM	Relapsed multiple myeloma
RNA	Ribonucleic acid
SCT	Stem cell transplant
SMM	Smouldering multiple myeloma

SSC	Side scatter
T	Thalidomide
TIL	Tumor-infiltrating lymphocytes
TMB	Tumor mutational burden
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
V	Bortezomib
VCD	Bortezomibf-cyclophosphamide-dexamethasone
VD	Bortezomib-dexamethasone
VRD	Bortezomib-lenalidomide-dexamethasone
VTD	Bortezomib-dexamethasone-thalidomide
WM	Waldenström's macroglobulinemia

ABSTRACT

Multiple myeloma is an incurable plasma cell malignancy with only 30% of patients surviving for more than 10 years. The bone marrow microenvironment is crucial to the survival, proliferation and growth of these malignant plasma cells and has also been heavily implicated in drug resistance. Therefore, therapeutic targeting of the microenvironment has gained interest in conjunction with targeting myeloma cells themselves.

Multiple myeloma is an extraordinarily complex hematological disease in regards to its ability to manipulate the cells in the bone marrow microenvironment, as well as its genesis and progression. Multiple myeloma is a malignancy that is heavily associated to relapse after therapy. The strong dependence malignant plasma cells have on the bone marrow microenvironment makes it extremely difficult to effectively treat this disease, with a small residual population of drug-resistant myeloma cells remaining within the bone marrow after nearly all cases of treatment. An important achievement in the immunotherapeutic treatment of cancer was the discovery of the PD-1/PD-L1 pathway, its function in the evasion of tumor immunity, and the development of targeted antibodies. The PD-1 pathway has been shown to be extraordinarily successful in slowing or clearing tumors in multiple human cancers. Although no definitive biomarker to predict success of PD-1 immunotherapy has been described, the pre-treatment density of $CD8^+$ T cell infiltration and expression of PD-1 or PD-L1 in the tumor microenvironment all correlate with responsiveness to PD-1 targeted therapies. Memory T cells likely play an important role in the response to tumor recurrence and metastases. Blocking PD-L1 may be a more effective therapeutic strategy than blocking PD-1, and that blocking both PD-1 and PD-L1 may be an effective combination. Although the majority of clinical effort has been put towards antibodies blocking PD-1, an antibody blocking PD-L1 interactions with both PD-1 and B7-1 has been approved in non-small cell lung cancer and bladder cancer. Anti-PD-1/PD-L1 antibody treatment could be clinically effective in MM patients by recovering T-cell cytotoxicity and inhibiting reverse signaling from PD-L1 on MM cells. Therefore, the use of combination therapies may significantly improve the impact of checkpoint inhibition as a treatment modality for selected patients. Flow cytometry may be a reliable, easy and value effective tool for the assessment of minimal residual disease in patients with multiple myeloma. Longer remissions that cannot be accurately evaluated with conventional techniques, such as immunofixation and electrophoresis, are achieved by novel drugs, which dramatically enhance patients' outcomes. Understanding the distribution of PD-1/PD-L1 molecules in the BM niche of patients with multiple myeloma and the contribution of immune resistance mechanisms to PD-1/PD-L1 blockade represents a critical step in order to identify the best patient subset that could benefit from this checkpoint blockade and to provide rationale for new combined therapeutic strategies.

INTRODUCTION

1.1. Multiple myeloma

In 1844, Dr. Samuel Solly published the first clinical case of multiple myeloma[1]. Myeloma is now considered as the second most frequent hematological malignancy diagnosed in the Western World[2].
In the bone marrow microenvironment, it is characterised by the accumulation and clonal proliferation of terminally differentiated $CD138^{+}CD38^{+}$ B-lymphocytes, known as plasma cells[3].
Myeloma is described by the excessive secretion of dysfunctional monoclonal immunoglobulin, commonly known as paraprotein. In turn, this characteristic can be used in the detection, diagnosis and post-treatment monitoring of multiple myeloma and its associated non-malignant precursor condition, monoclonal gammopathy of undetermined significance (MGUS). Myeloma clinically reveals in end-organ damage that leads to renal impairment, hypercalcaemia, anemia, recurrent infections and the formation of bone lesions, which are induced by the catastrophic manipulation of the homeostatic process of bone remodelling[4].
Although patient response rates have risen as a result of the development of new targeted therapies, this malignancy is still incurable, with about half of patients surviving for less than 5 years after diagnosis[5].
Myeloma cells clearly rely substantially on the bone marrow microenvironment, which contributes to increased proliferation, survival, and resistance to therapeutic treatment[6].
Drug resistant cells frequently accumulate in this microenvironment, possibly leading to patient relapse, with the duration of remission typically decreasing with each treatment course a patient receives[7,8]. To prevent these protective effects, it is crucial to understand the interactions and mechanisms behind the malignant plasma cells' reliance on the bone marrow. This could result in the discovery of novel therapeutic targets and provide a rationale for the development of more efficient treatment protocols for myeloma patients.

1.1.1. History of multiple myeloma

Dr. Samuel Solly described the first clinical case of multiple myeloma in 1844. Sarah Newbury, a 39-year-old housewife with significant back pain, was the patient. She died 4 years after her symptoms began, and a red substance had replaced the cancellous section of her sternum, as well as both femurs, according to a postmortem investigation. Fractures of the right radius and ulna, left tibia and fibula, and both femurs were discovered during the autopsy.

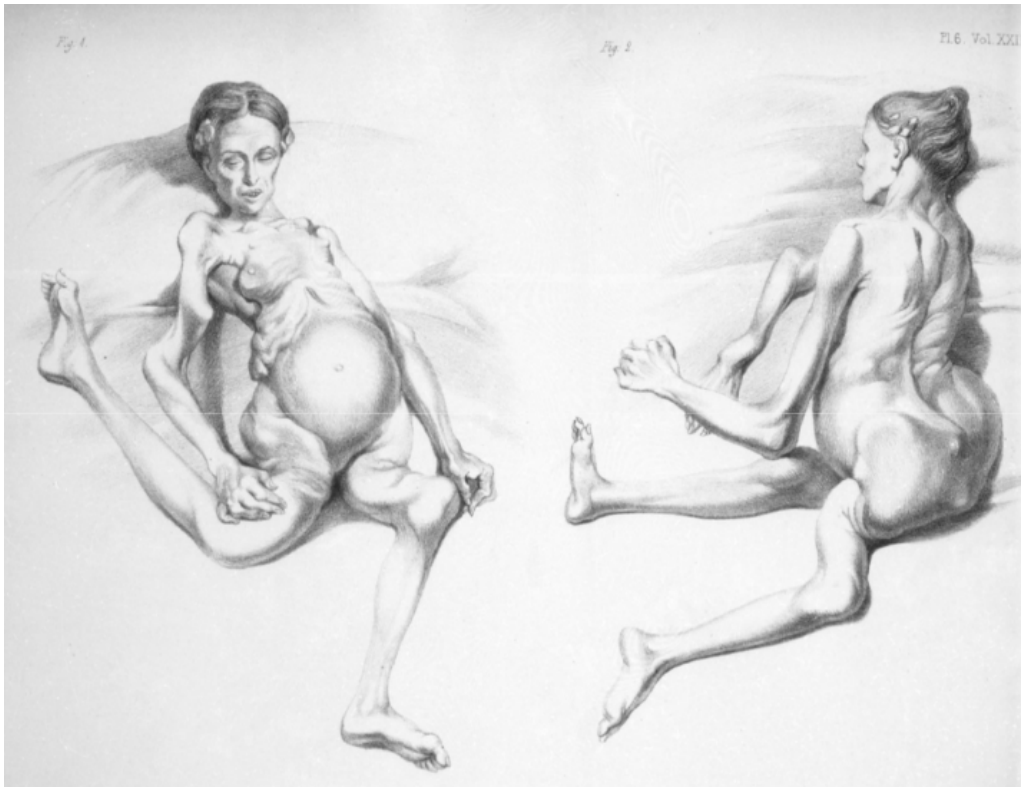


Figure 1. Sarah Newbury, the first reported patient with multiple myeloma[1].

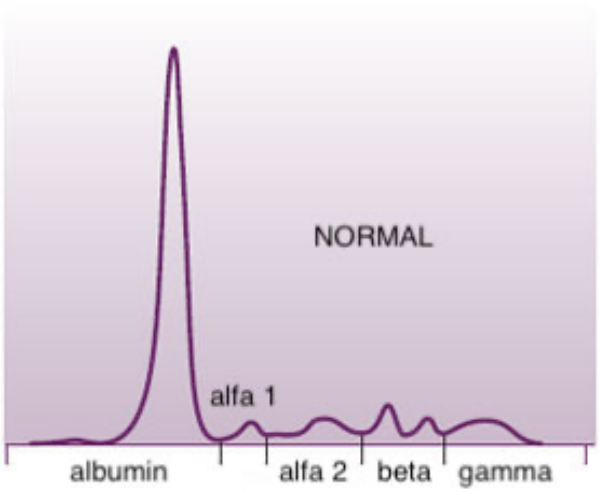
In 1873, while working at Professor von Recklinghausen's institute, von Rustizky coined the expression "multiple myeloma."
During a patient's autopsy, he discovered eight different bone marrow malignancies that were mushy in consistency and reddish in color and were diagnosed as "multiple myeloma".
In 1889, Kahler described the symptoms of multiple myeloma in a 46-year-old physician, including skeletal pain, albuminuria, pallor, anemia, a precipitable urine protein, and necroscopy results.

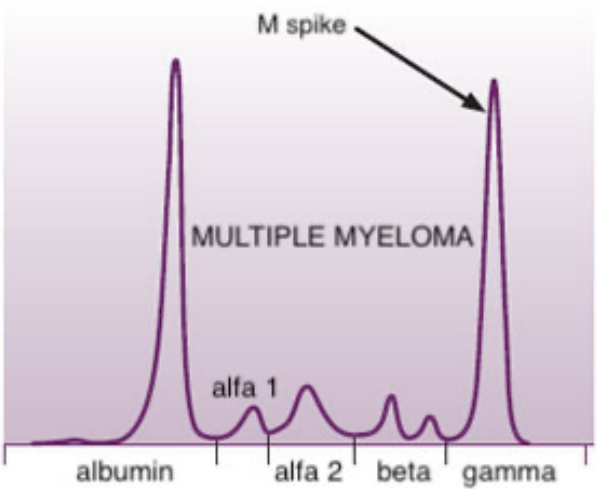
William MacIntyre, Henry Bence Jones, and John Dalrymple evaluated a patient of MacIntyre admitted to the hospital with nonspecific but continuous pain in the chest, back, and pelvis in 1845 at St. George's Hospital in London. MacIntyre contacted Henry Bence Jones and asked him to perform a urine test on the patient.

Bence Jones discovered a chemical in his urine that was precipitated when nitric acid was added. He observed that the precipitate was soluble in boiling water but that it reformed when the urine was cooled.
The patient had "albumosuria," he said. The patient died shortly after the urine was examined. Jones decided that the protein was a "oxide of albumen" and that the "hydrated deutoxide of albumen" was the result of the final analysis. On January 2, 1846, the patient passed away. An autopsy by Dalrymple revealed that the sternum, cervical, thoracic, and lumbar vertebrae were delicate, fragile, and easily breakable, and that they could be cut with a knife.
Furthermore, numerous hemorrhagic cavities in bones were discovered throughout the body. "Atrophy from albuminuria" was stated as the reason of death.
Albuminuria was the term used at the time to refer to proteinuria in general, while Fleischer coined the name "Bence Jones protein" in 1880.
Weber hypothesized in 1898 that the Bence Jones protein is produced in the bone marrow.
Jacobson and Walters discovered Bence Jones proteins in the bloodstream in 1917 and 1921, respectively, and concluded that they were most likely produced from blood proteins by aberrant cells in the bone marrow.

Longsworth and colleagues used electrophoresis to analyze multiple myeloma in 1939, demonstrating the towering narrow-based "church spire" peak.

In 1953 Grabar and Williams first described immunoelectrophoresis, which makes it easier to diagnose multiple myeloma.
Wilson first described immunofixation, also known as direct immunoelectrophoresis, in 1964. Serum and urine protein electrophoresis, followed by immunofixation, are used to detect monoclonal immunoglobulins.
Small monoclonal light chains, which are not visible on electrophoresis, can be detected by immunoelectrophoresis, immunofixation, or direct immunoelectrophoresis.
The principle behind protein electrophoresis is that proteins migrate at different rates based on their electrical charge.
During electrophoresis, immunoglobulins migrate to the gamma area, and the presence of monoclonal immunoglobulin causes a characteristic "M-spike" in the gamma region that is not detected in normal people[9].





Fractions	%	Ref. %	g/dl	Ref. g/dl
Albumin	69.9	52.9–66.9	4.9	3.7–4.9
Alpha	3.5	3.0–5.8	0.2	0.2–0.4
Alpha 2	8.8	7.5–13.4	0.6	0.5–1.0
Beta	9.3	8.5–13.7	0.7	0.6–1.0
Gamma	8.5	8.8–19.2	0.6	0.6–1.4

Fractions	%	Ref. %	g/dl	Ref. g/dl
Albumin	40.2	52.9–66.9	3.3 L	3.7–4.9
Alpha	4.8	3.0–5.8	0.4	0.2–0.4
Alpha 2	11.0	7.5–13.4	0.9	0.5–0.9
Beta	7.8	8.5–13.7	0.6	0.6–1.0
Gamma	36.2	8.8–19.2	3.0 H	0.6–1.4

Figure 2. Serum protein electrophoresis, normal and with M spike in gamma region.

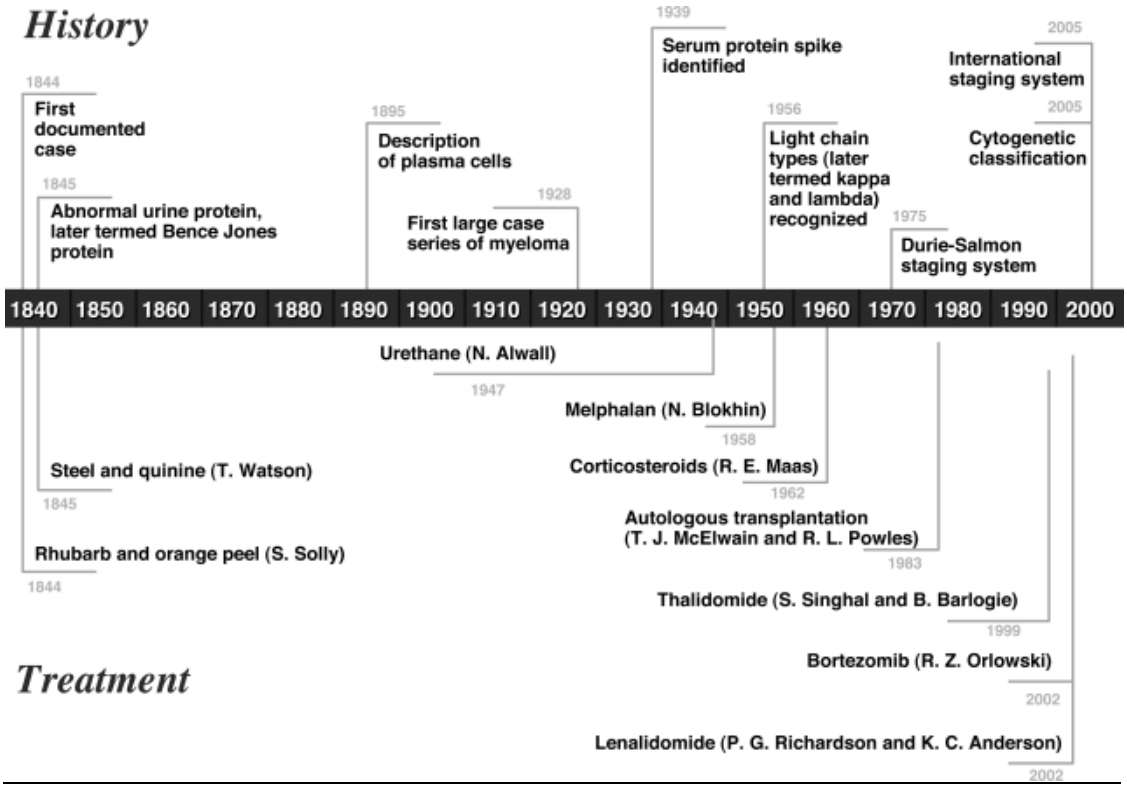


Figure 3. Timeline description of the history and treatment of multiple myeloma from 1844 to 2005. (Kyle R.A. and Rajkumar S.V. Multiple myeloma. Blood. 2008 Mar 15; 111(6): 2962–2972)

1.1.2. Epidemiology of myeloma

Although multiple myeloma is a relatively uncommon neoplasm, accounts for 13% of diagnoses of blood cancer, with an average age-adjusted incidence rate of about 8 cases per 100.000 persons per year.
Myeloma is responsible for 0.8% of all cancer diagnoses and 1% of all cancer deaths worldwide. The frequency of myeloma varies greatly by ethnicity, with black people being diagnosed at a rate of 2:1 more often than white people[2].
Australasia, Europe, and North America have the highest rates of myeloma in the world[10]. Asian populations have the lowest rates of myeloma, but recent studies have found considerable increases in incidence in Asian countries[11]. Myeloma diagnoses are also more common in males than females (58% vs 42%)[2]. Whilst the exact causes of this discrepancy are widely unknown, there is evidence to suggest that there are gender-dependent differences in primary genetic aberrations observed in myeloma; one study showed that males experienced a greater frequency of hyperdiploidy (62% vs 50%), whilst females had a higher incidence of immunoglobulin heavy chain gene (IgH) translocations (32% vs 50%)[12].
The average age of myeloma patients at diagnosis is 66, with 38% of diagnoses occurring in patients over the age of 70. Myeloma is considerably rarer in younger patients, with just 2% of diagnoses occurring in patients under the age of 40 years old[13,14.] The current overall 5-year survival rate of patients is 51.6% for symptomatic patients. Patients younger than 50 years old at diagnosis, had more favorable prognostic characteristics and have considerably higher 5-year survival rates than patients older than 50 years[15].

1.1.3. Etiology of myeloma

Since its discovery, the exact origin and etiology of multiple myeloma have been debated. Symptomatic myeloma is now widely understood to be the result of clinical progression from the asymptomatic disease monoclonal gammopathy of undetermined significance[16]. However, to date there has been no definitive identification of an individual aetiological event attributed to the origin of myeloma or its asymptomatic precursors. Obesity and a poor diet are among the environmental, lifestyle, and occupational risk factors linked to the development of myeloma[17].
According to some research, farmers had a higher risk of developing myeloma, which could be attributed to high levels of pesticide exposure in agriculture[18,19]. To date, little is known about hereditary associations in myeloma etiology. Overall it is not considered to be an explicitly inherited malignancy. However some studies have reported familial links that lead to a significant increase in risk of myeloma development in family members with first degree relatives affected by myeloma[20,21].

1.1.4. Plasma cell biology

Plasma cells are terminally differentiated post-germline cells of the B-lymphocyte line that reside and mature within the bone marrow microenvironment, which plays a crucial role in ensuring their prolonged survival[22]. Despite existing in very small proportions – representing just 1-3% of cells within the bone marrow – plasma cells are responsible for all antigen-specific antibody secreted in circulation[23]. Follicular B-cells and marginal-zone B-cells are two types of B-lymphocytes that are activated in a T-cell dependent or independent fashion, respectively. Upon activation, these B-cells proliferate within germinal centres in the lymph nodes and spleen, with a small number of these cells actively dividing to become short-lived antibody-secreting plasmablasts that ultimately differentiate into long-lived plasma cells in the bone marrow[24]. These cells are largely responsible for the secretion of monoclonal antibodies into the peripheral circulation, which are classified as IgG, IgA, IgM, IgE, and IgD isotypes. These isotypes are inferred by the immunoglobulin heavy chain (IgH) sequence after the process of class switch recombination and can also be further categorised by light-chain classification as either kappa (κ) or lambda (λ)[25].

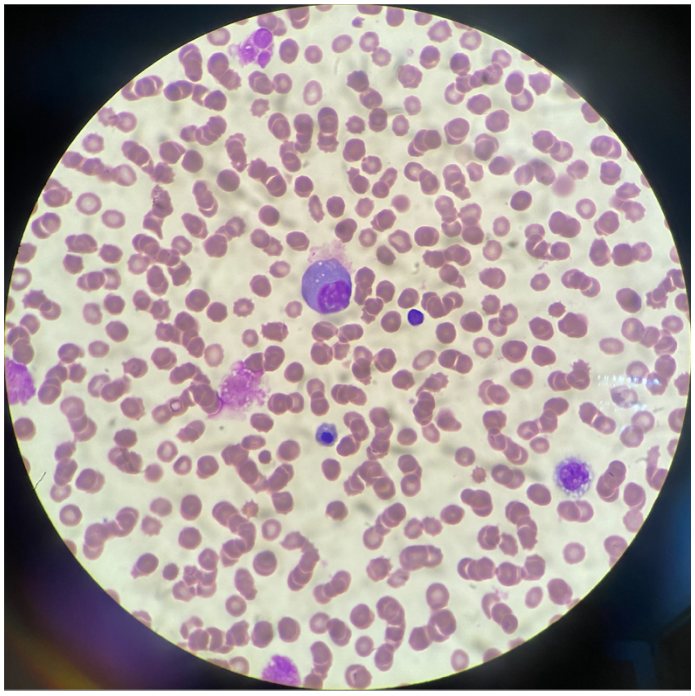


Figure 4. Plasma cell from bone marrow aspirate. (May-Grunwald Giemsa, 100X)

The majority of myeloma patients present with either IgG (52%) or IgA (21%) paraprotein[13]. IgM-myeloma is a relatively rare kind of plasma cell neoplasm that is linked to a poor prognosis. It shares numerous diagnostic characteristics with Waldenström's macroglobulinemia (WM) making it difficult to differentiate between these two disorders. However, end organ damage seen in myeloma, such as the creation of bone lesions, is unique to myeloma and not to WM[26]. A cytogenetic link

has also been shown between the presence of the t(11;14) translocation and IgM myeloma, which could distinguish it from WM[27].

The presentation of IgD and IgE-myeloma are also considerably rarer than IgG, IgA and light-chain secretory myeloma. When compared to more prevalent IgH subtypes, IgD-myeloma accounts for about 2% of all diagnoses and is associated with diagnosis at younger age, more aggressive illness, and a poorer prognosis[28,29]. The incidence of IgE-myeloma is incredibly uncommon, with only around 50 cases being reported in the literature[30]. It has been reported that up to 7% of myeloma patients are classified as non-secretors, despite the fact that the majority of these cases were oligo-secretors after the advent of the serum free light-chain assay. As a result, real non-secretors currently account for about 1-2% of newly diagnosed multiple myeloma patients[31,32].

IgH classification	Light-chain classification	Proportion of myeloma patients (%)
IgG	κ	34
	λ	18
IgA	κ	13
	λ	8
IgD	κ	1
	λ	1
IgM	κ	0.3
	λ	0.2
Free light chain (Bence Jones protein)	κ	9
	λ	7
Non-secretory	-	7

Table 1: Proportions of heavy and light-chain immunoglobulin distributions in myeloma patients. Data obtained from Kyle. RA *et al*, 2003[13].

1.1.5. Surface phenotype of myeloma cells

The expression of surface markers is essential to identify cells that cannot be distinguished purely by the evaluation of morphological characteristics.
The expression of surface markers is key to determining the identity of cells that are not able to be distinguished solely through the assessment of morphological features. This means that cells may be accurately identified by the analysis of their particular surface expression profile, which is crucial in the diagnosis of myeloma because it verifies the proportion of malignant plasma cells present in the bone marrow. Although myeloma is a cancer with significant heterogeneity, plasma cells share characteristics that can be used to predict the disease progression and response to therapy[33].

This is particularly helpful in the identification of residual myeloma cells in the bone marrow after treatment, known as minimal residual disease (MRD).
Syndecan-1, also known as CD138, is a membrane-bound receptor of the heparin sulphate proteoglycan family[34]. It acts as an extracellular matrix receptor and plays a key role in plasma cell adesion to the bone marrow extracellular matrix[35,36]. Amongst cells of hematopoietic origin, CD138 is exclusively expressed on plasmablasts and mature plasma cells, following differentiation from B-lymphocytes[37].
This characteristic is expressed by malignant plasma cells, which makes CD138 an excellent marker for the identification of myeloma cells in the bone marrow and peripheral blood[38]. Loss of CD138 through membrane shedding, which results in an increased level of soluble CD138, is associated with poor prognosis in patients[39].
In a number of myeloma-related studies, CD138neg myeloma cells have been found in both primary malignant plasma cells derived from patients and myeloma cell lines[40,41].
According to these studies, CD138neg myeloma cells have greater clonogenic capability and are more resistant to existing therapies[42-44]. These cells have also been speculated to possess stem cell-like properties and, considering the inevitable relapse of myeloma patients after treatment, it has been hypothesised that CD138neg cells are responsible for the regrowth of myeloma tumor sites within the bone marrow[45].

The expression of CD38 is another key phenotypic characteristic of myeloma cells, to such an extent that it has been recently highlighted for therapeutic targeting using the monoclonal antibody treatment, known as daratumumab[46]. Whilst the expression of this marker is also found on a number of other cells of hematopoietic origin, it is much more highly expressed on the surface of myeloma cells. Its combination of expression with CD138 is highly specific to the myeloma surface phenotype. These two markers are often recommended to be used in the primary gating strategy during flow cytometric analysis of myeloma cells[47].
Despite these well-established markers which can be detected using flow cytometry to identify malignant plasma cells in patients, the heterogenic nature of this malignancy inherently means that the phenotype of these cells can differ between patients and alter throughout the course of treatment. Other markers also recommended for neoplastic plasma cell identification include CD45, a pan-leucocyte marker which is known to be expressed at varying levels in neoplastic plasma cells, CD19 which is lost from the surface of myeloma cells following terminal differentiation from mature B-cells[48], and CD56, an adhesion marker which is found to be expressed on malignant plasma cells in up to 80% of patients. Lack of CD56 expression is indicative of late stage disease and poor prognosis[49]. CD27, CD81, CD200, and CD117 are additional markers that can be used in MRD analysis; they have all been previously identified as markers that frequently deviate from the phenotype of normal plasma cells[50].

1.1.6. Development and progression of myeloma

Multiple myeloma is a bone marrow residing plasma cell neoplasm that develops from a pre-malignant state (MGUS) and, in some cases, eventually evolves into a symptomatic disease (Figure 5). Rarely, patients may also develop extramedullary disease and plasma cell leukemia, in which plasma cells escape the bone marrow microenvironment and invade peripheral circulation before settling in other tissues and organs like the liver and kidneys. This frequently happens in relapsed/refractory patients, and it predicts a very poor prognosis[51].

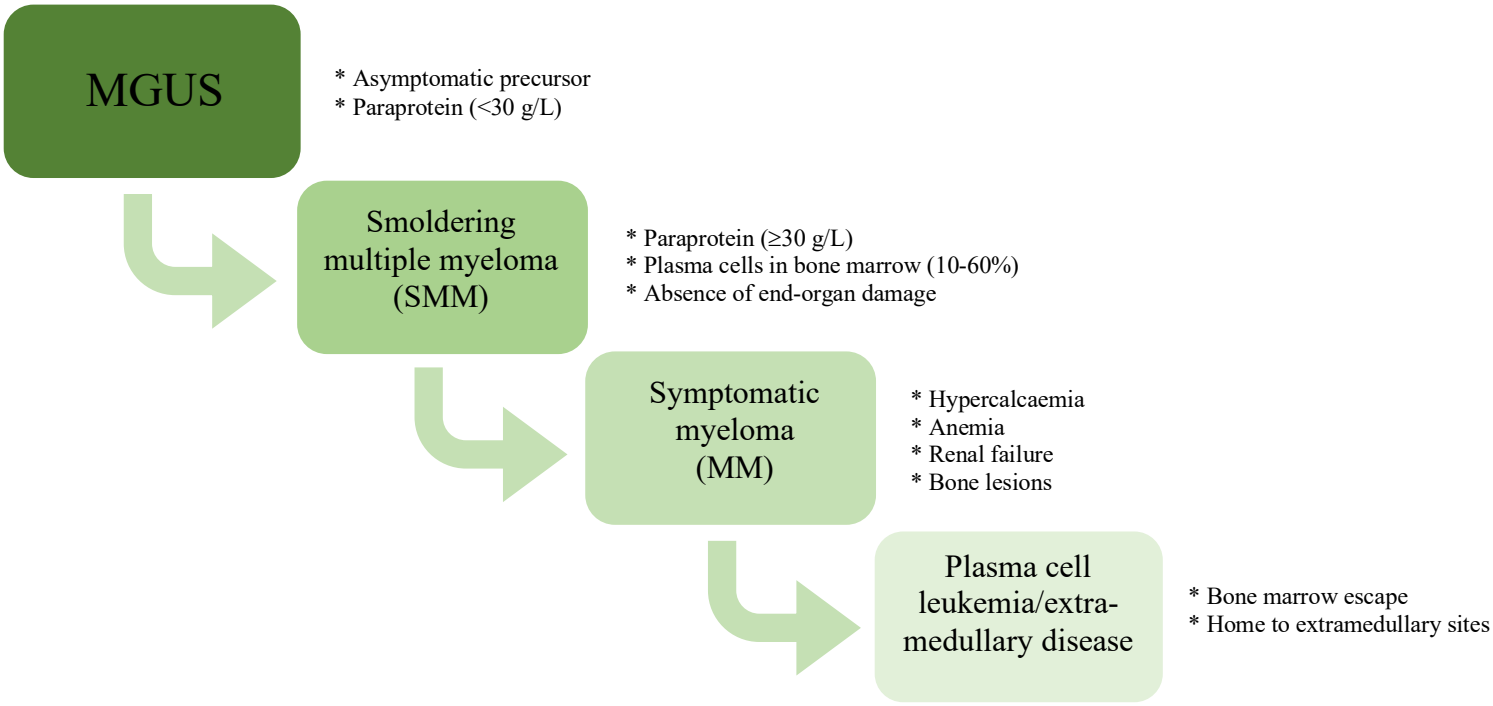


Figure 5. Stages of multiple myeloma development. The plasma cell gammopathies that play a role in the progression from MGUS to symptomatic myeloma and end-stage disease, with associated symptoms, physiological and diagnostic characteristics.

1.1.6.1. Monoclonal Gammopathy of Undetermined Significance (MGUS)

Monoclonal gammopathy of undetermined significance (MGUS) is an asymptomatic condition that represents an accumulative life-long risk of progression to symptomatic myeloma. Whilst not all cases of MGUS progress to symptomatic myeloma, it is well established that myeloma is consistently preceded by MGUS[16]. The incidence of progression from MGUS to symptomatic myeloma is 1.5% per year[52]. In the general population, MGUS is more frequent than myeloma and affects about 3.2% of people over 50 years of age[53]. It is often detected accidentally when patients present with other unrelated co-morbidities. As a result, it is reasonable to assume that current epidemiological data relating to MGUS might be biased to an extent that could suggest that a much greater proportion of patients across the country could be clinically classified as having MGUS.
MGUS is clinically diagnosed in patients demonstrating elevated levels of serum paraprotein <30 g/L, a bone marrow plasma cell proportion of <10% and an absence of end organ damage that is commonly associated with symptomatic myeloma[54]. Patients with MGUS are not recommended for treatment and are only required to be monitored for disease progression to smoldering or symptomatic myeloma. However, because MGUS is frequently diagnosed as a coincidental finding in association with other co-morbidities, determining the extent to which MGUS may be a contributing factor to such clinical presentations is difficult.
Although there is evidence that patients with MGUS have a higher risk of infections, osteoporosis, thrombosis, and other related malignancies such as myelodysplastic syndrome (MDS)[55], more research is needed.
This is rather unsurprising, considering that MGUS is defined as a clinically significant clonal proliferation of plasma cells that, while not causing end-organ damage like renal failure or anemia, can certainly contribute to increased bone fragility and thrombotic risk.

Whilst the exact cause(s) of transition from MGUS to myeloma is currently unknown, there are factors that have been taken into consideration that account for relative risk of progression. Abnormal kappa/lambda serum free light-chain ratios (normal reference: 0.26-1.65mg/L) have been shown to elude to an increased risk of progression to myeloma from MGUS[56,57]. The type and quantity of paraprotein in the blood has also been considered a risk factor, with a non-IgG subtype coupled with a paraprotein count >15g/L being linked with greater risk of progression from MGUS to myeloma[57].
According to the International Myeloma Working Group (IMWG), low-risk patients (paraprotein 15g/L, IgG subtype, normal FLC ratio (0.26-1.65)) should be monitored every 2-3 years, while intermediate to high-risk patients (paraprotein >15g/L, non-IgG subtype, abnormal FLC ratio (0.26-1.65)) should be monitored 6 months after diagnosis, followed by annual follow-up[58].

1.1.6.2. Smoldering Multiple Myeloma (SMM)

Smoldering multiple myeloma, which develops from MGUS and precedes symptomatic myeloma, is an intermediate and frequently asymptomatic disease. SMM is characterised by the detection of serum paraprotein (≥30 g/L) and an increased bone marrow plasma cell count (≥10%), but like MGUS, it does not cause end-organ damage[54]. In contrast to the rate of progression from MGUS to myeloma, the rate of progression from SMM to myeloma is time-dependent[52]. The probability of progression from SMM to myeloma is around 10% in the first 5 years following diagnosis, 3% in the next 5 years, and 1% per following year[59]. Approximately 3.2% of patients diagnosed with SMM possess a clonal plasma cell count ≥60%. Between 80-95% of patients in this category progressed to symptomatic myeloma within 2 years, according to reports, and had a significantly poorer prognosis when compared to individuals with a clonal plasma cell count <60%[60,61]. There is evidence that an abnormal serum free light-chain ratio (>100) indicates an indipendent prognostic factor that increases risk of progression to symptomatic myeloma[62,63]. Additionally, it has been demonstrated that these patients have two or more focal bone lesions, as identified through MRI scanning.
These biomarkers of elevated risk of progression from smoldering to symptomatic myeloma were added to the criteria for the diagnosis of symptomatic myeloma as a result, and these criteria are now known as the SLiM-CRAB criteria[64]. Therefore, patients with SMM who are at very high-risk of developing symptomatic myeloma are now recommended to undergo appropriate treatment[64].

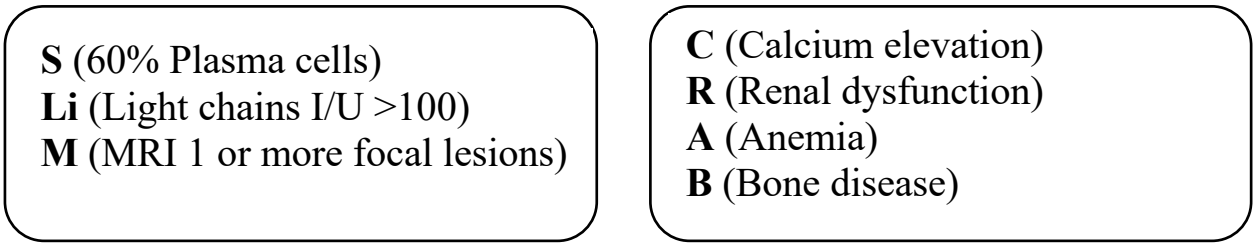


Figure 6: SLiM-CRAB criteria defining active myeloma.

1.1.6.3. Symptomatic myeloma

Diagnosis of myeloma is defined by the presence of paraprotein in the serum or urine of patients (≥30 g/L) and an elevated bone marrow clonal plasma cell count (≥10% or ≥60%). Myeloma is a clinicopathological disorder and requires evidence of end organ damage in order to fulfil a diagnosis. Hypercalcaemia, renal failure, anemia, and osteolytic bone lesion development are all symptoms of end organ damage, also known as CRAB[64]. The International Myeloma Working Group (IMWG) determined the most recent update to the diagnostic criteria for myeloma, which includes the involved:uninvolved serum-free light chain ratio ≥100 as a diagnostic factor[64]. Fluorescence in situ hybridisation (FISH) on CD138-selected bone marrow plasma cells is also recommended to identify genetic aberrations that are linked to disease prognosis.
The most common symptom of myeloma is bone pain, which affects 80% of patients[13]. This is principally caused by myeloma-induced upregulation of osteoclasts' bone-resorbing activity and their combined downregulation of osteoblasts' bone-producing activity. As a result, there is an overall increase in bone resorption, which causes patients to develop painful bone lesions. These painful bone lesions usually result in spontaneous fractures, which frequently occur at sites of red bone marrow, such as the ribs, spine, skull, and pelvis[65]. The presence of these osteolytic bone lesions are monitored by MRI scans that are able to quantify their number and distribution throughout the skeleton[64]. Hypercalcaemia, or a high calcium level in the blood, is a symptom that develops as a result of osteoclast-mediated bone resorption, which results in the excessive efflux of calcium into the serum. While it is recognized that lowering tumor burden has a direct impact on bone lesion formation and hypercalcaemia, these myeloma-related morbidities are also treated with bisphosphonates such as zoledronic acid and pamidronate, which limit osteoclast activity by inducing apoptosis in these cells[66,67].

Around 20% of myeloma patients have renal failure at diagnosis, with up to 50% of patients having decreased renal function over the course of their disease[68]. This is measured by an increase in the levels of serum creatinine (>20mg/L). Cast nephropathy is the leading cause of renal damage in myeloma patients and is attributed to 90% of these cases. The cause is excessive production of free light chains into the blood, which puts immense physiological pressure on the kidney's filtration mechanisms, resulting in nephrotic injury. Free light chains are typically filtered through the glomerulus and reabsorbed in the proximal tubules of the nephron, but in myeloma, the resorptive capacity of this mechanism is greatly exceeded, resulting in the production of protein casts in the distal tubules[69]. Through the identification of free light chains in the urine, also known as Bence Jones protein, this characteristic can be used as a diagnostic criterion for myeloma. Dialysis can be recommended for patients who come with more severe cases of renal failure in order

to restore abnormal kidney function. Decrease in tumor burden with established therapies has been attributed to reducing these symptoms[70]. Recurrent bacterial and viral infections are also common in myeloma patients, and infection is the major cause of death. After one year after diagnosis, approximately 22% of myeloma-related deaths are caused by infection[71]. Immunodeficiency is caused by a decrease in the number of $CD19^+$ B-cells, $CD4^+$ and $CD8^+$ T-cells, as well as impairments in dendritic and natural killer cell function, which leads to infections[72]. Despite considerable advancements in myeloma treatment, more intensive regimens integrating newer innovative drugs have been found to impair immune function whilst also inducing positive responses in reducing tumor burden[73].

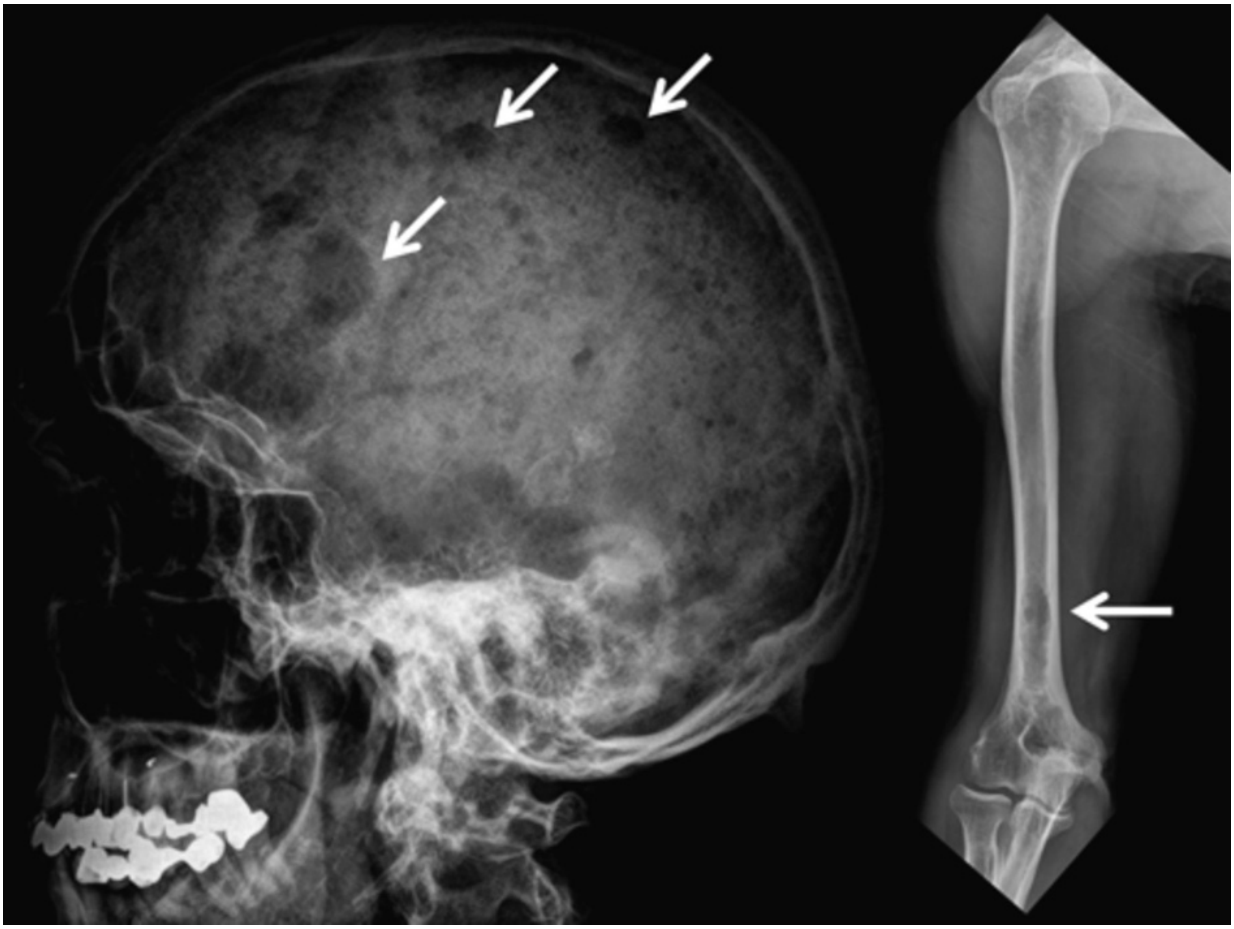


Figure 7. Osteolytic bone lesions of skull and right humerus in a patient with MM. (Ronald C. Walker et al. Imaging of multiple myeloma and related plasma cell dyscrasias. Journal of Nuclear Medicine July 2012, 53 (7) 1091-1101)

1.1.6.4. Extramedullary disease

Myeloma cells that have escaped the bone marrow, invaded the peripheral blood and manifest as plasma cell leukemia are known as extramedullary disease (EMD). Soft-tissue plasmacytomas can develop as a result of the invasion of external tissue by these circulating myeloma cells[74].

Plasma cell leukemia (PCL) is a very aggressive dyscrasia that can be classified as either primary or secondary, depending on whether it is detected at the time of diagnosis or emerges as part of end-stage leukemic transformation from multiple myeloma following relapse from treatment. Malignant progression from multiple myeloma occurs in very high-risk patients, resulting in both primary and secondary classifications, with the proportions of plasma cell leukemia patients that develop either primary or secondary disease being approximately 1:1. Plasma cell leukemia affects about 4% of myeloma patients, has an extremely poor prognosis due to a high-risk genetic signature, and is associated with short remissions, with median survival reported as low as 1.3 months[75]. Malignant plasma cells escape from the bone marrow microenvironment into peripheral circulation with a percentage proportion of ≥20% and an absolute count of $\geq 2 \times 10^9$ circulating plasma cells[76]. Secondary plasma cell leukemia occurs at the end-stage of myeloma disease and in patients who have been heavily pre-treated thus becoming refractory to treatment. Therefore, given the extremely short survival times of these patients, emphasis is generally placed on appropriate supportive care and palliative treatment[77].

Myeloma cells can invade other tissues to cause plasmacytomas on bone tissue near the primary tumor or in distant soft tissue organs after escaping from the bone marrow microenvironment.

The location of these soft-tissue secondary tumor sites can vary between patients, with the most common appearing in the skin, liver and lymph nodes[78]. Studies have shown that patients with soft tissue plasmacytomas have a significantly shorter overall survival than patients with localized bone plasmacytomas and patients without extramedullary disease (EMD), with an average lifespan of just 5 months[79].

1.1.7. Genetic aberrations

Pathogenesis and progression of all known cancers have been linked to genetic abnormalities. In myeloma, these genetic alterations are usually divided into two categories: primary events, which are thought to play a role in disease onset and subsequent progression from MGUS - and secondary events - which are thought to accumulate over the course of the disease and play a role in relapse and therapy resistance (Figure 8)[80]. Both of these subgroups have an effect on patient prognosis, with over 90% of patients having at least 1 chromosomal aberration that can be detected by FISH analysis[81,82].
Both chromosomal translocations involving the immunoglobulin heavy chain gene locus and hyperdiploidy, which is characterized by trisomies of 2 or more odd-numbered chromosomes, are primary genetic starting events. Although 10% of patients show both of these abnormalities, this percentage has been shown to overlap[80,81].
Upregulation of cyclin D proteins, which allows myeloma cells to replicate indefinitely, is the most prevalent primary translocation event associated with myeloma. This is induced by translocations like t(11;14) and t(6;14), which have an impact on the activity of cyclin D1 and D3, respectively. In almost 20% of all cases of myeloma, these are the initial pathogenesis-related events[83].
t(4;14), which increases the expression of FGFR3 and MMSET, and t(14;16), which increases c-MAF activity, are two other major translocations.
Both of these translocations, which affect cyclin D2 activity, are present in about 15% and 5% of patients, respectively[83-85].

The most frequent genetic abnormality in myeloma, which has been found in about 50% of patients, is the deletion of chromosome 13[86]. Additionally, there is proof that up to 40% of MGUS patients also have this chromosomal loss[87,88].
This suggests that this genetic event might occur early in the pathophysiology of myeloma or that it might accumulate later on as the disease progresses.
It can manifest either as a deletion – del(13q) – or through monosomy of chromosome 13[89].
Furthermore, 90% of patients with a primary t(4;14) translocation have also been shown to have a chromosome 13 abnormality, indicating that these two conditions are closely related[90,91].

Del(17p), which causes the loss of p53 expression and is present in about 11% of patients, is arguably the most unfavorable secondary genetic aberration connected to myeloma and indicates a very poor prognosis for patients[82].
Secondary genetic abnormalities include activating mutations in RAS oncogenes, which have been found in 7% of MGUS patients, 25% of symptomatic myeloma

patients, and 45% of relapsed patients and have been linked to increased tumor burden and poorer prognosis[92].
Rearrangements in the c-myc oncogene are seen in up to 15% of myeloma patients, and they have been demonstrated to increase the activity of c-myc during myeloma progression[93].
Additionally, it was found that these re-arrangements were associated with high levels of β2-microglobulin, which suggests more aggressive disease and a poorer diagnosis[94].
Myeloma is also characterized by mutations in genes encoding for NF-κB pathway components, with 17 % of patients reportedly carrying such mutations that result in constitutive activation of both canonical and non-canonical branches of this pathway[95]. NF-κB signaling is activated as a result of mutations in the genes that encode NIK, CD40, TACI, p50, and p52. Other mutations, such as those that affect the genes encoding the NF-κB regulators TRAF2/3, CYLD, and cIAP1/2, result in their inactivation. Overall however, these mutations favor the propagation of non-canonical pathways[95,96].

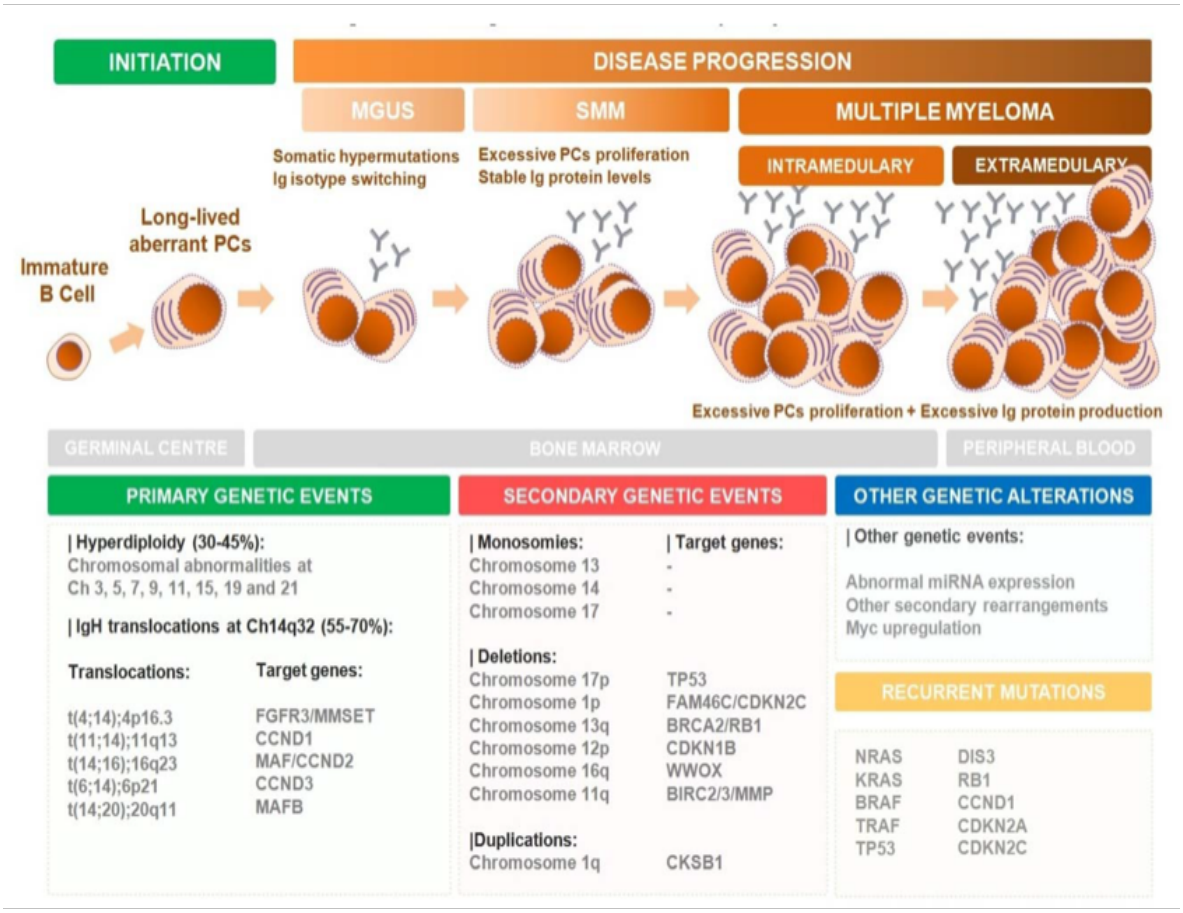


Figure 8. Genetic abnormalities associated with disease initiation and progression.
(Vanessa Pinto et al. Multiple myeloma: available therapies and causes of drug resistance. *Cancers* 2020, *12*, 407; doi:10.3390/cancers12020407)

1.1.8. Myeloma staging and prognostic factors

Although myeloma patients have a median survival of about 4.9 years after diagnosis, the disease is cytogenetically heterogeneous, which accounts for a variation of post-diagnosis survival durations. This emphasizes the need for a globally accepted staging system that incorporates a range of physiological and genetic traits to aid in patient outcome prediction. Durie and Salmon described the first such system in 1975, associating tumor burden to the presence of clinical symptoms[98].
However, in 2005 the measurement of serum albumin and β2-microglobulin led to a simplification of myeloma staging. The International Staging System (ISS) for myeloma prognosis was developed using these two different prognostic markers to identify 3 sub-groups of patient outcomes (Table 2)[99]. Although widely accepted and used, this staging method was criticized for not considering the prognostic relevance of myeloma-specific genetic abnormalities. Since high-risk myeloma was identified by del(17p) and/or translocation t(4;14) and/or t(14;16), this staging approach was modified in 2015 (R-ISS) to include LDH serum quantification and situ hybridization (FISH) analysis (Table 3). It was found that 28% of patients, 62% of patients, and 10% of patients, respectively, were staged at R-ISS I, R-ISS II, and R-ISS III[100].

ISS Stage	Prognostic Criteria (ISS)	Median OS (months)
I	Serum albumin ≥ 35g/L β_2-microglobulin <3.5g/dL	62
II	Neither ISS Stage I or III	44
III	β_2-microglobulin >5.5mg/L	29

Table 2: Prognostic criteria and overall survival of patients defined in the International Staging System for patients diagnosed with multiple myeloma.
Table adapted from data published in International Staging System for Multiple Myeloma[99].

(R-)ISS Stage	Prognostic Criteria (Revised-ISS)	PFS (months)	Median OS (months)
I	ISS Stage I Standard risk FISH Normal serum LDH	66	Not reached
II	Neither R-ISS Stage I or III	42	83
III	ISS Stage III High-risk FISH and/or high serum LDH	29	43

Table 3: Prognostic criteria and overall survival of patients defined in the Revised International Staging System for patients diagnosed with multiple myeloma.
Table adapted from data published in Revised International Staging System for Multiple Myeloma[100].

1.1.9. Multiple myeloma drugs

As standard practice, patients with myeloma are typically advised to start therapy as soon as a diagnosis is made.
With the exception of high-risk SMM patients who have a clonal bone marrow plasma cell count ≥60%, a high FLC ratio >100 (providing tumor FLC >100), or 2 or more asymptomatic lytic lesions on cross sectional imaging, patients with MGUS and SMM are monitored for disease progression but are not advised to start treatment until the diagnostic CRAB criteria indicating the presence of symptomatic multiple myeloma are met[64].
Since the late 1960s, prednisone, a glucocorticoid, and the alkylating drug melphalan have been used often in combination regimens to treat myeloma. Clinical care has made relatively little progress since then[101].
However, over the past 20 years, there have been a huge increase in the number of therapies that are available to patients, which has improved clinical outcomes[102].
These novel treatments include immunomodulatory drugs (iMIDs), proteasome inhibitors, and corticosteroids.
The following is a brief description of the main mechanisms of action for each of these classes of drug:

• Lenalidomide, a less toxic and more potent analogue of thalidomide, is one immunomodulatory drug that has a complex action but is known to work through multiple mechanisms, including immune cell modulation through T-cell activation, inhibition of pro-inflammatory cytokine secretion, and inhibition of angiogenesis. These therapies not only target myeloma cells directly, but they also influence the surrounding supportive microenvironment, inhibiting the proliferation of the cancer[103].

• The cellular degradation of ubiquitinated proteins is carried out by complex protein structures called proteasomes[104]. It is well known that malignant cells depend more on these structures to eliminate abnormal proteins, which are present in much greater abundance in comparison to normal cells. Since bortezomib was the first proteasome inhibitor to be clinically licensed for the treatment of myeloma, this has been proven by the ability of proteasome inhibitors to induce tumor-specific toxic effects[105].

• Corticosteroids such as dexamethasone is one of the long-established therapies for myeloma that has had great success in the clinic. Although it is known that these treatments inhibit the glucocorticoid receptor, which is their target receptor, there is still debate over the downstream effects of this inhibition, which cause apoptosis. However, numerous studies have linked corticosteroids such as dexamethasone to the inhibition of transcription factors including NF-κB and AP-1[106].

Patients can now receive treatment on a more customized basis to meet the heterogeneity of this condition thanks to this development in the myeloma therapeutic arsenal. Decisions made about a patient's treatment are stratified depending on tumor burden, disease stage, cytogenetics, age and treatment history, all factors that can be used as markers of a patient's ability to tolerate and respond to treatment[107].
Induction therapy, autologous stem cell transplant (if eligible), maintenance therapy, and the treatment of relapsed/refractory disease are the most important steps of myeloma treatment (Figure 9)[5].

Approximately two thirds of people with myeloma are over 65 when they are first diagnosed, making it a disease that typically affects the elderly[2]. Age is regarded to be an independent prognostic factor in myeloma. Younger patients have better survival rates and are better able to tolerate a wider variety of treatments at higher doses, including autologous stem cell transplant (ASCT)[15,108].
While younger, fitter people are more typically chosen for stem cell transplant, there is evidence that older patients can tolerate high dose treatment and ASCT and have clinical outcomes that are comparable to younger patients[109]. This emphasizes how crucial it is to evaluate a patient's general health in addition to age when evaluating treatment alternatives. Co-morbidities that influence renal, hepatic, and cardiac function are one of these, and it's also important to take into account a patient's fragility due to fatigue and low levels of physical activity.
These factors assist physicians in identifying patients who have the lowest risk of transplant-related complications and can therefore tolerate the procedure enough[110].

1.1.9.1. Current therapies

After a myeloma diagnosis, it is advised that treatment begin as soon as possible. NICE advises an induction therapy regimen to be used in newly diagnosed patients who are eligible for ASCT with the aim of lowering tumor burden while also preserving a recoverable population of $CD34^+$ hematopoietic stem cells from peripheral circulation for a subsequent transplant.
The suggested regimen for induction therapy combines bortezomib, thalidomide, and dexamethasone known as VTD[111].
The VTD triplet regimen has been demonstrated to be clinically superior than the VD doublet regimen[112,113].

Where thalidomide treatment is not practicable, evidence suggests that adding cyclophosphamide (VCD) or lenalidomide (VRD) - a 2nd generation thalidomide analogue – to the treatment is clinically preferable than VD alone[114,115]. Based on t(4;14) and del(17p) chromosomal abnormalities, the latter has been associated with a higher progression-free survival in high-risk patients[116].

For the vast majority of patients who are eligible for a stem cell transplant, an autologous procedure is used. When compared to patients who received only conventional chemotherapy, high dosage chemotherapy followed by ASCT has been demonstrated to significantly prolong median survival by up to 12 months[117,118]. In order to be transplanted at a later timepoint, stem cells are taken from the patient and cryogenically frozen prior to the administration of high dosage therapy. Patients undergo a conditioning treatment immediately prior to transplantation in order to achieve the best response rate possible. The current standard practice for this is treatment with 200mg/m^2 melphalan[119]. Transplantation can then take place soon after conditioning therapy[120].

Compared to an autologous stem cell transplant, allogeneic stem cell transplantation offers the advantage of eradicating any possible tumor cell contamination from the infused stem cells, but it is much less frequently administered.
This is because graft vs. host disease and a higher risk of infection are more frequently linked to higher levels of treatment-related mortality[121].
When comparing the success of allogeneic transplantation to autologous transplantation, there are some contradicting data. Two studies evaluated the outcomes of patients who received an auto-auto SCT against the ones of patients who received an auto-allo SCT. One study showed no significant difference between the two tandem transplants[122], whilst the other demonstrated a superior response in the auto-allo patient cohort[123]. The general consensus amongst clinicians is that allogeneic stem cell transplantation is not recommended as conventional therapy and should only be considered as a treatment option for younger patients with high-risk disease following a first or second relapse[121].

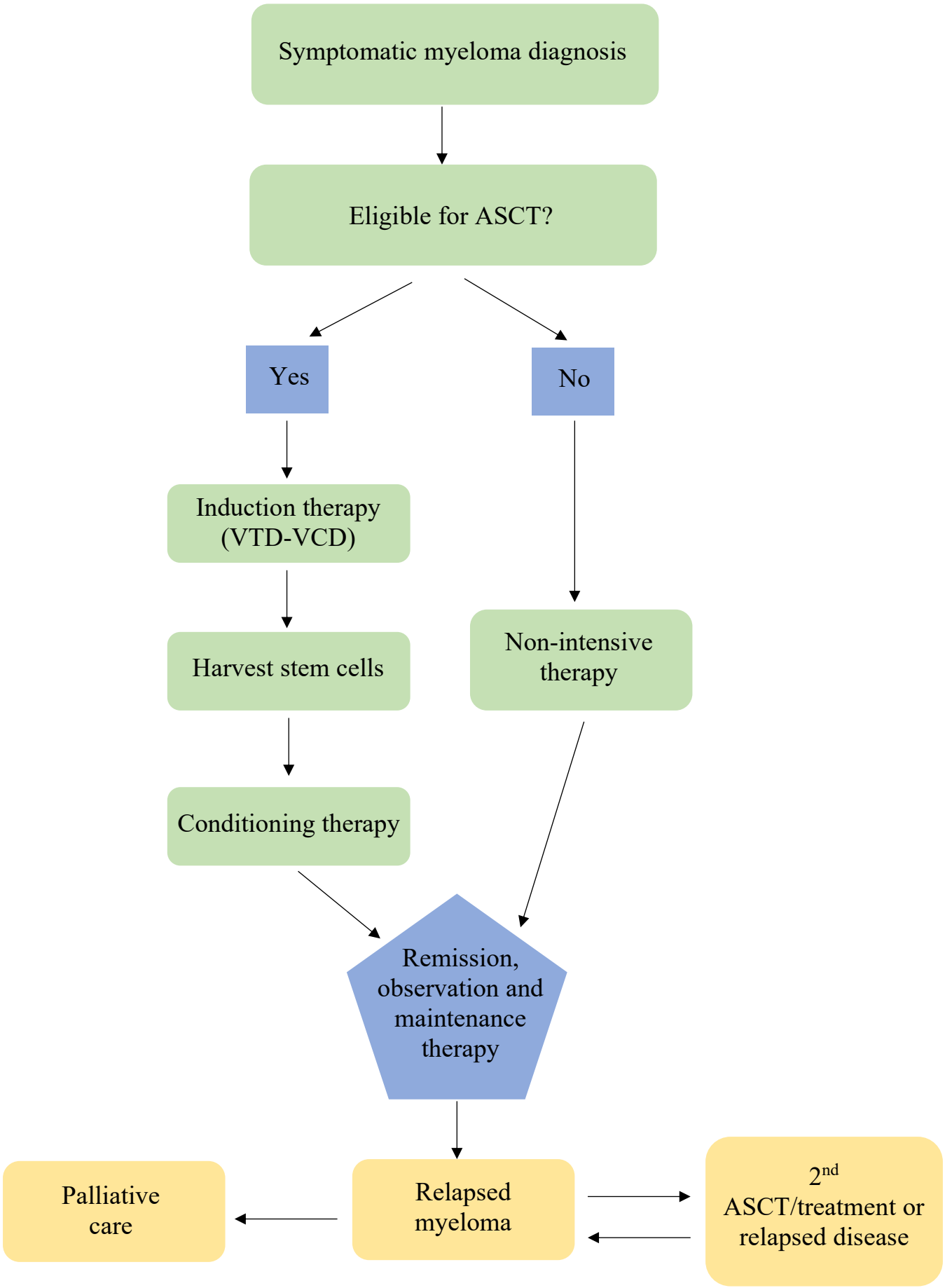


Figure 9: Treatment options for patients with multiple myeloma.

The majority of these patients are ineligible for ASCT since about two thirds of patients are older than 65. The major treatments for these transplant-ineligible individuals are lenalidomide/dexamethasone or melphalan and prednisone combination with bortezomib (VMP), with the latter showing promising outcomes in high-risk patients, similar to the responses reported in standard-risk patients[124,125]. The duration of remission is known to affect overall survival, with longer remission periods following first therapy being linked with greater survival rates[126]. However, the development of drug-resistant sub-clones causes post-treatment relapse in the vast majority of myeloma patients[127,128]. As a result, the rationale for providing patients with maintenance therapy after induction treatment is to prolong the length of remission. With several studies indicating a significant increase in PFS in lenalidomide-treated patients and one study further demonstrating a significantly improved OS, lenalidomide treatment represents a promising maintenance strategy following ASCT[129-131]. These conclusions have been supported by data from the ongoing Myeloma XI study, which also demonstrates that maintaining lenalidomide monotherapy increases PFS in both transplant-eligible and ineligible patients and is associated with better outcomes in high-risk patients, particularly those with del(17p) cytogenetics[132]. However, these studies did note an increased risk of developing secondary malignancies and a considerable number of hematological adverse events[133]. Prior to administering lenalidomide to a patient, the risks and benefits must be carefully considered, however it is generally accepted that the favorable treatment response in patients outweighs the relatively low risk of secondary malignancy development.

Bortezomib, a post-ASCT maintenance therapy that has been proven to increase PFS and OS, particularly in high-risk patients, is another option[134]. Additionally, there is evidence that bortezomib and lenalidomide were used in a triplet regimen that also included dexamethasone (RVD), which has shown promising results of PFS and OS in high-risk patients[135]. In patients who are ineligible for transplants, the use of bortezomib in maintenance therapy can also increase progression-free survival. An induction quadruplet of bortezomib, melphalan, prednisone, and thalidomide (VMPT) followed by a maintenance regimen of bortezomib and thalidomide (VT), resulted in a progression-free survival rate that was higher than only VMP alone[136].

1.1.9.2. Future treatments

Progressive or relapsed disease is defined by an increase in serum paraprotein of more than 25% and an increase in bone marrow plasma cell count to >10%. Additional signs include the discovery of new bone lesions or the development of end-organ damage[137]. Patients with myeloma who relapsed have a variety of treatment-related challenges because the disease frequently becomes resistant to the therapies they have already received. New therapies are frequently approved for relapse in myeloma, giving patients a wider range of treatment options at this stage of the disease course.

Peripheral neuropathy, nausea, vomiting, severe diarrhoea, and skin irritation are just a few of the major adverse effects that myeloma patients experience as a direct result of treatment[138]. To make these treatments more comfortable for patients, researchers must create novel medicines with increased potency and specificity targeting malignant plasma cells while reducing toxicity in non-malignant cells.

Carfilzomib and ixazomib, the next generation of proteasome inhibitors, as well as pomalidomide, a third-generation immunomodulatory drug, are now approved for treatment in relapsed disease[139-141]. Pomalidomide has been shown to produce favourable clinical responses in patients who have been previously treated with lenalidomide and low-dose dexamethasone[142,143]. In relapsed/refractory myeloma, the proteasome inhibitors carfilzomib and ixazomib have shown promising activity as single treatments and in combination regimens with lenalidomide and dexamethasone[144-146].

The aim of immunotherapy in myeloma, which has also shown encouraging effects, is to strengthen the host immune response against malignant cells. Recently, two monoclonal antibodies were authorized for the treatment of relapsed myeloma. Elotuzumab targets SLAM-F7 and has shown clinical efficacy in a triplet regimen with lenalidomide and dexamethasone after failing to produce favourable single agent activity[147]. In combination with pomalidomide and dexamethasone, it has recently enhanced clinical outcomes in patients with relapsed myeloma[148]. Daratumumab is an anti-CD38 monoclonal antibody that has demonstrated extremely promising single agent activity in relapsed patients[149,150]. It has also demonstrated promising results when combined with lenalidomide and dexamethasone, and it was recently authorized by NICE for use in combination with bortezomib and dexamethasone in relapsed patients[151-153]. Daratumumab has not yet been approved by NICE for induction therapy, however it is commonly used in standard practice and will certainly be approved for this by NICE in the near future.

Another immunotherapeutic alternative is chimeric antigen receptor (CAR) T-cell therapy, which has generated significant clinical results in patients with relapsed ALL

and CLL and a surge of interest when used to treat myeloma[154,155]. CARs are artificial receptors, independent of HLA, redirecting T-cell specificity and function toward a cell surface tumor target. This is a very potent principle that has been applied to the therapy of cancer to induce an incredibly focused immune response to tumor-specific targets[156]. To ensure that bioengineered CAR T-cells respond specifically to the tumor, it is crucial to choose the appropriate target antigens.
Myeloma research is now focusing on a number of targets, with clinical studies evaluating surface antigens such as B-cell maturation antigen (BCMA), CD19, CD38, CD138, and SLAMF7[157]. In patients with relapses after heavily treatment, CAR-BCMA T-cell infusions revealed good overall response rates of 83%, according to data from a recent phase 1 trial. The patients experienced a partial anti-myeloma response or greater achieved minimal residual disease-negative status[158].

1.2. Bone marrow microenvironment in multiple myeloma

Bone marrow tissue can be found within the majority of bones of the human skeleton. The primary functions of the bone marrow, including hematopoiesis and the development and subsequent maintenance of bones, are made possible by the complex network of cellular and non-cellular compartments that compose this microenvironment[159].
Numerous different cell types, which include the hematopoietic stem cells, stromal cells (BMSC), endothelial cells (EC), osteoblasts, and osteoclasts, compose the cellular compartment of the bone marrow microenvironment.
Myeloma cells depend on the bone marrow's cells for easier adherence and homing to this microenvironment which ensure the survival, proliferation, propagation of angiogenesis and resistance to chemotherapeutic intervention, the latter mediated both through cell-cell contact and soluble factor signalling.
Extracellular matrix (ECM) proteins and a liquid environment rich in cytokines, chemokines, and growth factors make up the bone marrow's non-cellular compartment, promoting myeloma cells adhesion to the bone marrow and survival within that milieu.
In myeloma, the involvement of the growth factor IL-6 is particularly crucial.
It is produced by myeloma cells, which in a paracrine manner also cause neighboring bone marrow cells to express it and secrete it. It is essential for the differentiation, maintenance, and proliferation of malignant plasma cells[160].
Collagen type 1 and fibronectin, two ECM proteins, bind to CD138 and CD49d, that are expressed on the surface of myeloma cells, respectively[161].
Additionally, this effect has been connected to the spread of the myeloma-specific cell adhesion-mediated drug resistance (CAM-DR) effect[162]. Myeloma patients can now be treated more thoroughly thanks to a therapeutic targeting method made possible by the fact that myeloma cells are dependent on the bone marrow microenvironment[163].

1.3. The role of PD-1/PD-L1 axis in cancer

Immune checkpoints have emerged as effective therapeutic targets in many solid malignancies (melanoma, non-small cell lung cancer, renal cell carcinoma, head and neck carcinoma) and Hodgkin's lymphoma in recent years[164,165]. Immunological checkpoints are a group of inhibitory and stimulatory molecules that are important for maintaining self-tolerance and controlling immune responses. Several stimulatory signals are involved in the activation and expansion of T cells, such as CD28/CD80, CD86, CD27/CD70, CD40/CD40L, and ICOS/ICOSL; on the other hand, we can find inhibitory pathways, such as PD-1/PD-L1 and PD-L2, CTLA-4/CD80 and CD86, A2AR/adenosine, and LAG3/Major histocompatibility complex (MHC) class II. However, tumor cells enhance the expression of checkpoint receptor ligands as an immune response escape mechanism, making checkpoint inhibition with monoclonal antibodies (mAbs) a novel cancer therapy option.

Programmed Death 1 (PD-1, CD279) is a type I transmembrane protein of 288 amino acids and a member of CD28/CTLA-4 family. It is encoded by PDCD1 gene located on 2q37.3 chromosome. PD-1 consists of an Ig-V like extracellular domain, a transmembrane domain, and a cytoplasmic domain with two tyrosine-based signaling motifs. Src family kinases phosphorylate the cytoplasmic immune-receptor tyrosine-based inhibitory motif (ITIM) and the immune-receptor tyrosine-based switch motif (ITSM). PD-1 is mostly expressed on activated /exhausted T and B cells[166,167].

Programmed Death Ligand 1 (PD-L1), also known as B7-H1 and CD274, is a 40kD Type I transmembrane glycoprotein that contains an extracellular domain that is similar to immunoglobulin (Ig)-V and Ig-C, a transmembrane domain, and a short cytoplasmic tail that lacks canonical signaling motifs[168].
PD-L1 and PD-L2 are encoded by CD274 and PDCD1LG2 genes respectively, located on chromosome 9p.24.1.
PD-L1 is expressed at low levels by myeloid cells such as macrophages and DCs, as well as vascular endothelial cells, pancreatic islet cells, and immunological privilege sites (placenta, testes, eye). After activation, the expression of PD-L2, is more restricted on DCs and macrophages[169].

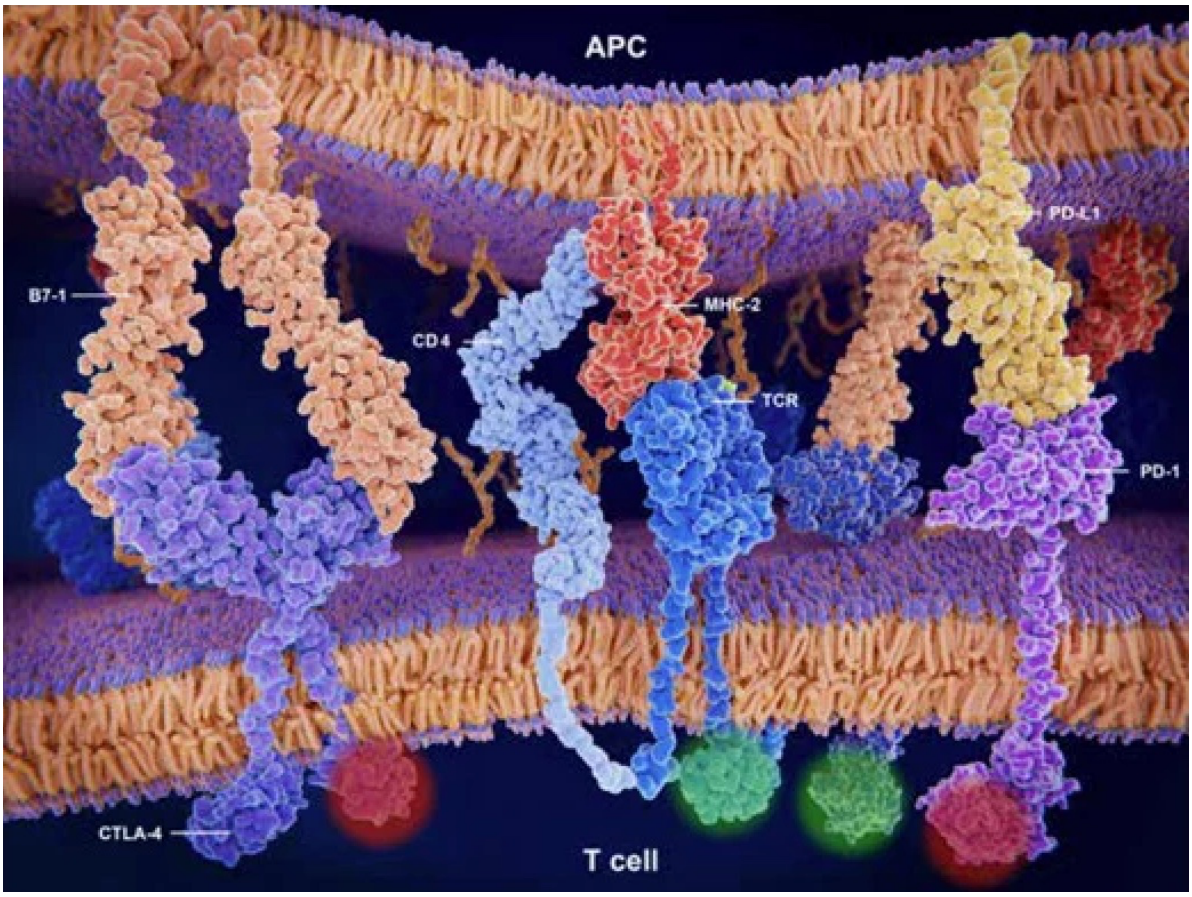


Figure 10. Interaction of PD-1 with its ligand PD-L1 between T cell and APC.

In light of its restricted protein expression and ubiquity of mRNA, several studies have focused on the control of PD-L1 expression, which appears to be mediated by both intrinsic and extrinsic processes.
The first are epigenetic and post-transcriptional alterations, such as deacetylation and microRNA regulation, which lower PD-L1 expression in tumor cells[170-172]. Several pro-inflammatory cytokines, including the most potent interferon gamma (IFN-γ), are involved in the extrinsic processes.
This factor stimulates PD-L1 expression at the post-transcriptional level and binds to two sites on interferon regulatory factor 1 in the PD-L1 promoter (200 and 320 base pairs upstream of transcriptional start site)[173].
The engagement of the PD-1 receptor with its ligands PD-L1 or PD-L2 activates PD-1 downstream of Src-homology 2-containing tyrosine phosphatase (SHP-2) and dephosphorylates ZAP70. This process inhibits T cell proliferation, survival and cytokine production, induces T-cell exhaustion, enhances Tregs development, and decreases NK cell cytotoxicity, granule exocytosis and IFN-γ secretion, through the interference with Protein kinase C (PKC)-θ, PhosphatidylInositol 3-Kinase (PI3K), extracellular signal-regulated kinase (ERK) and AKT activation[174,175].
It's worth noting that PD-L1 interacts with CD80 on T cells, limiting their proliferation[176]. According to a recent study by Bar et al., in vitro PD-L1 inhibition

promotes both monocyte-derived DC differentiation and CD40L-driven DC maturation in healthy donors[177]. It also boosts the production of inflammatory cytokines such as IL-6, IL-8, tumor necrosis factor (TNF)-α and IL-1. These effects were only seen with PD-L1 inhibition, not anti-PD-1 mAbs, implying that PD-L1 has a second role in regulating the inflammatory phenotype of myeloid cells and antigen presentation in DCs, as evidenced by in vivo results[177].
Aside from its action on effector immune cells, PD-L1 also sends an anti-apoptotic intracellular signal to cancer cells, offering resistance to T cell-mediated death without relying on PD-1-dependent T cell suppression[178].
However, nothing is known about how these emerging pro-survival signals are transmitted intracellularly from cell surface PD-L1.
In some tumor cells, this has been demonstrated to stimulate cancer initiation, epithelial to mesenchymal transition (EMT), invasion and metastasis, treatment resistance, and glucose metabolism.
A study by Chang et al. described the role of the PD-L1/PD-1 axis in metabolic competition between tumor cells and tumor-infiltrating T lymphocytes[179].
The extracellular acidification rate (ECAR), an indicator of aerobic glycolysis, was higher in the progressing tumor and inversely proportional to the metabolism of TILs isolated from that tumor in experimental data, implying that a more aggressive tumor consumes more glucose and limits its availability in the microenvironment.
TILs in the progressing tumors were also PD-1 higher, indicating that they were hyporesponsive.
Anti-PD-1 antibodies enhanced ECAR and oxygen consumption rate (OCR) in progressing-TILs to values equal to or higher than those seen in regressing-TILs, showing that the treatment improves the TILs' metabolic fitness. PD-L1 antibodies, on the other hand, enhanced aerobic glycolysis in TILs rather than OCR[179].
PD-L1 expression is also known to stimulate glycolysis and Akt/mammalian target of rapamycin (mTOR) activation in tumor cells while inhibiting this pathway in T cells. The involvement of PD-1 on activated T cells enhances endogenous lipid fatty acid oxidation rather than glycolysis or amino acid metabolism[180,181].
T cells get polarized towards a regulatory and exhausted phenotype as a result of this occurrence[29]. According to these findings, the treatment with PD-L1 inhibiting antibodies suppressed tumor development and glucose uptake in tumor cells while increasing T cell mTOR activity[179].
The hypoxia-inducible transcription factor 1(HIF-1), which also maintains fatty acid and protein synthesis to support malignant cell survival, promotes Akt/mTOR signaling activation[182].
The number of Food and Drug Administration (FDA)-approved agents that block the PD-L1/PD-1 axis is rapidly growing, with indications for treatment of a wide range of cancers, including Hodgkin lymphoma, head and neck squamous cell carcinoma (HNSCC), melanoma, and urothelial cancers, both as monotherapy and in combination with other agents.

Pembrolizumab, an anti-PD-1 antibody, was recently approved for the treatment of all solid cancers with a tumor mutational burden (TMB) of 10 mutations/megabase or above as determined by the FoundationOne CDx assay.
Cancers with a high TMB have more immunogenic neoantigens ("In 2019, the FDA approved a third oncology treatment that targets a key genetic cause of cancer rather than a specific type of tumor") and tumor neoantigen identification by host T cells is one of the key determinants of immunotherapy response[183].
However, even in tumors with relatively low mutational burden, a good response to anti-PD-1 antibody has been shown, suggesting that mutation quality is more essential than mutation quantity. Furthermore, PD-1 inhibitor sensitivity differs between inflamed and non-inflamed tumors[184], and cancer stemness and intra-tumoral heterogeneity may have a stronger impact on immune response and better predict immunotherapy results than TMB[185].

1.3.1. PD-L1/PD-1 distribution in MM microenvironment

The relevance of the PD-L1/PD-1 axis in MM is still debated among hematological malignancies.
Numerous research groups have investigated the expression profile of the PD-L1/PD-1 axis in MM; however, the use of mAbs to block this pathway is still up for debate, at least in part due to conflicting data on PD-L1/PD-1 distribution on malignant plasmacells (PCs) or immune effector cells within the BM microenvironment.
In vitro studies on MM models demonstrated that PCs express PD-L1 and, as in the other tumors, PD-L1$^+$ PCs suppress cytotoxic T cell lymphocyte (CTL) activity, contributing to immunological escape.
Studies on human myeloma cell lines (HMCLs) found that CD138$^+$PD-L1$^+$ cells have a more aggressive phenotype, with higher proliferation rates and resistance to anti-MM drugs including dexamethasone, melphalan, and bortezomib, which is mediated by the PI3K/AKT signaling pathway[186,187].
In PD-L1$^+$ myeloma cells, expression of cell cycle-related genes CCND3 and CDK6, as well as anti-apoptotic markers BCL2 and MCL1, was elevated[187].
Cross-talk between MM cells and BM stromal cells (MSCs) has been shown to increase tumor survival by inhibiting CD4$^+$ T cell activation through the PD-L1/PD-1 axis[188].
The scientists found that PD-L1 shRNA in BM MSCs significantly reversed BM MSC-mediated suppression of IFN-γ and stimulation of IL-4 and TGF-β production in CD4$^+$ T cells, correcting Th1/Th2 and Th17/Treg decrease. Furthermore, PD-L1 knockdown decreased BM MSCs' promotion of 5TGM1 cell proliferation, suggesting a function for PD-L1 in BM MSCs-induced MM growth[188]. In addition, BM MSCs cause MM cells to produce PD-L1, resulting in an aggressive phenotype[186].
Ex vivo investigations, on the other hand, reveal several inconsistencies.
PD-L1 expression is limited to PCs (assessed as CD138$^+$/CD38$^+$ cells) from MM patients and missing in HDs, according to several studies[186,189].
PD-L1 expression has also been found to be higher in MM PCs than in MGUS[186,189].
Other research groups, on the other hand, found no variations in PC PD-L1 expression between MM, MGUS, and healthy donors (HDs)[190]. SMM patients are still understudied. High PD-L1 expression on PCs was related with disease progression in patients with MGUS and asymptomatic MM, according to a study from Dhodapkar et al.[191]. PD-L1 on PCs increased from SMM diagnosis to the development of active MM after 2 years, according to a minor study on bone biopsies[192]. Lee BH et al.[193] developed a prognostic nomogram, finding that a combination of PD-L1 expression in PCs evaluated by the quantitative immunofluorescence (QIF) method and clinical parameters (age, cytogenetics, and lactate dehydrogenase) effectively predicted poor prognosis in newly diagnosed MM.
Furthermore, SMM and active MM patients have a comparable PD-L1/PD-1 BM immunological profile, according to a recent study[194].

Moreover, high PD-L1 levels in relapsed or refractory MM patients suggest that the PD-L1/PD-1 axis is implicated in the establishment of clonal resistance[186].
Paiva et al.[190] also discovered PD-L1 overexpression in patients with minimal residual disease (MRD), suggesting that residual PD-L1$^+$ myeloma cells had a higher potential to survive and evade immunosurveillance.
The majority of the research looked at PD-L1 using a standard flow panel to detect PCs (CD138$^+$/CD38$^+$ cells), with only one study focused on PC clonality with κ/λ staining[191], which found no differences between clonal and non-clonal PCs. To select and examine only PCs with abnormal phenotype, two studies used a more extensive panel containing CD45/CD19/CD56[190].
Immunohistochemistry found PD-L1 expression on PCs from patients with extra-medullary disease, together with PD-1$^+$ T cells infiltrating the extra-medullary lesions, suggesting a possible link between the PD-L1/PD-1 axis and a poor prognosis[195]. However, more studies are needed to find whether this checkpoint is involved in the onset of extra-medullary disease. Myeloid cells, such as monocytes, dendritic cells (DCs), and myeloid-derived suppressor cells (MDSCs), express PD-L1 in the MM BM microenvironment. Ray et al.[196] discovered that plasmacytoid DCs (pDCs), which play a key role in MM cell growth and survival, express PD-L1 at higher levels than MM PCs, and that blocking PD-L1/PD-1 interactions between pDC–T cells/NK cells inhibit MM proliferation.
Moreover, unlike myeloid CD141$^+$ DCs, which are positively connected with PD-L1$^+$ PCs %, PD-L1$^+$ DCs are mostly localized in the BM of MM patients, with a small fraction found in the peripheral blood. Immune dysfunction has been linked to PD-L1$^+$ DCs' reduced ability to induce T cell responses[197]. MDSCs, particularly myeloid MDSCs in newly diagnosed MM (NDMM) and granulocytic MDSCs in relapsed MM (RMM), appear to express PD-L1 at high levels, as Gorgun et al. showed[198].
In contrast, Castella et al found no differences in total PD-L1$^+$ MDSCs % between NDMM, RMM, and MM patients in remission[199].
On the other hand, data on PD-L1 expression by MDSCs in patients with asymptomatic myeloma are still limited.
Dhodapkar's group recently discovered that PD-L1 was higher in the myeloid compartment of MGUS and MM patients as compared to HDs using single-cell mass cytometry analysis of bone marrow mononuclear cells; however, no differences were observed between MGUS and MM patients[200].
A study from An et al. demonstrated PD-L1 up-regulation during in vitro osteoclastogenesis, suggesting that osteoclasts (OCs) in the myeloma microenvironment have an immune-suppressive function. OCs, in turn, stimulated PD-L1 expression in MM cell lines via an APRIL-dependent mechanism, providing OCs with further immunological inhibition[201]. In terms of PD-1 distribution, multiple studies have found that T cells from MM patients have higher PD-1 expression levels than HDs, which is followed by a loss of function on both circulating T cells and BM CD8$^+$ T and NK cells[198].
In MM patients, PD-1 expression was also linked to T cell exhaustion/senescence[202].

Paiva et al. found no variations in T and NK cell expression between MM, MGUS, and HDs, but found a significant increase in PD-1 expression on both $CD4^+$ and $CD8^+$ cells in MRD^+ and RMM patients compared to NDMM[190].
In contrast to these findings, Kwon M et al.[203] examined the % $CD8^+PD\text{-}1^+$ cells in MGUS/SMM and NDMM which displayed a higher percentage as compared with the other group. PD-1 expression has also been found on the anergic BM Vg9Vd2 T cell subset from MGUS patients, and it was found to be increased in MM after clinical remission[199]. A soluble form of PD-L1 has also been found in BM plasma of MM patients. It has been shown how PD-L1 soluble levels predict therapy responsiveness and progression-free survival (PFS) in newly diagnosed MM patients[204]; furthermore, elevated PD-L1 soluble levels in MM patients have been associated to lower overall survival (OS) rates and poorer outcomes after autologous stem cell transplantation (ASCT)[205]. There was no significant association between soluble PD-L1 levels and cytogenetic risk. However, the mechanisms that cause soluble PD-L1 are still unknown.

1.3.2. Mechanisms of PD-L1 regulation in MM

Several studies have examined the possible mechanisms that regulate PD-L1 expression in MM. IFN-γ, like other malignancies, has been shown to play a role in upregulating PD-L1 expression via IRF1[173]. IFN-γ, produced by activated Th1, macrophages, NK, and natural killer T (NKT) cells in the BM microenvironment, activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) as well as mitogen-activated protein kinase (MEK)/ERK pathways, strongly inducing PD-L1 expression[189]. Toll-like receptors (TLRs) such as TLR2, TLR4, TLR7, and TLR9, which are widely expressed in MM cells[206], also induce STAT1 activation via the MyD88/TRAF6 pathway. Inhibition of the TLR pathway adaptor proteins MyD88 and TRAF6 blocked not only PD-L1 expression caused by TLR ligands but also that mediated by IFN-γ[189].
Other mechanisms mediated by the phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/PI3K/AKT/mTOR pathway have been described in solid tumors, where loss of PTEN promoted cell proliferation, cell invasion, and a significant increase in the levels of phospho-AKT and phospho-mTOR, resulting in increased PD-L1 protein translation[170].
Through cell-to-cell interaction and the release of soluble factors, BM MSCs improve PC proliferation and survival in the myeloma microenvironment.
PD-L1 upregulation on MM cells is similarly mediated by BM MSCs[186]. In MM cells, PD-L1 was found to be downregulated after treatment with the JAK inhibitor ruxolitinib[207].
Binding between the proliferation-inducing ligand (APRIL), released by eosinophils, OCs, and myeloid cells, and B-cell maturation antigens (BCMAs) on MM cells is another method of PD-L1 up-regulation in MM.
MEK1/2 is phosphorylated as a result of this interaction, which causes PD-L1 upregulation in MM[201]. Several studies revealed the effect of different anti-MM therapies on PD-L1 expression. Immunomodulatory drugs (IMiDs), with the exception of thalidomide, have been shown to induce PD-L1 expression in IMiDs-resistant HMCLs and primary PCs from relapsed/refractory MM patients (RR-MM). The BCMA–APRIL pathway was responsible for this effect. In fact, IMiDs promote APRIL production in MM cells via Ikaros degradation, which is known to up-regulate PD-L1 expression[208].
In contrast, an in vitro study on primary cells from RR-MM treated with lenalidomide found that the drug reduced PD-L1 surface expression on malignant PCs and more significantly on monocytes/macrophages or myeloid MDSCs.
Furthermore, in vitro treatment with lenalidomide and pomalidomide significantly reduced PD-1 surface expression on $CD4^+$ and $CD8^+$ T cells, as well as NK cells[198].
Bortezomib, carfilzomib, and ixazomib, proteasome inhibitors, also impact PD-L1 levels in MM by up-regulating it. Moreover, Ray et al. found that treatment with histone deacetylase inhibitors (HDACis) increased PD-L1 expression in MM cells[209].
Finally, Stocker et al. demonstrated that when monocytes, myeloid cells, and pDCs

are treated with bortezomib-thalidomide-dexamethasone (VTD), PD-L1 expression increases; however, daratumumab blocks this effect[210]. All of these findings together give a rationale for therapeutic combinations to improve PD-L1/PD-1 blockade clinical activity in MM.
However, the FDA has put on hold clinical trials in MM using anti-PD-1/PD-L1 mAbs and IMiDs due to significant adverse effects, whereas HDACi combinations are only available for patients with advanced melanoma (NCT02935790 and NCT02032810).
In MM patients, a phase I clinical trial (MK-3475-023/KEYNOTE-023) investigated the effect of pembrolizumab in combination with standard treatment, including lenalidomide and carfilzomib.
However, no findings from combining pembrolizumab with the proteasome inhibitor are currently available.

1.3.3. The immune suppressive role of PD-L1/PD-1 axis in MM microenvironment: preclinical and clinical evidence

In MM, preclinical studies on PD-L1/PD-1 inhibition showed promising results.
In vitro, PD-L1/PD-1 blockade inhibited BM MSC-mediated MM development and increased anti–MM responses in NK and T cells[198].
When compared to PD-L1–negative myeloma cells, PD-L1–expressing MM cells can block the function of CTLs, gaining a proliferative advantage that leads to immune evasion and resistance to anti-myeloma treatments[186].
Furthermore, after treatment with PD-L1/PD-1 blocking mAbs, the ability of PD-$L1^+$ pDCs to generate cytotoxic activity of T cells and NK cells against MM PCs was restored[196]. PD-L1 blockage increased animals' life after autologous (syngeneic) stem-cell transplantation combined with the administration of a cell-based vaccination or after irradiation in in vivo investigations on the 5T33 murine MM models[210].
By acting primarily on $CD4^+$ or $CD8^+$ T cells, PD-1 inhibition also improved survival in disseminated myeloma-bearing mice[210].
In these models, PD-1 expression on both $CD8^+$ and $CD4^+$ T cells was higher in mice with advanced MM compared to non-tumor bearing mice; additionally, a correlation was discovered between the tumor burden and the percentages of PD-1^+ T cells that were defective for the production of pro-inflammatory cytokines (IFN- and IL-2) after in vitro stimulation. In addition, these cells showed higher levels of exhausted T cell marker, TIM-3[211].
Gorgun et al. demonstrated that lenalidomide therapy increases the cytotoxic effects of PD-L1/PD-1 inhibition in RR-MM[198].
Overall, these studies suggested that PD-L1/PD-1 inhibition, alone or in combination with other anti-MM therapeutic strategies, could be an effective treatment for MM.
Several phase III trials using pembrolizumab alone or in combination with IMiDs were designed and achieved a 44 % or 60 % overall response rate (ORR) in RR-MM patients, respectively.
However, the FDA put them on hold in 2017 due to a higher rate of adverse events such as neutropenic sepsis, myocarditis, and Stevens-Johnson syndrome, which could be linked to an excessive autoimmune reaction[212].
Nonetheless, results from the KEYNOTE 183 (pomalidomide + dexamethasone + pembrolizumab) and KEYNOTE 185 (lenalidomide + dexamethasone + pembrolizumab) suggested that anti-PD-1 mAbs are more effective than NDMM in patients with immune system activation; however, it is still unclear which combination, dose, and regimen is best to avoid toxicity while increasing the anti-tumor effect of this class of treatments.

1.3.4. CD38 and its role in MM microenvironment

The growing relevance of CD38 in the biology of MM and as a therapeutic target has been recognized in recent studies. CD38 is a 45-kDa type II transmembrane glycoprotein that works as a receptor and an ectoenzyme[213].
CD38 is overexpressed in myeloma PCs and activates T and NK cells. CD38 is implicated in T cell proliferation, B cell differentiation, and neutrophil chemotaxis[213]. In addition, IFN- γ stimulates monocyte CD38 expression and plays a role in their activation and adhesion pathways[214].
A study showed that CD38 is expressed on the surface of early OC progenitors, but it is lost during in vitro progression toward an osteoclastogenic phenotype[215]. Moreover, in vitro experiments showed that the completely humanized anti-CD38 mAb daratumumab (DARA) reduces OC production and activity in MM patients, confirming the role of CD38 in bone remodeling[215].
Through IFN- γ signaling, CD38 also controls the migration, survival, and Th-1 polarizing capacity of mature monocyte-derived dendritic cells[216].
CD38 also interacts with the non-substrate ligand CD31, which is expressed by endothelial cells. CD38 and CD31 co-expression was also seen in MM cells, but not in PC leukemia[217]. A recent study reported that CD38 expression can be lost in extramedullary MM cells[218]. It's unclear if this effect is related to a drug-induced alteration in the microenvironment or to the selective survival and proliferation of an antigen negative subpopulation.
CD38 is an ectoenzyme that plays a role in the extracellular conversion of Nicotinamide adenine dinucleotide $(NAD)^+$ to calcium signaling regulators such the immuno-suppressive factor adenosine (ADO)[219].
This effect is mediated through the alternative axis that includes other ectoenzymes such as CD73 and CD203a, bypassing the conventional pathway mediated by CD39, and it is dependent from the pH status[220]. According to literature data, MM patients have higher BM plasma levels of ADO than asymptomatic monoclonal gammopathies such as MGUS and SMM; additionally, ADO levels correlated with International Staging System (ISS) staging in patients with active disease, indicating that ADO is produced in the MM niche by an ectoenzymatic CD38 network and partially identifying the source of ADO generation in the MM microenvironment[221].
In vitro research showed that interactions between MM PCs and other BM niche cells, including as OCs, osteoblasts (OBs), and stromal cells, result in the production of ADO.
ADO was not observed in isolated BM microenvironment cells, implying that MM cells play a role in this pathway[181]. A study has recently explored the expression and function of ectoenzymes on microvesicles (MVs) isolated from BM plasma samples from MM patients. When MVs produced from MM patients were compared to MGUS and SMM, the percentage of MVs expressing high levels of ectoenzymes had increased. The MV immunophenotype of MM patients revealed high levels of expression of CD38, CD39, CD73, and CD203a ectoenzymes, which were also seen

in $CD138^+$ PCs. Finally, they demonstrated that MVs from MM patients had higher ATP, NAD^+, ADPR, and AMP to ADO catabolism than controls. This suggests that, in comparison to MGUS and SMM, the ectoenzymes expressed by MVs isolated from BM samples of MM patients were functionally active and involved in increased ADO production. ADO release is additionally promoted by the hypoxic and acidic conditions of MM BM niche. Because aerobic glycolysis is the primary source of cell energy, hypoxia stimulates the Warburg effect. The subsequent increase of lactic acid activates ectonucleotidases, which decrease ATP and enhance NAD^+, the substrate of the non-canonical CD38/CD203a/CD73 pathway for ADO synthesis. The anergic immune state that emerges from this accumulation in the BM niche promotes tumor survival. ADO's immune suppressive role has been thoroughly investigated. By acting on A2b signaling, one of the particular G protein-coupled receptors, ADO limits DCs ability to stimulate and enhance Th1 immune responses in favor of a pro-angiogenic and tolerogenic Th2[222].

Furthermore, combining ADO with the other receptor A2a on T cells reduces T cell growth and release of many components, as well as causing T cell anergy.

In activated T cells, A2a activation also inhibits the mitogen-activated protein kinase (MAPK) pathway, causing them to polarize toward a $LAG3^+$ regulatory phenotype[223].

In vitro experiments on murine models indicated that A2a signaling can increase PD-1 expression on both effector and regulatory T cells, confirming ADO's immune suppressive role[224].

In light of these findings, new therapeutic strategies targeting ADO-mediated immunosuppression via CD73 and A2a receptor were developed and have entered in phase I clinical trials in different solid tumors, including non-small-cell lung carcinoma (NSCLC), melanoma, and renal cell carcinoma[225].

1.3.5. The possible link between CD38 and PD-L1 in MM

Resistance to anti-PD-1/PD-L1 antibodies is mediated by the up-regulation of CD38 caused by the production of both all-trans retinoid acid and IFN-β in preclinical models of several solid tumors, according to a recent study[226].
CD38 expression by cancer cells is thought to promote immunological suppression via ADO synthesis and its effect on $CD8^+$ cytotoxic T cells, according to the authors. Indeed, it has previously been demonstrated that ADO inhibits $CD8^+$ T-cell activity by interacting with ADO receptors ADORA2a and ADORA2b[227].
CD38-expressing tumor cells impair $CD8^+$ T-cell function and proliferation, according to mouse studies; however, treatment with ADO receptor antagonists effectively reversed the CD38 suppressive effect on tumor infiltrating $CD8^+$ cells, indicating that CD38-mediated ADO production inhibits $CD8^+$ T-cell proliferation via adenosine receptor signaling. These preclinical findings were validated in human lung and melanoma cancer specimens, which revealed a strong link between CD38 expression and the presence of a cytolytic T cell tumor infiltration[226].
In addition, Ng HH et al. discovered that CD38 expression on immune cells, particularly macrophages, predicts sensitivity to PD-1/PD-L1 blocking therapy in hepatocellular cancer patients[228].
Moreover, suppressing ADO production or signaling via CD73 or A2AR improved tumor sensitivity to anti–PD-1 treatments. ADO, on the other hand, increases PD-1 levels in $CD8^+$ T cells[229]. All of these evidences indicate the existence of a vicious loop in tumors between CD38/ADO and the PD-1/PD-L1 axis; however, this approach has not been well investigated in MM patients. In MM patients, treatment with anti-CD38 mAb daratumumab (DARA) has been shown to reduce the increase in PD-L1 expression on antigen-presenting cells caused by conventional treatment without DARA[230].
Furthermore, it has been demonstrated that MM cells enhance PD-1 expression by NK and PD-L1 expression by monocytes, and that the PD-1/PD-L1 axis suppresses the antibody-dependent cellular cytotoxicity (ADCC) mediated by the anti-CD38 mAb isatuximab. Isatuximab combined with anti-PD-L1 or anti-PD-1 antibodies consistently improved the killing of MM cells[231]. Isatuximab, but not DARA, inhibits CD38 enzymatic activity, reducing ADO synthesis[232]. Isatuximab-mediated ADO reduction and PD-1/PD-L1 inhibition could thus help to reverse immunological suppression in MM patients. BM MSCs, which have immune suppressive activities in the MM microenvironment, are involved in ADO release via the CD31/CD73/CD203a pathway expressed on their surface, as well as in promoting MM cell proliferation and T cell inactivation via the PD-L1/PD-1 axis[186].
Blocking both CD38 and PD-L1 could thereby reverse the effects of BM MSCs and prevent myeloma growth. Surprisingly, Verkleij et al. found that long-term therapy with anti-mouse PD-1 mAb significantly increased anti-mouse CD38 ADCC in vivo in the murine $CD38^+$ myeloma model J558 and other $CD38^+$ cancers[233].

Overall, these findings support the rationale for combining anti-CD38 and anti-PD-1/PD-L1 blocking antibodies to increase anti-tumor activity in both solid tumors and MM. In RR-MM patients, phase I–II trials with DARA and anti-PD-1 mAbs pembrolizumab[234], nivolumab (NCT03184194, NCT01592370) or anti-PD-L1, durvalumab (FUSION-MM-005) and atezolizumab are currently in progress (NCT02431208). In contrast to prior trials utilizing DARA in monotherapy, which showed CMV and herpes zoster reactivation due to NK cell depletion[235], preliminary data from FUSION-MM-005 revealed a low rate of viral reactivation (1 out of 18 patients). These findings suggest that combining anti-PD-L1/PD-1 mAbs with CD38 blocking Abs may result in less infection-related damage than combining IMiDs; however, more data from ongoing clinical trials will be needed to confirm this.

AIM OF THE PROJECT

Current research aims to identify biomarkers that can predict which patients will benefit from PD-1 pathway interference, with the aim to effectively guide treatment selection and improve patient outcome.

PD-L1 expression is heterogenous among cancer and immune cells, and is highly dynamic depending on signals from the tumor microenvironment. Because patients with PD-L1 negative tumors have been shown to respond to anti-PD-1 therapy. Identifying tumor growth-inhibitory effects of antibody-mediated PD-1 blockade at the level of the myeloma cell might help refine PD-1-targeted therapies to further improve outcome in patients.

MATERIALS AND METHODS

2.1. Flow cytometry

According to the literature review, CDs are frequently employed to identify populations that share similar phenotypical characteristics and to determine the functional status of a cell. The expression of receptors on the surface of granulocytes has been linked to the granulocytic functions examined in the literature study. When membrane-bound receptors are activated, they may undergo up- or down-regulation, and since some receptors are found on granule surfaces, their mobility projects the receptors to the cell membrane. The receptors can then be measured using flow cytometry and surface staining.

Flow cytometry is a highly complex technique that allows simultaneous measurement of various physical characteristics of particles (cells) from heterogeneous populations, as they are interrogated by laser beams through a fluid stream. By combining fluidic, optic and electronic systems, flow cytometry provides information on each particle's size, internal complexity and relative fluorescence intensity. To be evaluated by the optical system, each cell must be sorted into a single file by the fluidics system. It does this by examining the dynamical characteristics of coaxial laminar flow and the physical principles of flow systems as described by Reynolds (1883).

In a nutshell, the sample is injected through a central channel that is surrounded by an outer sheath that contains fluid moving at a higher velocity, causing a drag effect (hydrodynamic focusing) on the central channel.

As the center tube narrows, a parabolic profile of flow velocities is generated, positioning each particle in the flow cell at the observation area at the optimal velocity. The lighting (laser), light collection, and detection systems make up a flow cytometer's optical system.
Light is deflected, scattered, emitted, or absorbed as the laser beam's path is interrupted by the flow of the cell.

The extent of forward scatter (FSC) determines the size of the cell, whereas the side scatter (SSC) is proportional to the nuclear structure, cytosolic complexity, and granularity of the cell. The measurement is primarily made from diffracted light detected in the axis of the laser beam (by a photodiode) or from light reflected and refracted at 90° to the laser beam (by photomultiplier tubes - PMT), respectively, making the detection of these properties independent of fluorescence.
When combined, the data from FSC and SSC offer sufficient details to distinguish between several cell types in a heterogeneous cell population.

The ability to couple light-excitable molecules (i.e. fluorophores) to a desired specific target, frequently through receptor-ligand qualities, allows for the detection of additional characteristics in a particle.
Fluorophores are excited to a higher, but very unstable, energy state following the laser interrogation.

The excited molecule loses the newly gained energy by emitting light at a greater wavelength than the one that excited it since it is unable to tolerate such excitation. These wavelengths are detected by fluorescence-exclusive detecting PMTs, which guarantee specificity through filters that allow passage of certain wavelengths while blocking others.

Scientists have developed single and tandem fluorochrome dyes, which are frequently combined with antibodies, to increase the number of properties that may be analyzed simultaneously. Tandem dye antibodies are composed of two covalently linked conjugated dyes with similar absorption spectra (30-50 nm).
A nearby dye receives the emission from an excited dye, which causes it to produce its fluorescent signal at a higher wavelength. Multicolour immunofluorescent staining is essential in identifying mixed cell populations or characterizing multiple parameters in single cells by flow cytometry or immunofluorescence microscopy. Tandem dyes expand the possibilities for fluorescence colour selection of antibodies for use in multicolour flow cytometry. These tandem dyes can provide a much higher wavelength fluorescence emission relative to the excitation wavelength and thus allow for multiple distinct emission ranges from a single laser. The electrical system subsequently processes the gathered emissions by translating the voltage produced into digital values.

Flow cytometers identify the detector (channel) numbers using a series of voltage amplifiers and analogue to digital converters, and then plot the values on a linear or logarithmic scale. Events of interest may be gated to enable more research on this particular subpopulation. This enables the simultaneous investigation of several parameters and subsets present in a heterogeneous solution (such as blood) and aids in improving the definition of subpopulations that are not well defined.

Flow cytometry is frequently employed in immunological research because to its capability to quickly analyze various parameters in each individual cell as well as to detect and sort different cell populations.
Early cytometers, which were created in the late 1960s, monitored three parameters: one fluorescent signal and two scatter light signals, FSC and SSC.
Modern flow cytometers can now measure more than 12 fluorescent signals thanks to technical advancements.

This study used a Navios EX Flow Cytometer (Beckman Coulter Life Sciences, Marseille, France) and a multi-colored panel to distinguish between various leucocyte populations and measure the expression of PD-1/PD-L1.

The Navios EX Flow Cytometer used contains three spatially separated lasers: (1) a violet laser that emits at a wavelenght of 405 nm; (2) a blue laser that emits light at a wavelength of 488 nm; and (3) a red laser that emits light at a wavelength of 638 nm. For this study, based on the capabilities of the flow cytometer, the fluorochromes used were as follows:

Fluorochrome	**Excitation (nm)**	**Emission (nm)**
Fluorescein isothiocyanate (FITC)	495	520
R-Phycoerythrin (PE)	480	575
R-Phycoerythrin-Texas Red-X (ECD)	480	620
R-Phycoerythrin-Cyanine 5.5 (PC5.5)	480	694
R-Phycoerythrin-Cyanine 7 (PC7)	480	767
Allophycocyanin (APC)	650	660
Allophycocyanin Alexa Fluor 700 (APC-A700)	650	719
Allophycocyanin Alexa Fluor 750 (APC-A750)	650	780
Pacific Blue (PB)	405	455
Krome Orange (KrO)	398	528

Table 4. Fluorochromes – excitation and emission wavelengths

2.1.1. Protocol analysis

Monoclonal antibodies that target particular CDs were used to identify immune cell groups utilizing CD antigens. Each CD was carefully selected to identify a particular cell population (table 5).

	Gene identity	Function and relation to plasma cell phenotype
CD3	T-cell surface glycoprotein CD3 delta chain	Aberrant expression of CD3 is extremely rare in plasma cell neoplasm (PCN), and only a few cases have been reported[237].
CD4	T-cell surface glycoprotein CD4	The frequency of cytotoxic CD4 T cells was negatively linked with the frequency of circulating plasma cells in MM patients. CD4 T cell-mediated cytotoxicity was present in MM patients naturally and might be used in antitumor therapies[238].
CD8	T-cell surface glycoprotein CD8 alpha chain	$CD8^+$ effector T cells, especially in the bone marrow, of myeloma patients are increased but cells are functionally severely impaired and display several features of exhaustion and senescence[202].
CD19	B-lymphocyte antigen CD19	Expressed by B-lymphocytes and is lost as a result of terminal differentiation to plasma cells. Evidence has reported $CD19^+$ cells being representative of cancer stem-like cells[236], with CAR T-cell therapy also being developed against CD19 to be used in treatment[239].
CD27	CD27L receptor	Highly expressed in MGUS and heterogeneous expression and low intensity in MM. Lack of CD27 is associated with shorter PFS and OS[240].
CD38	Cyclic ADP ribose hydrolase	Surface receptor that is uniformly expressed on the surface of plasma cells and is used extensively in the identification of myeloma cells alongside CD138[47]. Novel immunotherapies have been designed to target this antigen in the treatment of myeloma, namely daratumumab[46].
CD45	Protein tyrosine phosphatase, receptor type, C	Marker related to adhesive function that is variably expressed on plasma cells. Increased expression is more commonly associated with plasma cells at an earlier stage of differentiation, an observation which is also replicated with disease stage[241].
CD56	Neural cell adhesion molecule	Adhesion marker used to distinguish identity of malignant plasma cells from normal plasma cells[242]. Loss of expression could also be linked with late stage disease, with CD56 expression being found to be

		inversely correlated with the number of myeloma cells in peripheral circulation[49].
CD81	Target of the Antiproliferative Antibody 1 (TAPA-1)	Associated with CD19 expression, CD81 possesses important roles in cell growth, motility and plasma cell homing. Whilst little is currently known about its role in myeloma pathology, there have been studies that have implicated CD81 expression in disease prognosis[243].
CD117	Mast/stem cell growth factor receptor	Growth factor receptor found to be expressed on a proportion of malignant plasma cells, which surprisingly infers good prognosis in patients and is used as a marker to distinguish between normal and malignant plasma cells[244].
CD138	Syndecan-1	Extracellular surface glycoprotein with adhesion-related roles, almost exclusively expressed on plasma cells and is used as the primary marker in the identification of malignant plasma cells. Correlates with loss of CD19 during B-lymphocyte terminal differentiation[35].
CD200	OX-2 membrane glycoprotein	Transmembrane receptor that has been highlighted as an independent prognostic marker in myeloma, where increased expression resulted in lower event-free survival rates[245].

Table 5. Summary of the identity and function of surface markers used in immunophenotyping of myeloma cells.

All samples came from patients of UOC Hematology of A. O. Ospedali Riuniti Marche Nord, Pesaro, who underwent immunophenotypic analysis as periodic checks. Negative control samples were obtained using patients who revealed negative for hematological disease. Bone marrow aspirate was collected into vacutainers (BD, Heidelberg, Germany) containing EDTA for anticoagulation.

Premixed, dry reagent cocktail (DuraClone RE PC antibody panel) as well as CD117 ECD antibody used for the assessment of residual abnormal plasma cells and T cells were obtained from Beckman Coulter (Marseille, France). To determine the number of leucocytes was used a hematology analyzer (DxH 900, Beckman Coulter).

DuraClone RE PC tubes contained dried antibodies and 5 μL of CD117 ECD antibody dosed to stain up to 20×10^3 leukocytes in 100 μL volume of bone marrow aspirate: CD81 FITC; CD27 PE; CD19 PC5.5; CD200 PC7; CD138 APC; CD56 APC-A750; CD38 PB; CD45 KrO.

A second tube was prepared with 5 µL of each antibody: CD62L FITC; CD279 PE; CD27 ECD; CD4 PC7; CD274 APC; CD8 APC-A700; CD3 APC-A750; CD45 RA-PB; CD45 KrO. 100 µL of bone marrow aspirate was added to the tube.

Both tubes were incubated at room temperature for 15 minutes.

Then, at room temperature for 15 minutes, erythrocytes were lysed with 2000 µL of VersaLyse solution (Beckman Coulter). After being centrifuged at 300 x g, cell suspensions were then resuspended in 2000 µL of phosphate buffered saline (PBS, Beckman Coulter) + fetal bovine serum (FCS, Euroclone); for two times.
The resulting pellet was resuspended in 500 µL PBS + FCS.

All these steps can be done automatically through the cell washer HT4150L (hta, Brescia, Italy).

Tube	CD Marker	Clone	Fluorophore
T1	CD81	JS64	FITC
	CD27	1A4CD27	PE
	CD19	J3-119	PC5.5
	CD200	OX-104	PC7
	CD138	B-A38	APC
	CD56	N901	APC-A750
	CD38	LS198-4-3	PB
	CD45	J33	KrO
	CD117	104D2D1	ECD
T2	CD62L	LECAM-1	FITC
	CD279	PD1.3	PE
	CD27	1A4CD27	ECD
	CD4	SFCI12T4D11	PC7
	CD274	PD-L1	APC
	CD8	B9.11	APC-A700
	CD3	UCHT1	APC-A750
	CD45	2H4LDH11LDB9	RA-PB
	CD45	J33	KrO

Table 6. Antibody panels used in 10 color multi-parameter flow cytometry protocol.

Using preset parameters, samples were acquired using 9-color, 3-laser NAVIOS flow cytometer (Beckman Coulter). By properly adjusting the FSC recording trigger, debris was excluded. Using just one CD117 staining and the eight DuraClone RE PC compensation tubes, the acquisition parameters were established in accordance with the manufacturer's instructions.

Target channels for all scatter and fluorescence detectors were defined using acquired photomultiplier tube (PMT) voltages and calibration bead particles (Flow-Set Pro beads, Beckman Coulter). To avoid target mismatch, matching of the target channels was checked every day using a new calibration run. Furthermore, using additional calibration bead particles, all instruments underwent daily verification of optical alignment and fluidics (Flow Check beads, Beckman Coulter).
The Navios^{TM} EX Cytometer software, version 2.2, was used to examine all data files that were acquired (Beckman Coulter).
Cell doublets were eliminated by choosing either the events with the greatest FSC peak signals or the events with the shortest FSC signal breadth.
Furthermore, a forward scatter time versus side scatter dot plot was used to remove cell debris from the data.

Plasma cells were characterized as events with a high density of CD138 and CD38 expression. Patients' abnormal phenotypes varied and were identified by a combination of the following characteristics: increased expression of CD56, asynchronous expression of CD117, and CD200, and decreased expression of CD19, CD27, CD38, CD45, and/or CD81.

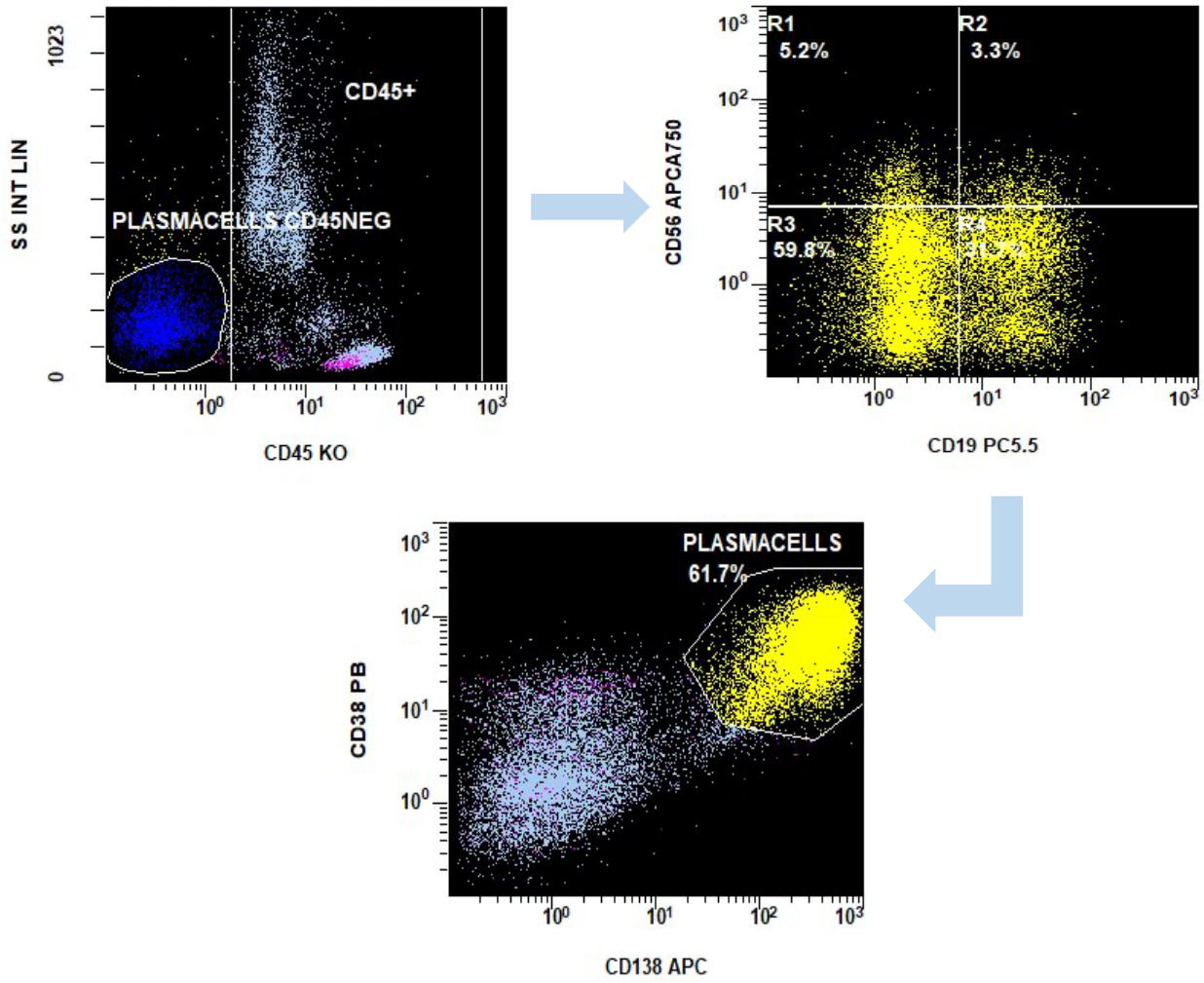


Figure 11. Gating strategy to identifying and defining plasma cells ($CD56^{\pm}/CD19^{-}/CD138^{+}/CD45^{-}$).

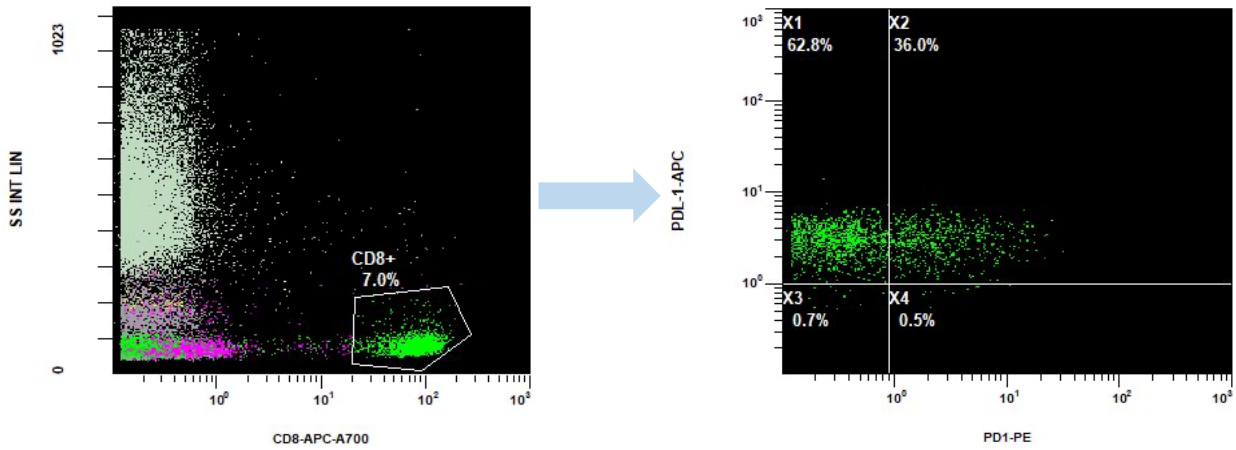


Figure 12. Expression of PD-1 and PD-L1 in $CD8^{+}$ cells.

RESULTS

A total of 14 diagnostic hematological patient samples were analysed using MFC panels encompassing 15 surface markers associated with MM (Table 6). The patients' median age was 70 years (range 51–84). The sex ratio (M/F) was 1.3. Cytogenetic test results were available for all patients and there were 5 patients with poor prognosis. Patient characteristics are listed in Table 7.

Age	Sex	Hb	ESR	LDH	Ca	B2M	Ig	FLC	Bence-Jones	% PC	cytogenetics mutation
67	M	9.3	40	109	8.90	3.74	IgG	lambda	+	8	
76	F	12.1	116	167	9.20	2.59	IgA	kappa	+	62	
76	M	13.5	6	571	9.50	11.69	IgG	lambda	+	3	
70	M	8.6	37	161	8.60	5.18	IgG	kappa	+	0.1	
77	M	15.4	14	467	9.40	3.30	IgG	kappa	-	10	
84	M	12.7	57	188	15.7	9.98	IgG	lambda	+	13	
51	F	10.1	43	146	9.50	2.76	IgG	kappa	+	17	
69	M	12.3	32	164	10.70	8.00	IgG	kappa	+	22	t(4;14)
74	F	10.3	70	121	8.70	2.64	IgG	kappa	+	28	
58	F	7.4	48	195	11.70	4.64	IgG	kappa	-	39	1q21
76	M	14.7	23	185	9.60	1.95	IgG	kappa	+	12	
73	F	12.4	12	145	9.40	3.00	IgG	kappa	-	5	t(4;14)
69	F	7.4	55	119	9.70	16.37	IgG	lambda	+	15	1q21
66	M	9.1	116	496	8.69	8.10	IgG	lambda	+	70	1q21

Table 7. Patients' characteristics (Hb: hemoglobin, ESR: erythrocyte sedimentation rate, LDH: lactate dehydrogenase, Ca: calcium, B2M: beta-2-microglobulin, Ig: immunoglobulins, FLC: serum free light chain, PC: plasma cells).

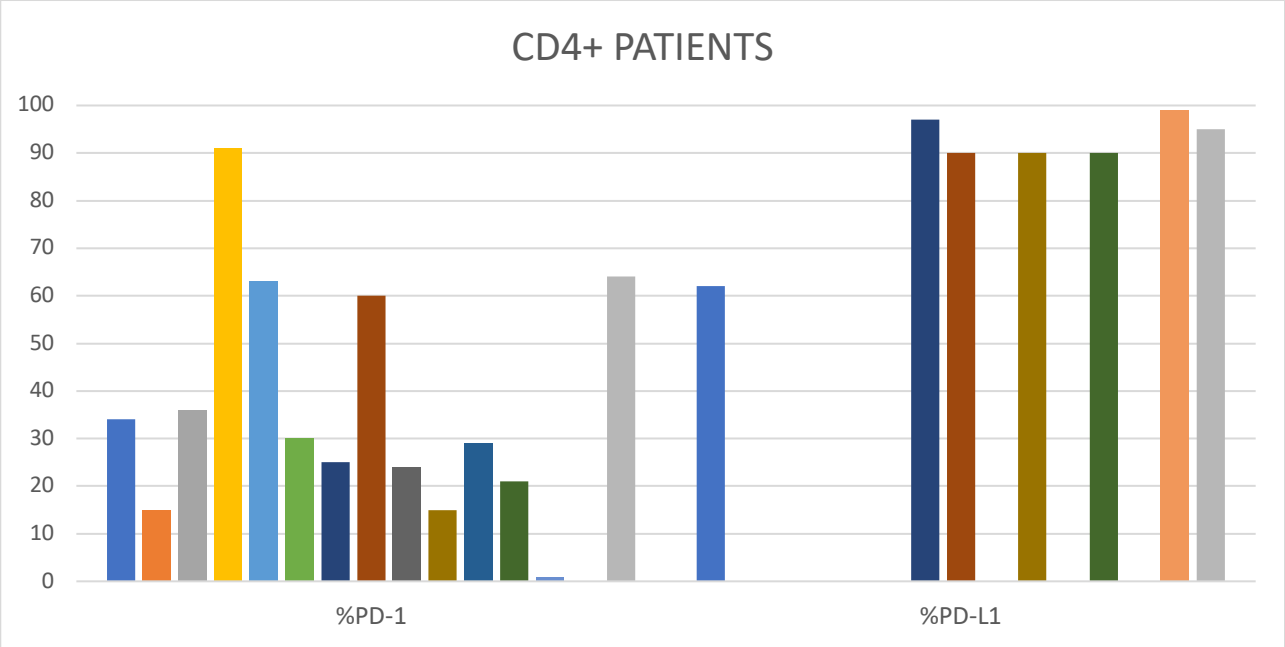


Figure 13. PD-1 and PD-L1 expression in CD4$^+$ patients' cells. Flow cytometry was utilized to assess the percentage of PD-1 and PD-L1 protein expression in CD4$^+$ patients' cells.

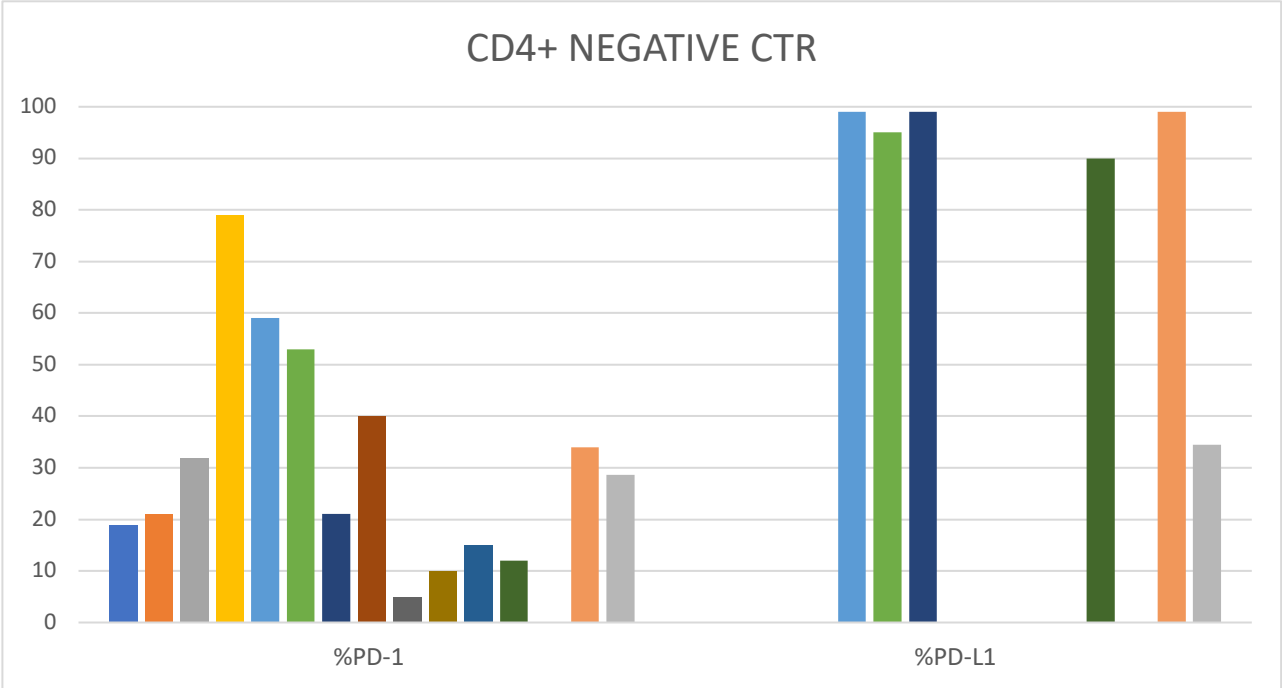


Figure 14. PD-1 and PD-L1 expression in CD4$^+$ negative control samples' cells. Flow cytometry was utilized to assess the percentage of PD-1 and PD-L1 protein expression in CD4$^+$ negative control samples' cells.

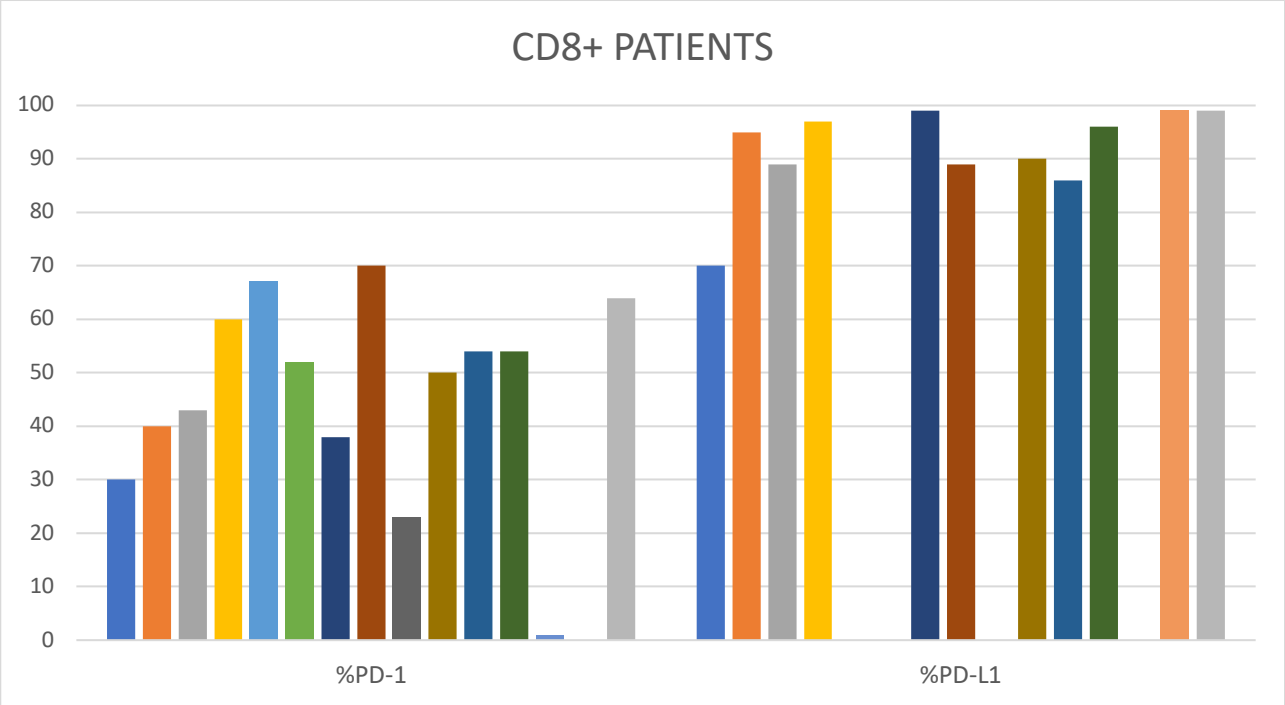


Figure 15. PD-1 and PD-L1 expression in CD8$^+$ patients' cells. Flow cytometry was utilized to assess the percentage of PD-1 and PD-L1 protein expression in CD8$^+$ patients' cells.

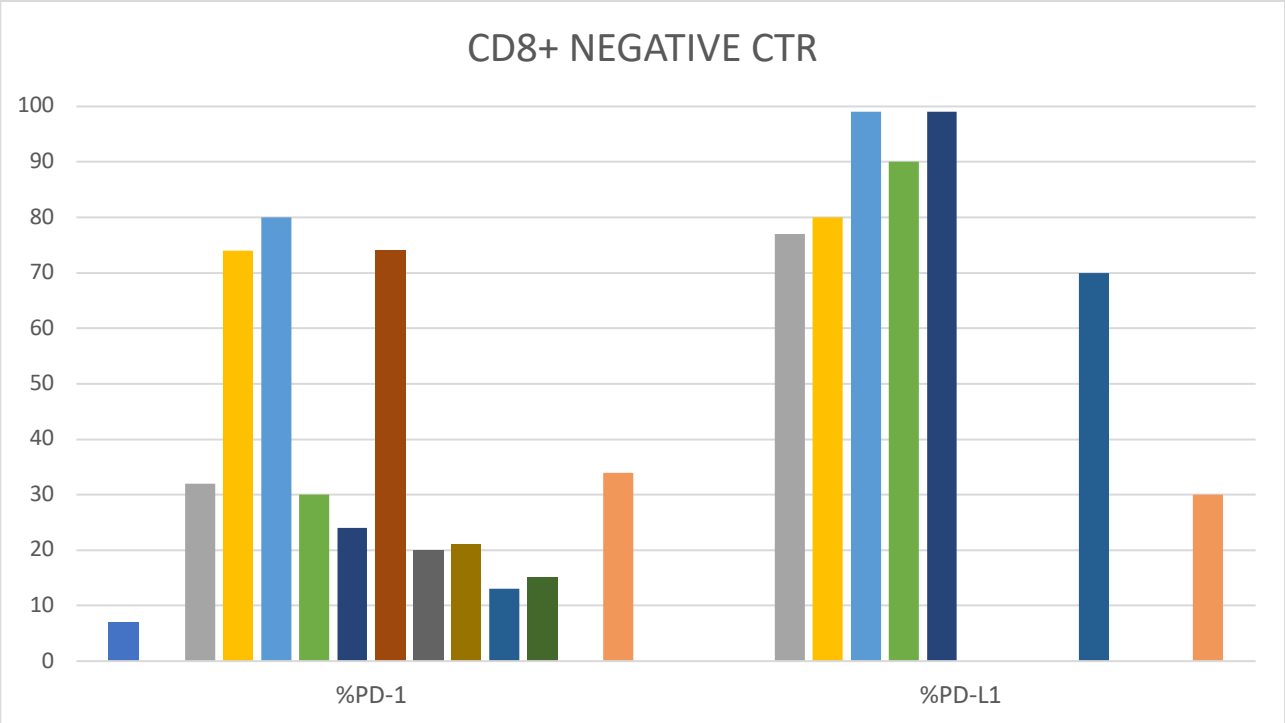


Figure 16. PD-1 and PD-L1 expression in CD8$^+$ negative control samples' cells. Flow cytometry was utilized to assess the percentage of PD-1 and PD-L1 protein expression in CD8$^+$ negative control samples' cells.

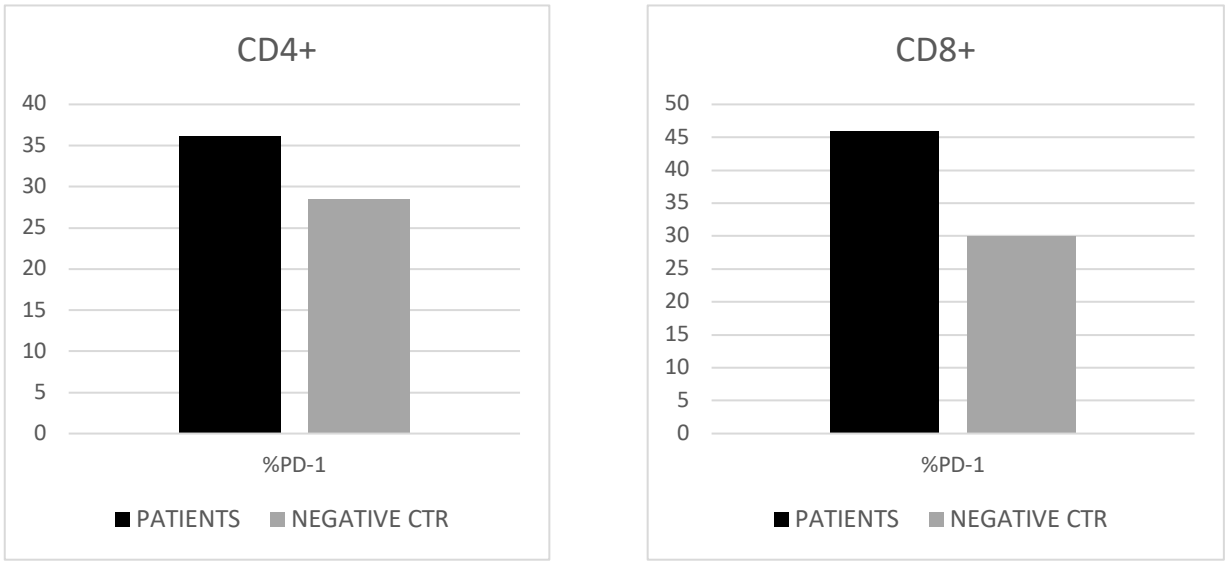


Figure 17. PD-1 is overexpressed in $CD4^+$ and $CD8^+$ patients' samples.

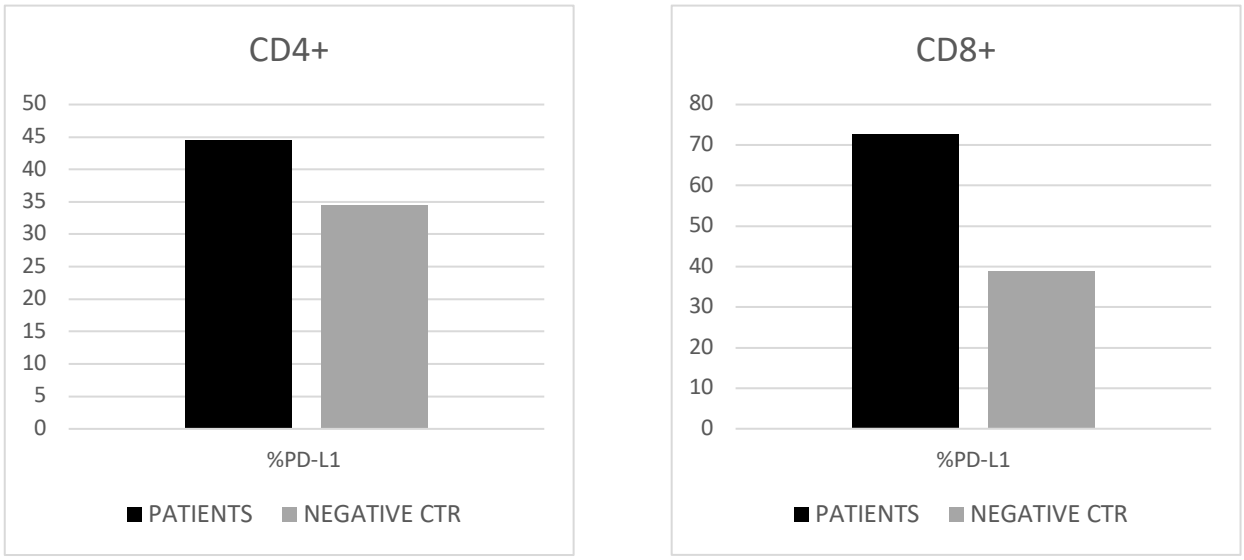


Figure 18. PD-L1 is overexpressed in $CD4^+$ and $CD8^+$ patients' samples.

Our study showed that, if compared with negative control samples, PD-1 and PD-L1 are more expressed in $CD4^+$ and $CD8^+$ cells of patients with multiple myeloma.

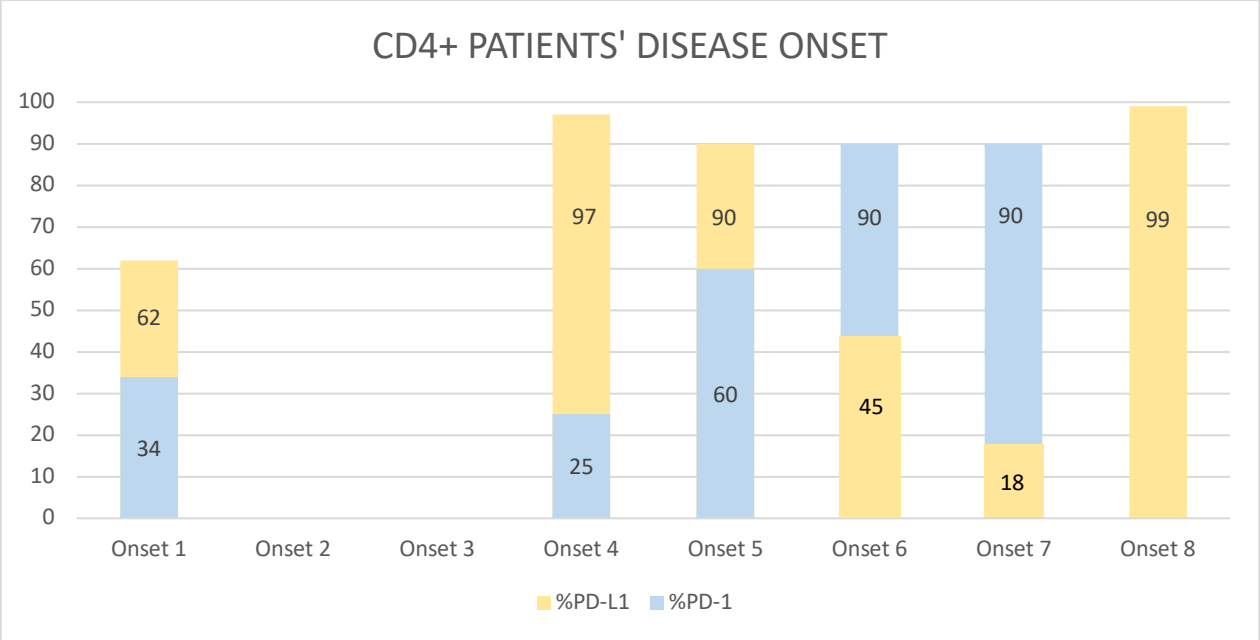


Figure 19. PD-1 and PD-L1 expression in CD4$^+$ patients at disease onset.

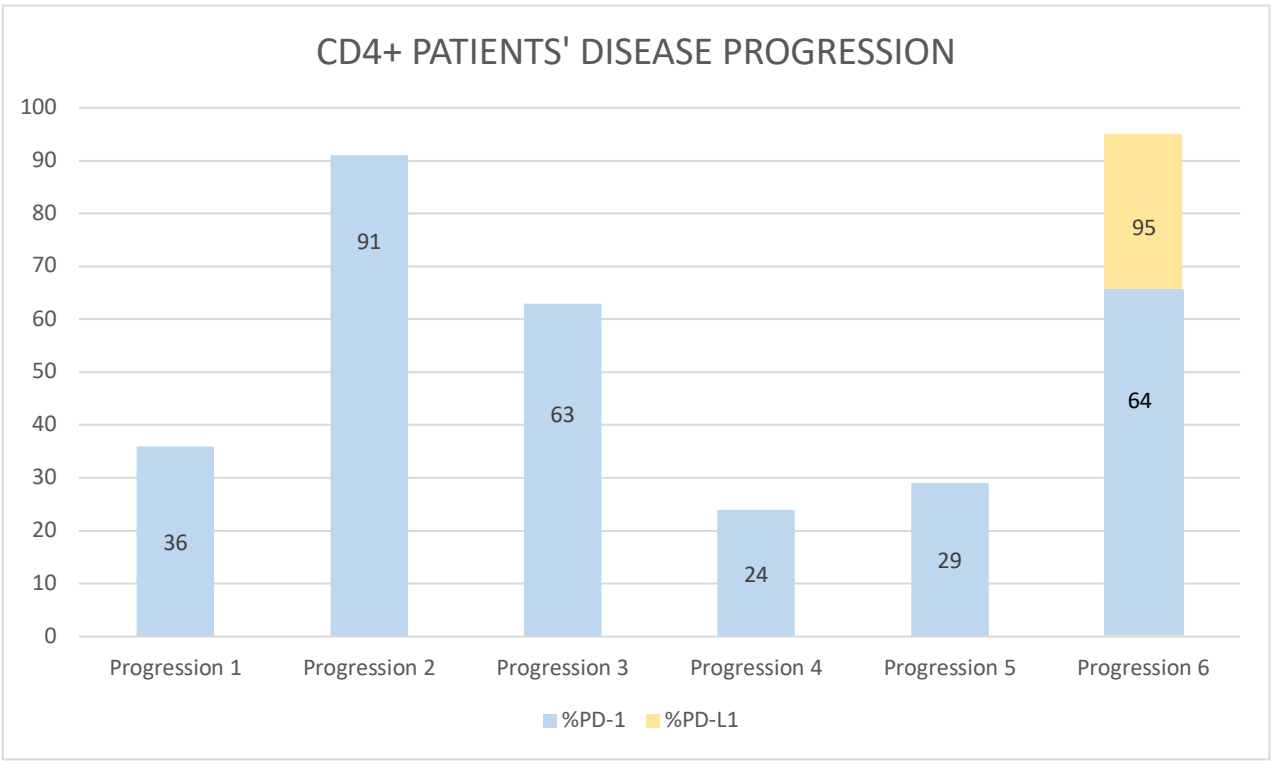


Figure 20. PD-1 and PD-L1 expression in CD4$^+$ patients with MM progression.

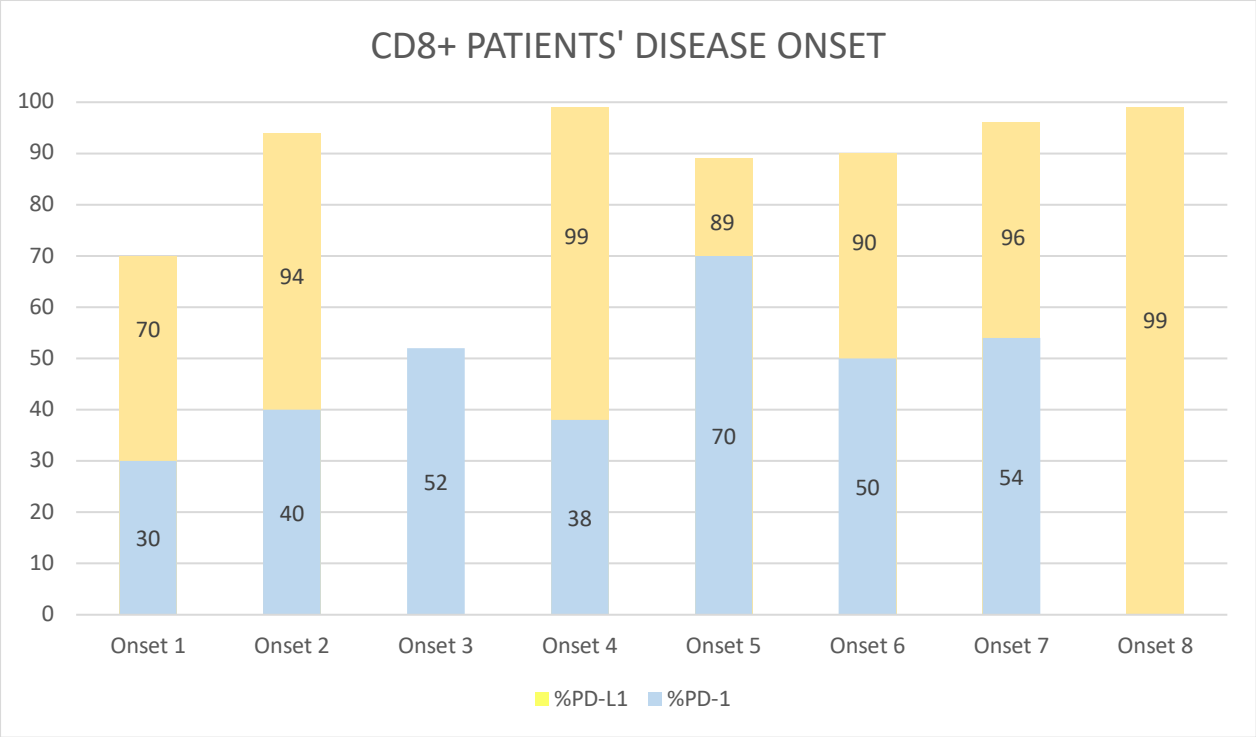


Figure 21. PD-1 and PD-L1 expression in CD8^{+} patients at disease onset.

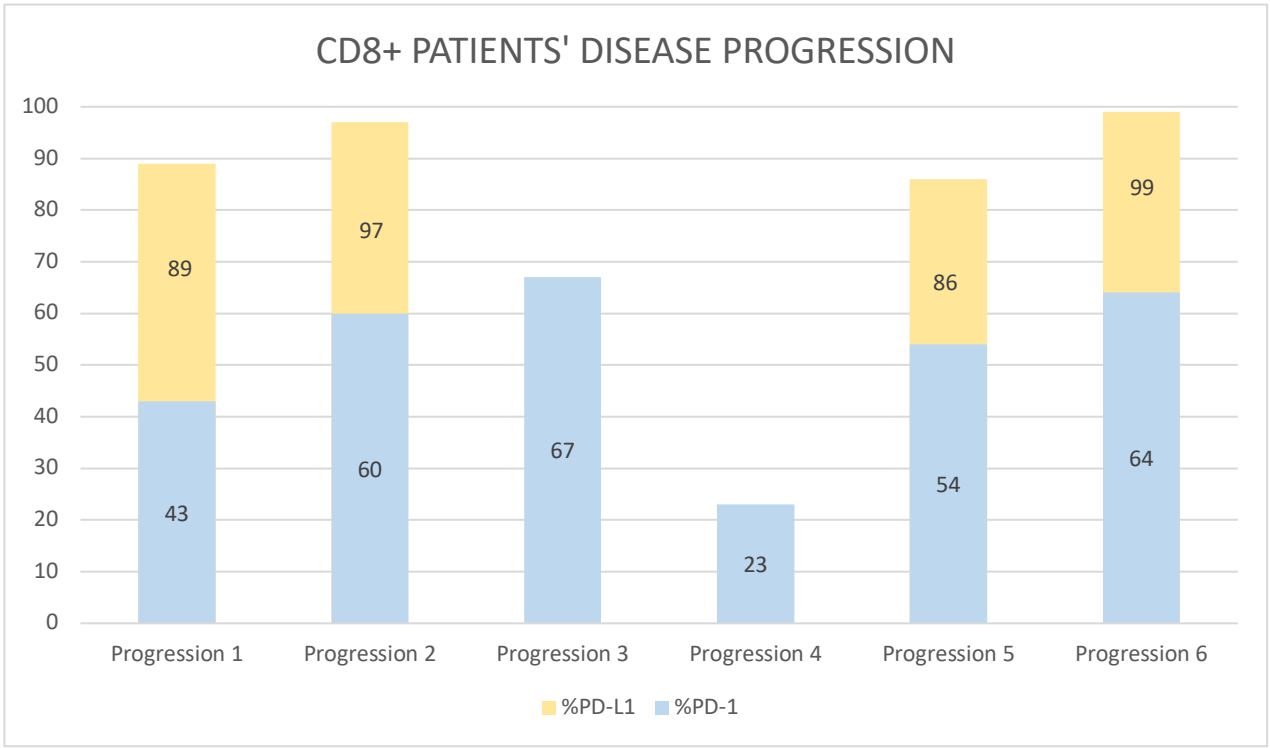


Figure 22. PD-1 and PD-L1 expression in CD8^{+} patients with MM progression.

Our study showed that PD-L1 is more expressed at disease onset and PD-1 during disease progression.

DISCUSSION

Multiple myeloma is an incurable plasma cell malignancy with only 30% of patients surviving for more than 10 years[4]. The bone marrow microenvironment is crucial to the survival, proliferation and growth of these malignant plasma cells and has also been heavily implicated in drug resistance[6]. Therefore, therapeutic targeting of the microenvironment has gained interest in conjunction with targeting myeloma cells themselves[246]. This highlights the importance of the microenvironment in supporting disease pathogenesis and progression[247].

Multiple myeloma is an extraordinarily complex hematological disease in regards to its ability to manipulate the cells in the bone marrow microenvironment, as well as its genesis and progression.
It is well documented in the literature that malignant plasma cells are dependent on the bone marrow milieu for their survival, proliferation, and ability to control angiogenic processes that ultimately facilitate metastasis and dissemination throughout the bone marrow. Migration is an essential process that facilitates the dissemination of malignant plasma cells throughout the bone marrow[396]. While the precise mechanics of this process are still up for debate, it is evident that myeloma cells can influence endothelial cells' ability to create microblood capillaries in the bone marrow, which subsequently facilitates their migratory capacity[248].

A lot of elucidations are still to be done regarding the precise subset of myeloma cells that are in charge of these processes, as well as the ways in which they carry out these abnormal tasks. In addition to the variability shown across patients, myeloma has been reported to exhibit intra-clonal heterogeneity, which means that there is a significant chance of genetic and phenotypic heterogeneity within a patient's malignancy[249]. This leads to further complications in the ability to tailor treatments that would be most effective for patients on an individual basis.

Malignant plasma cells can be identified using a combination of phenotypic markers that are widely used in this field[250]. Strategies for determining these cells must fulfil specific criteria that, first, distinguish them from healthy plasma cells and, second, distinguish them from other cell types[242].

This has made immunophenotyping an useful technique to aid diagnosis and subsequently monitor the progression of the disease and the response of the patient to therapy.

Multiple myeloma is a malignancy that is heavily associated to relapse after therapy[137]. The strong dependence malignant plasma cells have on the bone marrow microenvironment makes it extremely difficult to effectively treat this disease, with a small residual population of drug-resistant myeloma cells remaining within the bone

marrow after nearly all cases of treatment[251]. It is evident that these chemo-resistant, malignant plasma cells are ultimately responsible for the eventual propagation of disease that causes inevitable relapse.

The presence of drug-resistant cells that are strongly reliant on the cells of the bone marrow microenvironment are responsible for re-populating this environment in a post-treatment scenario. Determination of the presence and load of minimal residual disease is directly associated with time until a patient's next relapse[252]. The identification of these chemo-resistant cells has been of interest in order to tackle the small number of malignant plasma cells that remain in the bone marrow after treatment, in order to fully eradicate the malignancy.

The PD-1 pathway has been shown to be extraordinarily successful in slowing or clearing tumors in multiple human cancers[253]. Although no definitive biomarker to predict success of PD-1 immunotherapy has been described, the pre-treatment density of $CD8^+$ T cell infiltration and expression of PD-1 or PD-L1 in the tumor microenvironment all correlate with responsiveness to PD-1 targeted therapies[254]. Memory T cells likely play an important role in the response to tumor recurrence and metastases.

The next great challenges in checkpoint blockade therapy are to extend the efficacy to additional patients and to define biomarkers that can be used to predict success prior to initiation of therapy or assess success early during treatment. In order to achieve these goals, we need a more mechanistic understanding of the anti-tumor immunity induced by PD-1 blockade.

Blocking PD-L1 may be a more effective therapeutic strategy than blocking PD-1, and that blocking both PD-1 and PD-L1 may be an effective combination. Indeed, this combination is currently being investigated in clinical trials. Although the majority of clinical effort has been put towards antibodies blocking PD-1, an antibody blocking PD-L1 interactions with both PD-1 and B7-1 has been approved in non-small cell lung cancer and bladder cancer[255]. Further studies will be needed to compare the efficacy of this and similar antibodies versus antibodies that block PD-1 interactions.

Anti-PD-1/PD-L1 antibody treatment could be clinically effective in MM patients by recovering T-cell cytotoxicity and inhibiting reverse signaling from PD-L1 on MM cells. This is because the PD-1/PD-L1 pathway may be connected to the pathogenesis of MM. Additionally, DCs in the MM microenvironment expressed PD-L1 and through the PD-1/PD-L1 pathway suppressed immune functions of T-cells and NK-cells that expressed PD-1[256]. In the bone marrow, PD-L1-expressing MM cells were found to be localized with elevated PD-L1 expression on plasmacytoid DCs, which

are crucial for the proliferation and extended survival of MM cells[197]. The therapeutic target of the PD-1/PD-L1 blockade may be PD-L1 on both these cells and MM cells.

Therefore, the use of combination therapies may significantly improve the impact of checkpoint inhibition as a treatment modality for selected patients. The increased response rates however were also accompanied by a sharp increase in systemic toxicity, and therefore, combination therapies with several different checkpoint inhibiting antibodies may present benefits only for restricted subgroups of patients.

Even though our study was based on a reduced number of patients, we are confident that the better comprehension of PD-1/PD-L1 mechanism could be very useful for the identification of the best therapeutic individual approach.

In addition to the pandemic diffusion of Covid 19, which impeded us to collect a wider casuistic of samples, their intra and interindividual variability is of crucial importance, considering that we analyzed patients of various subsets and not standardized cell lines.

CONCLUSIONS

An important achievement in the immunotherapeutic treatment of cancer was the discovery of the PD-1/PD-L1 pathway, its function in the evasion of tumor immunity, and the development of targeted antibodies.

Additional investigation of these patients could lead to the identification of more novel targets that could further unravel the mechanisms that contribute to myeloma disease pathology. In order to maximize the clinical impact of anti-PD-1 therapy, it may be possible to find additional immunomodulatory pathways and immunosuppressive variables by understanding the molecular and cellular mechanisms underlying myeloma-PD-1-driven tumor immune evasion.

Secondly, cancer cell-expressed PD-1 was identified as a novel tumor cell-intrinsic growth-promoting mechanism, including in the absence of immunity.
The possible link between cancer cell-expressed PD-1 and hyperactivation of oncogenic pathways, including PI3K/AKT/mTOR, PD-L1 expression, cancer cell metabolism, cell cycle progression, metastasis, and therapeutic resistance could critically enhance the basic understanding of cancer initiation and growth. Combining anti-PD-1 Abs with therapies that target oncogenic pathways downstream of cancer cell-expressed PD-1 might work synergistically to further improve the clinical efficacy of PD-1 inhibition.
This could also help in the discovery of additional targets downstream of PD-1 expressed by cancer cells, which might also function as indicators of response or targets for therapeutic intervention.

Flow cytometry may be a reliable, easy and value effective tool for the assessment of minimal residual disease in patients with multiple myeloma. Longer remissions that cannot be accurately evaluated with conventional techniques, such as immunofixation and electrophoresis, are achieved by novel drugs, which dramatically enhance patients' outcomes.

Understanding the distribution of PD-1/PD-L1 molecules in the BM niche of patients with multiple myeloma and the contribution of immune resistance mechanisms to PD-1/PD-L1 blockade represents a critical step in order to identify the best patient subset that could benefit from this checkpoint blockade and to provide rationale for new combined therapeutic strategies.

REFERENCES

1. Solly S. Remarks on the pathology of mollities ossium; with cases. Med Chir Trans. 1844;27:435-498.438.
2. Noone AM, Howlader N, Krapcho M, et al. SEER Cancer Statistics Review 1975-2015, National Cancer Institute. Bethesda, MD; 2018.
3. Kyle RA, Rajkumar SV. Multiple myeloma. N Engl J Med. 2004;351(18):1860-1873.
4. Palumbo A, Anderson K. Multiple myeloma. N Engl J Med. 2011;364(11):1046-1060.
5. Moreau P, Attal M, Facon T. Frontline therapy of multiple myeloma. Blood. 2015;125(20):3076-3084.
6. Manier S, Sacco A, Leleu X, Ghobrial IM, Roccaro AM. Bone marrow microenvironment in multiple myeloma progression. J Biomed Biotechnol. 2012;2012:157496.
7. Anderson KC, Auclair D, Kelloff GJ, et al. The Role of Minimal Residual Disease Testing in Myeloma Treatment Selection and Drug Development: Current Value and Future Applications. Clin Cancer Res. 2017;23(15):3980-3993.
8. Kumar SK, Therneau TM, Gertz MA, et al. Clinical course of patients with relapsed multiple myeloma. Mayo Clin Proc. 2004;79(7):867-874.
9. Ribatti D. A historical perspective on milestones in multiple myeloma research Eur J Haematol. 2018;100:221–228.
10. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893-2917.
11. Kim K, Lee JH, Kim JS, et al. Clinical profiles of multiple myeloma in Asia-An Asian Myeloma Network study. Am J Hematol. 2014;89(7):751-756.
12. Boyd KD, Ross FM, Chiecchio L, et al. Gender disparities in the tumor genetics and clinical outcome of multiple myeloma. Cancer Epidemiol Biomarkers Prev. 2011;20(8):1703-1707.
13. Kyle RA, Gertz MA, Witzig TE, et al. Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc. 2003;78(1):21-33.
14. Kazandjian D. Multiple myeloma epidemiology and survival: A unique malignancy. Semin Oncol. 2016;43(6):676-681.
15. Ludwig H, Durie BG, Bolejack V, et al. Myeloma in patients younger than age 50 years presents with more favorable features and shows better survival: an analysis of 10 549 patients from the International Myeloma Working Group. Blood. 2008;111(8):4039-4047.
16. Landgren O, Kyle RA, Pfeiffer RM, et al. Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. Blood. 2009;113(22):5412-5417.
17. Teras LR, Kitahara CM, Birmann BM, et al. Body size and multiple myeloma

mortality: a pooled analysis of 20 prospective studies. Br J Haematol. 2014;166(5):667-676.
18. Alexander DD, Mink PJ, Adami HO, et al. Multiple myeloma: a review of the epidemiologic literature. Int J Cancer. 2007;120 Suppl 12:40-61.
19. Khuder SA, Mutgi AB. Meta-analyses of multiple myeloma and farming. Am J Ind Med. 1997;32(5):510-516.
20. Landgren O, Linet MS, McMaster ML, Gridley G, Hemminki K, Goldin LR. Familial characteristics of autoimmune and hematologic disorders in 8,406 multiple myeloma patients: a population-based case-control study. Int J Cancer. 2006;118(12):3095-3098.
21. Schinasi LH, Brown EE, Camp NJ, et al. Multiple myeloma and family history of lymphohaematopoietic cancers: Results from the International Multiple Myeloma Consortium. Br J Haematol. 2016;175(1):87-101.
22. Chu VT, Berek C. The establishment of the plasma cell survival niche in the bone marrow. Immunol Rev. 2013;251(1):177-188.
23. Fairfax KA, Kallies A, Nutt SL, Tarlinton DM. Plasma cell development: from B-cell subsets to long-term survival niches. Semin Immunol. 2008;20(1):49-58.
24. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. Nat Rev Immunol. 2015;15(3):160-171.
25. Stavnezer J, Guikema JE, Schrader CE. Mechanism and regulation of class switch recombination. Annu Rev Immunol. 2008;26:261-292.
26. Schuster SR, Rajkumar SV, Dispenzieri A, et al. IgM multiple myeloma: disease definition, prognosis, and differentiation from Waldenstrom's macroglobulinemia. Am J Hematol. 2010;85(11):853-855.
27. Avet-Loiseau H, Garand R, Lodé L, Robillard N, Bataille R. 14q32 Translocations discriminate IgM multiple myeloma from Waldenstrom's macroglobulinemia. Semin Oncol. 2003;30(2):153-155.
28. Jancelewicz Z, Takatsuki K, Sugai S, Pruzanski W. IgD multiple myeloma. Review of 133 cases. Arch Intern Med. 1975;135(1):87-93.
29. Pisani F, Petrucci MT, Giannarelli D, et al. IgD multiple myeloma a descriptive report of 17 cases: survival and response to therapy. J Exp Clin Cancer Res. 2012;31:17.
30. Pandey S, Kyle RA. Unusual myelomas: a review of IgD and IgE variants. Oncology (Williston Park). 2013;27(8):798-803.
31. Rajkumar SV. Multiple myeloma: 2016 update on diagnosis, risk-stratification, and management. Am J Hematol. 2016;91(7):719-734.
32. Corso A, Mangiacavalli S. Non-Secretory Myeloma: Ready for a new Definition? Mediterr J Hematol Infect Dis. 2017;9(1):e2017053.
33. de Mel S, Lim SH, Tung ML, Chng WJ. Implications of heterogeneity in multiple myeloma. Biomed Res Int. 2014;2014:232546.
34. Mali M, Jaakkola P, Arvilommi AM, Jalkanen M. Sequence of human syndecan indicates a novel gene family of integral membrane proteoglycans. J Biol Chem. 1990;265(12):6884-6889.

35. O'Connell FP, Pinkus JL, Pinkus GS. CD138 (syndecan-1), a plasma cell marker immunohistochemical profile in hematopoietic and nonhematopoietic neoplasms. Am J Clin Pathol. 2004;121(2):254-263.
36. Koda JE, Rapraeger A, Bernfield M. Heparan sulfate proteoglycans from mouse mammary epithelial cells. Cell surface proteoglycan as a receptor for interstitial collagens. J Biol Chem. 1985;260(13):8157-8162.
37. Sanderson RD, Lalor P, Bernfield M. B lymphocytes express and lose syndecan at specific stages of differentiation. Cell Regul. 1989;1(1):27-35.
38. Wijdenes J, Vooijs WC, Clément C, et al. A plasmocyte selective monoclonal antibody (B-B4) recognizes syndecan-1. Br J Haematol. 1996;94(2):318-323.
39. Seidel C, Sundan A, Hjorth M, et al. Serum syndecan-1: a new independent prognostic marker in multiple myeloma. Blood. 2000;95(2):388-392.
40. Reid S, Yang S, Brown R, et al. Characterisation and relevance of CD138-negative plasma cells in plasma cell myeloma. Int J Lab Hematol. 2010;32(6 Pt 1):e190-196.
41. Witzig TE, Kimlinger T, Stenson M, Therneau T. Syndecan-1 expression on malignant cells from the blood and marrow of patients with plasma cell proliferative disorders and B-cell chronic lymphocytic leukemia. Leuk Lymphoma. 1998;31(1-2):167-175.
42. Matsui W, Huff CA, Wang Q, et al. Characterization of clonogenic multiple myeloma cells. Blood. 2004;103(6):2332-2336.
43. Matsui W, Wang Q, Barber JP, et al. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. Cancer Res. 2008;68(1):190-197.
44. Kawano Y, Fujiwara S, Wada N, et al. Multiple myeloma cells expressing low levels of CD138 have an immature phenotype and reduced sensitivity to lenalidomide. Int J Oncol. 2012;41(3):876-884.
45. Franqui-Machin R, Wendlandt EB, Janz S, Zhan F, Tricot G. Cancer stem cells are the cause of drug resistance in multiple myeloma: fact or fiction? Oncotarget. 2015;6(38):40496-40506.
46. de Weers M, Tai YT, van der Veer MS, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. J Immunol. 2011;186(3):1840-1848.
47. Rawstron AC, Orfao A, Beksac M, et al. Report of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders. Haematologica. 2008;93(3):431-438.
48. Tembhare PR, Yuan CM, Venzon D, et al. Flow cytometric differentiation of abnormal and normal plasma cells in the bone marrow in patients with multiple myeloma and its precursor diseases. Leuk Res. 2014;38(3):371-376.
49. Rawstron A, Barrans S, Blythe D, et al. Distribution of myeloma plasma cells in peripheral blood and bone marrow correlates with CD56 expression. Br J Haematol. 1999;104(1):138-143.
50. Rawstron AC, Child JA, de Tute RM, et al. Minimal residual disease assessed by multiparameter flow cytometry in multiple myeloma: impact on outcome in the

Medical Research Council Myeloma IX Study. J Clin Oncol. 2013;31(20):2540-2547.
51. Short KD, Rajkumar SV, Larson D, et al. Incidence of extramedullary disease in patients with multiple myeloma in the era of novel therapy, and the activity of pomalidomide on extramedullary myeloma. Leukemia. 2011;25(6):906-908.
52. Kyle RA, Therneau TM, Rajkumar SV, Larson DR, Plevak MF, Melton LJ. Long-term follow-up of 241 patients with monoclonal gammopathy of undetermined significance: the original Mayo Clinic series 25 years later. Mayo Clin Proc. 2004;79(7):859-866.
53. Kyle RA, Therneau TM, Rajkumar SV, et al. Prevalence of monoclonal gammopathy of undetermined significance. N Engl J Med. 2006;354(13):1362-1369.
54. Group IMW. Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. Br J Haematol. 2003;121(5):749-757.
55. van de Donk NW, Palumbo A, Johnsen HE, et al. The clinical relevance and management of monoclonal gammopathy of undetermined significance and related disorders: recommendations from the European Myeloma Network. Haematologica. 2014;99(6):984-996.
56. Katzmann JA, Clark RJ, Abraham RS, et al. Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains. Clin Chem. 2002;48(9):1437-1444.
57. Rajkumar SV, Kyle RA, Therneau TM, et al. Serum free light chain ratio is an independent risk factor for progression in monoclonal gammopathy of undetermined significance. Blood. 2005;106(3):812-817.
58. Kyle RA, Durie BG, Rajkumar SV, et al. Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. Leukemia. 2010;24(6):1121-1127.
59. Kyle RA, Remstein ED, Therneau TM, et al. Clinical course and prognosis of smoldering (asymptomatic) multiple myeloma. N Engl J Med. 2007;356(25):2582-2590.
60. Rajkumar SV, Larson D, Kyle RA. Diagnosis of smoldering multiple myeloma. N Engl J Med. 2011;365(5):474-475.
61. Rajkumar SV, Landgren O, Mateos MV. Smoldering multiple myeloma. Blood. 2015;125(20):3069-3075.
62. Dispenzieri A, Kyle RA, Katzmann JA, et al. Immunoglobulin free light chain ratio is an independent risk factor for progression of smoldering (asymptomatic) multiple myeloma. Blood. 2008;111(2):785-789.
63. Kastritis E, Terpos E, Moulopoulos L, et al. Extensive bone marrow infiltration and abnormal free light chain ratio identifies patients with asymptomatic myeloma at high risk for progression to symptomatic disease. Leukemia. 2013;27(4):947-

953.
64. Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. Lancet Oncol. 2014;15(12):e538-548.
65. Hameed A, Brady JJ, Dowling P, Clynes M, O'Gorman P. Bone disease in multiple myeloma: pathophysiology and management. Cancer Growth Metastasis. 2014;7:33-42.
66. Drake MT, Clarke BL, Khosla S. Bisphosphonates: mechanism of action and role in clinical practice. Mayo Clin Proc. 2008;83(9):1032-1045.
67. Terpos E, Roodman GD, Dimopoulos MA. Optimal use of bisphosphonates in patients with multiple myeloma. Blood. 2013;121(17):3325-3328.
68. Clark AD, Shetty A, Soutar R. Renal failure and multiple myeloma: pathogenesis and treatment of renal failure and management of underlying myeloma. Blood Rev. 1999;13(2):79-90.
69. Finkel KW, Cohen EP, Shirali A, Abudayyeh A, Forum ASoNO-N. Paraprotein-Related Kidney Disease: Evaluation and Treatment of Myeloma Cast Nephropathy. Clin J Am Soc Nephrol. 2016;11(12):2273-2279.
70. Dimopoulos MA, Kastritis E, Rosinol L, Bladé J, Ludwig H. Pathogenesis and treatment of renal failure in multiple myeloma. Leukemia. 2008;22(8):1485-1493.
71. Blimark C, Holmberg E, Mellqvist UH, et al. Multiple myeloma and infections: a population- based study on 9253 multiple myeloma patients. Haematologica. 2015;100(1):107-113.
72. Pratt G, Goodyear O, Moss P. Immunodeficiency and immunotherapy in multiple myeloma. Br J Haematol. 2007;138(5):563-579.
73. Nucci M, Anaissie E. Infections in patients with multiple myeloma in the era of high-dose therapy and novel agents. Clin Infect Dis. 2009;49(8):1211-1225.
74. Varettoni M, Corso A, Pica G, Mangiacavalli S, Pascutto C, Lazzarino M. Incidence, presenting features and outcome of extramedullary disease in multiple myeloma: a longitudinal study on 1003 consecutive patients. Ann Oncol. 2010;21(2):325-330.
75. Tiedemann RE, Gonzalez-Paz N, Kyle RA, et al. Genetic aberrations and survival in plasma cell leukemia. Leukemia. 2008;22(5):1044-1052.
76. Fernández de Larrea C, Kyle RA, Durie BG, et al. Plasma cell leukemia: consensus statement on diagnostic requirements, response criteria and treatment recommendations by the International Myeloma Working Group. Leukemia. 2013;27(4):780-791.
77. van de Donk NW, Lokhorst HM, Anderson KC, Richardson PG. How I treat plasma cell leukemia. Blood. 2012;120(12):2376-2389.
78. Bladé J, Fernández de Larrea C, Rosiñol L, Cibeira MT, Jiménez R, Powles R. Soft-tissue plasmacytomas in multiple myeloma: incidence, mechanisms of extramedullary spread, and treatment approach. J Clin Oncol. 2011;29(28):3805-3812.
79. Pour L, Sevcikova S, Greslikova H, et al. Soft-tissue extramedullary multiple

myeloma prognosis is significantly worse in comparison to bone-related extramedullary relapse. Haematologica. 2014;99(2):360-364.
80. Rajan AM, Rajkumar SV. Interpretation of cytogenetic results in multiple myeloma for clinical practice. Blood Cancer J. 2015;5:e365.
81. Kumar S, Fonseca R, Ketterling RP, et al. Trisomies in multiple myeloma: impact on survival in patients with high-risk cytogenetics. Blood. 2012;119(9):2100-2105.
82. Avet-Loiseau H, Attal M, Moreau P, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myélome. Blood. 2007;109(8):3489-3495.
83. Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. Blood. 2005;106(1):296-303.
84. Hurt EM, Wiestner A, Rosenwald A, et al. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. Cancer Cell. 2004;5(2):191-199.
85. Keats JJ, Reiman T, Maxwell CA, et al. In multiple myeloma, t(4;14)(p16;q32) is an adverse prognostic factor irrespective of FGFR3 expression. Blood. 2003;101(4):1520-1529.
86. Fonseca R, Oken MM, Harrington D, et al. Deletions of chromosome 13 in multiple myeloma identified by interphase FISH usually denote large deletions of the q arm or monosomy. Leukemia. 2001;15(6):981-986.
87. Fonseca R, Bailey RJ, Ahmann GJ, et al. Genomic abnormalities in monoclonal gammopathy of undetermined significance. Blood. 2002;100(4):1417-1424.
88. Avet-Loiseau H, Facon T, Daviet A, et al. 14q32 translocations and monosomy 13 observed in monoclonal gammopathy of undetermined significance delineate a multistep process for the oncogenesis of multiple myeloma. Intergroupe Francophone du Myélome. Cancer Res. 1999;59(18):4546-4550.
89. Avet-Louseau H, Daviet A, Sauner S, Bataille R, Myélome IFd. Chromosome 13 abnormalities in multiple myeloma are mostly monosomy 13. Br J Haematol. 2000;111(4):1116-1117.
90. Avet-Loiseau H, Li JY, Morineau N, et al. Monosomy 13 is associated with the transition of monoclonal gammopathy of undetermined significance to multiple myeloma. Intergroupe Francophone du Myélome. Blood. 1999;94(8):2583-2589.
91. Fonseca R, Bergsagel PL, Drach J, et al. International Myeloma Working Group molecular classification of multiple myeloma: spotlight review. Leukemia. 2009;23(12):2210-2221.
92. Chng WJ, Gonzalez-Paz N, Price-Troska T, et al. Clinical and biological significance of RAS mutations in multiple myeloma. Leukemia. 2008;22(12):2280-2284.
93. Chng WJ, Huang GF, Chung TH, et al. Clinical and biological implications of MYC activation: a common difference between MGUS and newly diagnosed multiple myeloma. Leukemia. 2011;25(6):1026-1035.

94. Avet-Loiseau H, Gerson F, Magrangeas F, et al. Rearrangements of the c-myc oncogene are present in 15% of primary human multiple myeloma tumors. Blood. 2001;98(10):3082-3086.
95. Keats JJ, Fonseca R, Chesi M, et al. Promiscuous mutations activate the noncanonical NF- kappaB pathway in multiple myeloma. Cancer Cell. 2007;12(2):131-144.
96. Demchenko YN, Glebov OK, Zingone A, Keats JJ, Bergsagel PL, Kuehl WM. Classical and/or alternative NF-kappaB pathway activation in multiple myeloma. Blood. 2010;115(17):3541-3552.
97. Agarwal A, Ghobrial IM. Monoclonal gammopathy of undetermined significance and smoldering multiple myeloma: a review of the current understanding of epidemiology, biology, risk stratification, and management of myeloma precursor disease. Clin Cancer Res. 2013;19(5):985-994.
98. Durie BG, Salmon SE. A clinical staging system for multiple myeloma. Correlation of measured myeloma cell mass with presenting clinical features, response to treatment, and survival. Cancer. 1975;36(3):842-854.
99. Greipp PR, San Miguel J, Durie BG, et al. International staging system for multiple myeloma. J Clin Oncol. 2005;23(15):3412-3420.
100. Palumbo A, Avet-Loiseau H, Oliva S, et al. Revised International Staging System for Multiple Myeloma: A Report From International Myeloma Working Group. J Clin Oncol. 2015;33(26):2863-2869.
101. Alexanian R, Bergsagel DE, Migliore PJ, Vaughn WK, Howe CD. Melphalan therapy for plasma cell myeloma. Blood. 1968;31(1):1-10.
102. Kumar SK, Dispenzieri A, Lacy MQ, et al. Continued improvement in survival in multiple myeloma: changes in early mortality and outcomes in older patients. Leukemia. 2014;28(5):1122-1128.
103. Kotla V, Goel S, Nischal S, et al. Mechanism of action of lenalidomide in hematological malignancies. J Hematol Oncol. 2009;2:36.
104. Lecker SH, Goldberg AL, Mitch WE. Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. J Am Soc Nephrol. 2006;17(7):1807-1819.
105. Hideshima T, Richardson PG, Anderson KC. Mechanism of action of proteasome inhibitors and deacetylase inhibitors and the biological basis of synergy in multiple myeloma. Mol Cancer Ther. 2011;10(11):2034-2042.
106. Greenstein S, Ghias K, Krett NL, Rosen ST. Mechanisms of glucocorticoid-mediated apoptosis in hematological malignancies. Clin Cancer Res. 2002;8(6):1681-1694.
107. Chng WJ, Dispenzieri A, Chim CS, et al. IMWG consensus on risk stratification in multiple myeloma. Leukemia. 2014;28(2):269-277.
108. Brenner H, Gondos A, Pulte D. Recent major improvement in long-term survival of younger patients with multiple myeloma. Blood. 2008;111(5):2521-2526.
109. Kumar SK, Dingli D, Lacy MQ, et al. Autologous stem cell transplantation in

patients of 70 years and older with multiple myeloma: Results from a matched pair analysis. Am J Hematol. 2008;83(8):614- 617.
110. Gertz MA, Dingli D. How we manage autologous stem cell transplantation for patients with multiple myeloma. Blood. 2014;124(6):882-890.
111. NICE. Myeloma: Diagnosis and Management: NICE Guidelines; 2016.
112. Kaufman JL, Nooka A, Vrana M, Gleason C, Heffner LT, Lonial S. Bortezomib, thalidomide, and dexamethasone as induction therapy for patients with symptomatic multiple myeloma: a retrospective study. Cancer. 2010;116(13):3143-3151.
113. Moreau P, Avet-Loiseau H, Facon T, et al. Bortezomib plus dexamethasone versus reduced- dose bortezomib, thalidomide plus dexamethasone as induction treatment before autologous stem cell transplantation in newly diagnosed multiple myeloma. Blood. 2011;118(22):5752-5758; quiz 5982.
114. Richardson PG, Weller E, Lonial S, et al. Lenalidomide, bortezomib, and dexamethasone combination therapy in patients with newly diagnosed multiple myeloma. Blood. 2010;116(5):679-686.
115. Reeder CB, Reece DE, Kukreti V, et al. Cyclophosphamide, bortezomib and dexamethasone induction for newly diagnosed multiple myeloma: high response rates in a phase II clinical trial. Leukemia. 2009;23(7):1337-1341.
116. Sonneveld P, Avet-Loiseau H, Lonial S, et al. Treatment of multiple myeloma with high-risk cytogenetics: a consensus of the International Myeloma Working Group. Blood. 2016;127(24):2955- 2962.
117. Attal M, Harousseau JL, Stoppa AM, et al. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Français du Myélome. N Engl J Med. 1996;335(2):91-97.
118. Child JA, Morgan GJ, Davies FE, et al. High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. N Engl J Med. 2003;348(19):1875-1883.
119. Moreau P, Facon T, Attal M, et al. Comparison of 200 mg/m(2) melphalan and 8 Gy total body irradiation plus 140 mg/m(2) melphalan as conditioning regimens for peripheral blood stem cell transplantation in patients with newly diagnosed multiple myeloma: final analysis of the Intergroupe Francophone du Myélome 9502 randomized trial. Blood. 2002;99(3):731-735.
120. Gertz MA, Lacy MQ, Inwards DJ, et al. Early harvest and late transplantation as an effective therapeutic strategy in multiple myeloma. Bone Marrow Transplant. 1999;23(3):221-226.
121. Rajkumar SV, Kumar S. Multiple Myeloma: Diagnosis and Treatment. Mayo Clin Proc. 2016;91(1):101-119.
122. Krishnan A, Pasquini MC, Logan B, et al. Autologous haemopoietic stem-cell transplantation followed by allogeneic or autologous haemopoietic stem-cell transplantation in patients with multiple myeloma (BMT CTN 0102): a phase 3 biological assignment trial. Lancet Oncol. 2011;12(13):1195-1203.
123. Bruno B, Rotta M, Patriarca F, et al. A comparison of allografting with

autografting for newly diagnosed myeloma. N Engl J Med. 2007;356(11):1110-1120.
124. San Miguel JF, Schlag R, Khuageva NK, et al. Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. N Engl J Med. 2008;359(9):906-917.
125. Benboubker L, Dimopoulos MA, Dispenzieri A, et al. Lenalidomide and dexamethasone in transplant-ineligible patients with myeloma. N Engl J Med. 2014;371(10):906-917.
126. Hoering A, Crowley J, Shaughnessy JD, et al. Complete remission in multiple myeloma examined as time-dependent variable in terms of both onset and duration in Total Therapy protocols. Blood. 2009;114(7):1299-1305.
127. Magrangeas F, Avet-Loiseau H, Gouraud W, et al. Minor clone provides a reservoir for relapse in multiple myeloma. Leukemia. 2013;27(2):473-481.
128. Yang WC, Lin SF. Mechanisms of Drug Resistance in Relapse and Refractory Multiple Myeloma. Biomed Res Int. 2015;2015:341430.
129. Attal M, Lauwers-Cances V, Marit G, et al. Lenalidomide maintenance after stem-cell transplantation for multiple myeloma. N Engl J Med. 2012;366(19):1782-1791.
130. Palumbo A, Cavallo F, Gay F, et al. Autologous transplantation and maintenance therapy in multiple myeloma. N Engl J Med. 2014;371(10):895-905.
131. McCarthy PL, Owzar K, Hofmeister CC, et al. Lenalidomide after stem-cell transplantation for multiple myeloma. N Engl J Med. 2012;366(19):1770-1781.
132. Jackson GH, Davies FE, Pawlyn C, et al. Lenalidomide maintenance versus observation for patients with newly diagnosed multiple myeloma (Myeloma XI): a multicentre, open-label, randomised, phase 3 trial. Lancet Oncol. 2019;20(1):57-73.
133. Jones JR, Cairns DA, Gregory WM, et al. Second malignancies in the context of lenalidomide treatment: an analysis of 2732 myeloma patients enrolled to the Myeloma XI trial. Blood Cancer J. 2016;6(12):e506.
134. Sonneveld P, Schmidt-Wolf IG, van der Holt B, et al. Bortezomib induction and maintenance treatment in patients with newly diagnosed multiple myeloma: results of the randomized phase III HOVON-65/ GMMG-HD4 trial. J Clin Oncol. 2012;30(24):2946-2955.
135. Nooka AK, Kaufman JL, Muppidi S, et al. Consolidation and maintenance therapy with lenalidomide, bortezomib and dexamethasone (RVD) in high-risk myeloma patients. Leukemia. 2014;28(3):690-693.
136. Palumbo A, Bringhen S, Larocca A, et al. Bortezomib-melphalan-prednisone-thalidomide followed by maintenance with bortezomib-thalidomide compared with bortezomib-melphalan- prednisone for initial treatment of multiple myeloma: updated follow-up and improved survival. J Clin Oncol. 2014;32(7):634-640.
137. Laubach J, Garderet L, Mahindra A, et al. Management of relapsed multiple myeloma: recommendations of the International Myeloma Working Group. Leukemia. 2016;30(5):1005-1017.

138. McCullough KB, Hobbs MA, Abeykoon JP, Kapoor P. Common adverse effects of novel therapies for multiple myeloma (MM) and their management strategies. Curr Hematol Malig Rep. 2018;13(2):114-124.
139. NICE. Carfilzomib for previously treated multiple myeloma.: NICE Guidelines; 2017.
140. NICE. Ixazomib with lenalidomide and dexamethasone for treating relapsed of refractory multiple myeloma.: NICE Guidelines; 2018.
141. NICE. Pomalidomide for multiple myeloma previously treated with lenalidomide and bortezomib.: NICE Guidelines; 2017.
142. Richardson PG, Siegel DS, Vij R, et al. Pomalidomide alone or in combination with low-dose dexamethasone in relapsed and refractory multiple myeloma: a randomized phase 2 study. Blood. 2014;123(12):1826-1832.
143. Miguel JS, Weisel K, Moreau P, et al. Pomalidomide plus low-dose dexamethasone versus high- dose dexamethasone alone for patients with relapsed and refractory multiple myeloma (MM-003): a randomised, open-label, phase 3 trial. Lancet Oncol. 2013;14(11):1055-1066.
144. Stewart AK, Rajkumar SV, Dimopoulos MA, et al. Carfilzomib, lenalidomide, and dexamethasone for relapsed multiple myeloma. N Engl J Med. 2015;372(2):142-152.
145. Moreau P, Masszi T, Grzasko N, et al. Oral Ixazomib, Lenalidomide, and Dexamethasone for Multiple Myeloma. N Engl J Med. 2016;374(17):1621-1634.
146. Siegel DS, Martin T, Wang M, et al. A phase 2 study of single-agent carfilzomib (PX-171-003-A1) in patients with relapsed and refractory multiple myeloma. Blood. 2012;120(14):2817-2825.
147. Lonial S, Dimopoulos M, Palumbo A, et al. Elotuzumab therapy for relapsed or refractory multiple myeloma. N Engl J Med. 2015;373(7):621-631.
148. Dimopoulos MA, Dytfeld D, Grosicki S, et al. Elotuzumab plus pomalidomide and dexamethasone for multiple myeloma. N Engl J Med. 2018;379(19):1811-1822.
149. Lonial S, Weiss BM, Usmani SZ, et al. Daratumumab monotherapy in patients with treatment- refractory multiple myeloma (SIRIUS): an open-label, randomised, phase 2 trial. Lancet. 2016;387(10027):1551-1560.
150. Usmani SZ, Weiss BM, Plesner T, et al. Clinical efficacy of daratumumab monotherapy in patients with heavily pretreated relapsed or refractory multiple myeloma. Blood. 2016;128(1):37-44.
151. Dimopoulos MA, Oriol A, Nahi H, et al. Daratumumab, lenalidomide, and dexamethasone for multiple myeloma. N Engl J Med. 2016;375(14):1319-1331.
152. Palumbo A, Chanan-Khan A, Weisel K, et al. Daratumumab, bortezomib, and dexamethasone for multiple myeloma. N Engl J Med. 2016;375(8):754-766.
153. NICE. Daratumumab with bortezomib and dexamethasone for previously treated multiple myeloma.: NICE Guidelines; 2019.
154. Park JH, Rivière I, Gonen M, et al. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. N Engl J Med. 2018;378(5):449-459.

155. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med. 2011;365(8):725-733.
156. June CH, Sadelain M. Chimeric Antigen Receptor Therapy. N Engl J Med. 2018;379(1):64-73.
157. Susanibar Adaniya SP, Cohen AD, Garfall AL. Chimeric antigen receptor T cell immunotherapy for multiple myeloma: A review of current data and potential clinical applications. Am J Hematol. 2019;94(S1):S28-S33.
158. Brudno JN, Maric I, Hartman SD, et al. T cells genetically modified to express an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of poor-prognosis relapsed multiple myeloma. J Clin Oncol. 2018;36(22):2267-2280.
159. Anthony BA, Link DC. Regulation of hematopoietic stem cells by bone marrow stromal cells. Trends Immunol. 2014;35(1):32-37.
160. Lauta VM. A review of the cytokine network in multiple myeloma: diagnostic, prognostic, and therapeutic implications. Cancer. 2003;97(10):2440-2452.
161. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. Blood. 2004;104(3):607-618.
162. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood. 1999;93(5):1658-1667.
163. Podar K, Chauhan D, Anderson KC. Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia. 2009;23(1):10-24.
164. Brahmer, J.; Reckamp, K.L.; Baas, P.; Crino, L.; Eberhardt, W.E.; Poddubskaya, E.; Antonia, S.; Pluzanski, A.; Vokes, E.E.; Holgado, E.; et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. N. Engl. J. Med. 2015, 373, 123–135.
165. Ansell, S.M.; Lesokhin, A.M.; Borrello, I.; Halwani, A.; Scott, E.C.; Gutierrez, M.; Schuster, S.J.; Millenson, M.M.; Cattry, D.; Freeman, G.J.; et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. N. Engl. J. Med. 2015, 372, 311–319.
166. Sun, C.; Mezzadra, R.; Schumacher, T.N. Regulation and Function of the PD-L1 Checkpoint. Immunity 2018, 48, 434–452.
167. Gibbons Johnson, R.M.; Dong, H. Functional Expression of Programmed Death-Ligand 1 (B7-H1) by Immune Cells and Tumor Cells. Front. Immunol. 2017, 8, 961.
168. Lin, D.Y.; Tanaka, Y.; Iwasaki, M.; Gittis, A.G.; Su, H.P.; Mikami, B.; Okazaki, T.; Honjo, T.; Minato, N.; Garboczi, D.N. The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors. Proc. Natl. Acad. Sci. USA 2008, 105, 3011–3016.
169. Boussiotis, V.A. Molecular and biochemical aspects of the PD-1 checkpoint pathway. N. Engl. J. Med. 2016, 375, 1767–1778.
170. Wang, Q.; Lin, W.; Tang, X.; Li, S.; Guo, L.; Lin, Y.; Kwok, H.F. The roles of microRNAs in regulating the expression of PD-1/PD-L1 immune checkpoint. Int.

J. Mol. Sci. 2017, 18, 2540.
171. Horita, H.; Law, A.; Hong, S.; Middleton, K. Identifying regulatory posttranslational modifications of PD-L1: a Focus on monoubiquitinaton. neoplasia 2017, 19, 346–353.
172. Lim, S.O.; Li, C.W.; Xia, W.; Cha, J.H.; Chan, L.C.; Wu, Y.; Chang, S.S.; Lin, W.C.; Hsu, J.M.; Hsu, Y.H.; et al. Deubiquitination and stabilization of PD-L1 by CSN5. Cancer Cell 2016, 30, 925–939.
173. Lee, S.J.; Jang, B.C.; Lee, S.W.; Yang, Y.I.; Suh, S.I.; Park, Y.M.; Oh, S.; Shin, J.G.; Yao, S.; Chen, L.; et al. Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-gamma-induced upregulation of B7-H1 (CD274). FEBS Lett. 2006, 580, 755–762.
174. Parry, R.V.; Chemnitz, J.M.; Frauwirth, K.A.; Lanfranco, A.R.; Braunstein, I.; Kobayashi, S.V.; Linsley, P.S.; Thompson, C.B.; Riley, J.L. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol. Cell. Biol. 2005, 25, 9543–9553.
175. Freeman, G.J.; Long, A.J.; Iwai, Y.; Bourque, K.; Chernova, T.; Nishimura, H.; Fitz, L.J.; Malenkovich, N.; Okazaki, T.; Byrne, M.C.; et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J. Exp. Med. 2000, 192, 1027–1034.
176. Butte, M.J.; Keir, M.E.; Phamduy, T.B.; Sharpe, A.H.; Freeman, G.J. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity 2007, 27, 111–122.
177. Bar, N.; Costa, F.; Das, R.; Duffy, A.; Samur, M.; McCachren, S.; Gettinger, S.N.; Neparidze, N.; Parker, T.L.; Bailur, J.K.; et al. Differential effects of PD-L1 versus PD-1 blockade on myeloid inflammation in human cancer. JCI Insight 2020, 5.
178. Azuma, T.; Yao, S.; Zhu, G.; Flies, A.S.; Flies, S.J.; Chen, L. B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. Blood 2008, 111, 3635–3643.
179. Chang, C.H.; Qiu, J.; O'Sullivan, D.; Buck, M.D.; Noguchi, T.; Curtis, J.D.; Chen, Q.; Gindin, M.; Gubin, M.M.; van der Windt, G.J.; et al. Metabolic Competition in the tumor microenvironment is a driver of cancer progression. Cell 2015, 162, 1229–1241.
180. Patsoukis, N.; Bardhan, K.; Chatterjee, P.; Sari, D.; Liu, B.; Bell, L.N.; Karoly, E.D.; Freeman, G.J.; Petkova, V.; Seth, P.; et al. PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. Nat. Commun. 2015, 6, 6692.
181. Ogando, J.; Saez, M.E.; Santos, J.; Nuevo-Tapioles, C.; Gut, M.; Esteve-Codina, A.; Heath, S.; Gonzalez-Perez, A.; Cuezva, J.M.; Lacalle, R.A.; et al. PD-1 signaling affects cristae morphology and leads to mitochondrial dysfunction in human CD8(+) T lymphocytes. J. Immunother. Cancer 2019, 7, 151.
182. Barsoum, I.B.; Koti, M.; Siemens, D.R.; Graham, C.H. Mechanisms of hypoxia-mediated immune escape in cancer. Cancer Res. 2014, 74, 7185–7190.
183. Schumacher, T.N.; Kesmir, C.; van Buuren, M.M. Biomarkers in cancer

immunotherapy. Cancer Cell 2015, 27, 12–14.
184. Samstein, R.M.; Lee, C.H.; Shoushtari, A.N.; Hellmann, M.D.; Shen, R.; Janjigian, Y.Y.; Barron, D.A.; Zehir, A.; Jordan, E.J.; Omuro, A.; et al. Tumor mutational load predicts survival after immunotherapy across multiple cancer types. Nat. Genet. 2019, 51, 202–206.
185. Miranda, A.; Hamilton, P.T.; Zhang, A.W.; Pattnaik, S.; Becht, E.; Mezheyeuski, A.; Bruun, J.; Micke, P.; de Reynies, A.; Nelson, B.H. Cancer stemness, intratumoral heterogeneity, and immune response across cancers. Proc. Natl. Acad. Sci. USA 2019, 116, 9020–9029.
186. Tamura, H.; Ishibashi, M.; Yamashita, T.; Tanosaki, S.; Okuyama, N.; Kondo, A.; Hyodo, H.; Shinya, E.; Takahashi, H.; Dong, H.; et al. Marrow stromal cells induce B7-H1 expression on myeloma cells, generating aggressive characteristics in multiple myeloma. Leukemia 2013, 27, 464–472.
187. Ishibashi, M.; Tamura, H.; Sunakawa, M.; Kondo-Onodera, A.; Okuyama, N.; Hamada, Y.; Moriya, K.; Choi, I.; Tamada, K.; Inokuchi, K. Myeloma drug resistance induced by binding of myeloma B7-H1 (PD-L1) to PD-1. Cancer Immunol. Res. 2016, 4, 779–788.
188. Chen, D.; Tang, P.; Liu, L.; Wang, F.; Xing, H.; Sun, L.; Jiang, Z. Bone marrow-derived mesenchymal stem cells promote cell proliferation of multiple myeloma through inhibiting T cell immune responses via PD-1/PD-L1 pathway. Cell Cycle 2018, 17, 858–867.
189. Liu, J.; Hamrouni, A.; Wolowiec, D.; Coiteux, V.; Kuliczkowski, K.; Hetuin, D.; Saudemont, A.; Quesnel, B. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. Blood 2007, 110, 296–304.
190. Paiva, B.; Azpilikueta, A.; Puig, N.; Ocio, E.M.; Sharma, R.; Oyajobi, B.O.; Labiano, S.; San-Segundo, L.; Rodriguez, A.; Aires-Mejia, I.; et al. PD-L1/PD-1 presence in the tumor microenvironment and activity of PD-1 blockade in multiple myeloma. Leukemia 2015, 29, 2110.
191. Dhodapkar, M.V.; Sexton, R.; Das, R.; Dhodapkar, K.M.; Zhang, L.; Sundaram, R.; Soni, S.; Crowley, J.J.; Orlowski, R.Z.; Barlogie, B. Prospective analysis of antigen-specific immunity, stem-cell antigens, and immune checkpoints in monoclonal gammopathy. Blood 2015, 126, 2475–2478.
192. Mussetti, A.; Pellegrinelli, A.; Cieri, N.; Garzone, G.; Dominoni, F.; Cabras, A.; Montefusco, V. PD-L1, LAG3, and HLA-DR are increasingly expressed during smoldering myeloma progression. Ann. Hematol. 2019, 98, 1713–1720.
193. Lee, B.H.; Park, Y.; Kim, J.H.; Kang, K.W.; Lee, S.J.; Kim, S.J.; Kim, B.S. PD-L1 expression in bone marrow plasma cells as a biomarker to predict multiple myeloma prognosis: Developing a nomogram-based prognostic model. Sci. Rep. 2020, 10, 12641.
194. Costa, F.; Vescovini, R.; Marchica, V.; Storti, P.; Notarfranchi, L.; Dalla Palma, B.; Toscani, D.; Burroughs-Garcia, J.; Catarozzo, M.T.; Giuliani, N. PD-L1/PD-1

pattern of expression within the bone marrow immune microenvironment in smoldering myeloma and active multiple myeloma patients. Front. Immunol. 2020, 11, 3398.
195. Crescenzi, A.; Annibali, O.; Bianchi, A.; Pagano, A.; Donati, M.; Grifoni, A.; Avvisati, G. PD-1/PD-L1 expression in extra- medullary lesions of multiple myeloma. Leuk. Res. 2016, 49, 98–101.
196. Ray, A.; Das, D.S.; Song, Y.; Richardson, P.; Munshi, N.C.; Chauhan, D.; Anderson, K.C. Targeting PD1-PDL1 immune checkpoint in plasmacytoid dendritic cell interactions with T cells, natural killer cells and multiple myeloma c cells. Leukemia 2015, 29, 1441–1444.
197. Chauhan, D.; Singh, A.V.; Brahmandam, M.; Carrasco, R.; Bandi, M.; Hideshima, T.; Bianchi, G.; Podar, K.; Tai, Y.T.; Mitsiades, C.; et al. Functional interaction of plasmacytoid dendritic cells with multiple myeloma cells: A therapeutic target. Cancer Cell 2009, 16, 309–323.
198. Gorgun, G.; Samur, M.K.; Cowens, K.B.; Paula, S.; Bianchi, G.; Anderson, J.E.; White, R.E.; Singh, A.; Ohguchi, H.; Suzuki, R.; et al. Lenalidomide enhances immune checkpoint blockade-induced immune response in multiple myeloma. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 2015, 21, 4607–4618.
199. Castella, B.; Foglietta, M.; Sciancalepore, P.; Rigoni, M.; Coscia, M.; Griggio, V.; Vitale, C.; Ferracini, R.; Saraci, E.; Omede, P.; et al. Anergic bone marrow Vgamma9Vdelta2 T cells as early and long-lasting markers of PD-1-targetable microenvironment-induced immune suppression in human myeloma. Oncoimmunology 2015, 4, e1047580.
200. Bailur, J.K.; McCachren, S.S.; Doxie, D.B.; Shrestha, M.; Pendleton, K.; Nooka, A.K.; Neparidze, N.; Parker, T.L.; Bar, N.; Kaufman, J.L.; et al. Early alterations in stem-like/resident T cells, innate and myeloid cells in the bone marrow in preneoplastic gammopathy. JCI Insight 2019, 5.
201. An, G.; Acharya, C.; Feng, X.; Wen, K.; Zhong, M.; Zhang, L.; Munshi, N.C.; Qiu, L.; Tai, Y.T.; Anderson, K.C. Osteoclasts promote immune suppressive microenvironment in multiple myeloma: Therapeutic implication. Blood 2016, 128, 1590–1603.
202. Zelle-Rieser, C.; Thangavadivel, S.; Biedermann, R.; Brunner, A.; Stoitzner, P.; Willenbacher, E.; Greil, R.; Johrer, K. T cells in multiple myeloma display features of exhaustion and senescence at the tumor site. J. Hematol. Oncol. 2016, 9, 116.
203. Kwon, M.; Kim, C.G.; Lee, H.; Cho, H.; Kim, Y.; Lee, E.C.; Choi, S.J.; Park, J.; Seo, I.H.; Bogen, B.; et al. PD-1 Blockade Reinvigorates Bone Marrow CD8(+) T cells from patients with multiple myeloma in the presence of TGFbeta inhibitors. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 2020, 26, 1644–1655.
204. Wang, L.; Wang, H.; Chen, H.; Wang, W.D.; Chen, X.Q.; Geng, Q.R.; Xia, Z.J.; Lu, Y. Serum levels of soluble programmed death ligand 1 predict treatment response and progression free survival in multiple myeloma. Oncotarget 2015, 6, 41228–41236.

205. Huang, S.Y.; Lin, H.H.; Lin, C.W.; Li, C.C.; Yao, M.; Tang, J.L.; Hou, H.A.; Tsay, W.; Chou, S.J.; Cheng, C.L.; et al. Soluble PD-L1: A biomarker to predict progression of autologous transplantation in patients with multiple myeloma. Oncotarget 2016, 7, 62490–62502.
206. Bohnhorst, J.; Rasmussen, T.; Moen, S.H.; Flottum, M.; Knudsen, L.; Borset, M.; Espevik, T.; Sundan, A. Toll-like receptors mediate proliferation and survival of multiple myeloma cells. Leukemia 2006, 20, 1138–1144.
207. Chen, H.M.; Li, M.J.; Xu, N.; Ng, N.; Sanchez, E.; Hekmati, T.; Wang, C.; Bujarski, S.; Tang, G.; Berenson, J. Ruxolitinib (RUX) reverses checkpoint inhibition by downregulating PD-L1 expression in both multiple myeloma (MM) tumor and stromal cells. Cl Lymph Myelom Leuk 2019, 19, E158–E159.
208. Ishibashi, M.; Yamamoto, J.; Ito, T.; Handa, H.; Inokuchi, K.; Takahashi, H.; Tamura, H. Upregulation of PD-L1 on myeloma cells by immunomodulatory agents potentiates the effect of durvalumab. Blood 2018, 132.
209. Ray, A.; Das, D.S.; Song, Y.; Hideshima, T.; Tai, Y.T.; Chauhan, D.; Anderson, K.C. Combination of a novel HDAC6 inhibitor ACY-241 and anti-PD-L1 antibody enhances anti-tumor immunity and cytotoxicity in multiple myeloma. Leukemia 2018, 32, 843–846.
210. Kearl, T.J.; Jing, W.; Gershan, J.A.; Johnson, B.D. Programmed death receptor-1/programmed death receptor ligand-1 blockade after transient lymphodepletion to treat myeloma. J. Immunol. 2013, 190, 5620–5628.
211. Hallett, W.H.; Jing, W.; Drobyski, W.R.; Johnson, B.D. Immunosuppressive effects of multiple myeloma are overcome by PD-L1 blockade. Biol. Blood Marrow Transplant. J. Am. Soc. Blood Marrow Transplant. 2011, 17, 1133–1145.
212. Mateos, M.V.; Blacklock, H.; Schjesvold, F.; Oriol, A.; Simpson, D.; George, A.; Goldschmidt, H.; Larocca, A.; Chanan-Khan, A.; Sherbenou, D.; et al. Pembrolizumab plus pomalidomide and dexamethasone for patients with relapsed or refractory multiple myeloma (KEYNOTE-183): A randomised, open-label, phase 3 trial. Lancet Haematol. 2019, 6, e459–e469.
213. Malavasi, F.; Deaglio, S.; Funaro, A.; Ferrero, E.; Horenstein, A.L.; Ortolan, E.; Vaisitti, T.; Aydin, S. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. Physiol. Rev. 2008, 88, 841–886.
214. Musso, T.; Deaglio, S.; Franco, L.; Calosso, L.; Badolato, R.; Garbarino, G.; Dianzani, U.; Malavasi, F. CD38 expression and functional activities are up-regulated by IFN-gamma on human monocytes and monocytic cell lines. J. Leukoc. Biol. 2001, 69, 605–612.
215. Costa, F.; Toscani, D.; Chillemi, A.; Quarona, V.; Bolzoni, M.; Marchica, V.; Vescovini, R.; Mancini, C.; Martella, E.; Campanini, N.; et al. Expression of CD38 in myeloma bone niche: A rational basis for the use of anti-CD38 immunotherapy to inhibit osteoclast formation. Oncotarget 2017, 8, 56598–56611.
216. Frasca, L.; Nasso, M.; Spensieri, F.; Fedele, G.; Palazzo, R.; Malavasi, F.; Ausiello, C.M. IFN-gamma arms human dendritic cells to perform multiple effector functions. J. Immunol. 2008, 180, 1471–1481.

217. Vallario, A.; Chilosi, M.; Adami, F.; Montagna, L.; Deaglio, S.; Malavasi, F.; Caligaris-Cappio, F. Human myeloma cells express the CD38 ligand CD31. Br. J. Haematol. 1999, 105, 441–444.
218. Accardi, F.; Notarfranchi, L.; Palma, B.D.; Manfra, I.; De Luca, F.; Mancini, C.; Martella, E.; Marchica, V.; Storti, P.; Bolzoni, M.; et al. The loss of CD38 expression by myeloma plasma cells may occur in the extramedullary disease. Haematologica 2018, 103, S54.
219. Aarhus, R.; Graeff, R.M.; Dickey, D.M.; Walseth, T.F.; Lee, H.C. ADP-ribosyl cyclase and CD38 catalyze the synthesis of a calcium-mobilizing metabolite from NADP. J. Biol. Chem. 1995, 270, 30327–30333.
220. Matsushita, H.; Vesely, M.D.; Koboldt, D.C.; Rickert, C.G.; Uppaluri, R.; Magrini, V.J.; Arthur, C.D.; White, J.M.; Chen, Y.S.; Shea, L.K.; et al. Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. Nature 2012, 482, 400–404.
221. Horenstein, A.L.; Quarona, V.; Toscani, D.; Costa, F.; Chillemi, A.; Pistoia, V.; Giuliani, N.; Malavasi, F. Adenosine generated in the bone marrow niche through a CD38-mediated pathway correlates with progression of human myeloma. Mol. Med. 2016, 22, 694–704.
222. Novitskiy, S.V.; Ryzhov, S.; Zaynagetdinov, R.; Goldstein, A.E.; Huang, Y.; Tikhomirov, O.Y.; Blackburn, M.R.; Biaggioni, I.; Carbone, D.P.; Feoktistov, I.; et al. Adenosine receptors in regulation of dendritic cell differentiation and function. Blood 2008, 112, 1822–1831.
223. Zarek, P.E.; Huang, C.T.; Lutz, E.R.; Kowalski, J.; Horton, M.R.; Linden, J.; Drake, C.G.; Powell, J.D. A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. Blood 2008, 111, 251–259.
224. Kinsey, G.R.; Huang, L.; Jaworska, K.; Khutsishvili, K.; Becker, D.A.; Ye, H.; Lobo, P.I.; Okusa, M.D. Autocrine adenosine signaling promotes regulatory T cell-mediated renal protection. J. Am. Soc. Nephrol. JASN 2012, 23, 1528–1537.
225. Emens, L.; Powderly, J.; Fong, L.; Brody, J.; Forde, P.; Hellmann, M.; Hughes, B.; Kummar, S.; Loi, S.; Luke, J.; et al. CPI-444, an oral adenosine A2a receptor (A2aR) antagonist, demonstrates clinical activity in patients with advanced solid tumors. Cancer Res. 2017, 77.
226. Chen, L.; Diao, L.; Yang, Y.; Yi, X.; Rodriguez, B.L.; Li, Y.; Villalobos, P.A.; Cascone, T.; Liu, X.; Tan, L.; et al. CD38-mediated immunosuppression as a mechanism of tumor cell escape from PD-1/PD-L1 blockade. Cancer Discov. 2018, 8, 1156–1175.
227. Morandi, F.; Morandi, B.; Horenstein, A.L.; Chillemi, A.; Quarona, V.; Zaccarello, G.; Carrega, P.; Ferlazzo, G.; Mingari, M.C.; Moretta, L.; et al. A non-canonical adenosinergic pathway led by CD38 in human melanoma cells induces suppression of T cell proliferation. Oncotarget 2015, 6, 25602–25618.
228. Ng, H.H.M.; Lee, R.Y.; Goh, S.; Tay, I.S.Y.; Lim, X.; Lee, B.; Chew, V.; Li, H.; Tan, B.; Lim, S.; et al. Immunohistochemical scoring of CD38 in the tumor

microenvironment predicts responsiveness to anti-PD-1/PD-L1 immunotherapy in hepatocellular carcinoma. J. Immunother. Cancer 2020, 8.
229. Vijayan, D.; Young, A.; Teng, M.W.L.; Smyth, M.J. Targeting immunosuppressive adenosine in cancer. Nat. Rev. Cancer 2017, 17, 765.
230. Stocker, N.; Gaugler, B.; Ricard, L.; de Vassoigne, F.; Marjanovic, Z.; Mohty, M.; Malard, F. Daratumumab prevents programmed death ligand-1 expression on antigen-presenting cells in de novo multiple myeloma. Cancer Med. 2020, 9, 2077–2084.
231. Zhu, C.; Song, Z.; Wang, A.; Srinivasan, S.; Yang, G.; Greco, R.; Theilhaber, J.; Shehu, E.; Wu, L.; Yang, Z.Y.; et al. Isatuximab acts through Fc-dependent, independent, and direct pathways to kill multiple myeloma cells. Front. Immunol. 2020, 11, 1771.
232. Martin, T.G.; Corzo, K.; Chiron, M.; Velde, H.V.; Abbadessa, G.; Campana, F.; Solanki, M.; Meng, R.; Lee, H.; Wiederschain, D.; et al. Therapeutic opportunities with pharmacological inhibition of CD38 with isatuximab. Cells 2019, 8, 1522.
233. Verkleij, C.P.M.; Jhatakia, A.; Broekmans, M.E.C.; Frerichs, K.A.; Zweegman, S.; Mutis, T.; Bezman, N.A.; van de Donk, N. Preclinical rationale for targeting the PD-1/PD-L1 axis in combination with a CD38 antibody in multiple myeloma and other CD38-positive malignancies. Cancers 2020, 12, 3713.
234. Paul, B.; Symanowski, J.; Osipoff, P.; Norek, S.; Ndiaye, A.P.; Robinson, J.; Atrash, S.; Bhutani, M.; Voorhees, P.M.; Usmani, S.Z. A Phase 2 trial of daratumumab and pembrolizumab in refractory multiple myeloma. Blood 2020.
235. Nahi, H.; Chrobok, M.; Gran, C.; Lund, J.; Gruber, A.; Gahrton, G.; Ljungman, P.; Wagner, A. K.; Alici, E. Infectious complications and NK cell depletion following daratumumab treatment of Multiple Myeloma. PLoS ONE 2019, 14, e0211927.
236. Hajek R, Okubote SA, Svachova H. Myeloma stem cell concepts, heterogeneity and plasticity of multiple myeloma. Br J Haematol. 2013;163(5):551-564.
237. Jai-Hyang Go. Aberrant CD3 Expression in a Relapsed Plasma Cell Neoplasm. Journal of Pathology and Translational Medicine 2018; 52: 202-205.
238. Xiaole Zhanga, Lei Gaoa, Kai Meng et al. Characterization of CD4+ T cell-mediated cytotoxicity in patients with multiple myeloma. Cell Immunol. 2018 May;327:62-67.
239. Garfall AL, Maus MV, Hwang WT, et al. Chimeric Antigen Receptor T Cells against CD19 for Multiple Myeloma. N Engl J Med. 2015;373(11):1040-1047.
240. M. Oyaert, M. Delforge, N. Boeckx. Use of multiparameter flow cytometry in multiple myeloma and other plasma cell neoplasms. Belg J Hematol 2015;6(2):46-53.
241. Kumar S, Rajkumar SV, Kimlinger T, Greipp PR, Witzig TE. CD45 expression by bone marrow plasma cells in multiple myeloma: clinical and biological correlations. Leukemia. 2005;19(8):1466-1470.
242. Flores-Montero J, de Tute R, Paiva B, et al. Immunophenotype of normal vs.

myeloma plasma cells: Toward antibody panel specifications for MRD detection in multiple myeloma. Cytometry B Clin Cytom. 2016;90(1):61-72.
243. Paiva B, Gutiérrez NC, Chen X, et al. Clinical significance of CD81 expression by clonal plasma cells in high-risk smoldering and symptomatic multiple myeloma patients. Leukemia. 2012;26(8):1862- 1869.
244. Schmidt-Hieber M, Pérez-Andrés M, Paiva B, et al. CD117 expression in gammopathies is associated with an altered maturation of the myeloid and lymphoid hematopoietic cell compartments and favorable disease features. Haematologica. 2011;96(2):328-332.
245. Moreaux J, Hose D, Reme T, et al. CD200 is a new prognostic factor in multiple myeloma. Blood. 2006;108(13):4194-4197.
246. Kawano Y, Moschetta M, Manier S, et al. Targeting the bone marrow microenvironment in multiple myeloma. Immunol Rev. 2015;263(1):160-172.
247. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nat Rev Cancer. 2007;7(8):585-598.
248. Podar K, Tai YT, Davies FE, et al. Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration. Blood. 2001;98(2):428-435.
249. Brioli A, Melchor L, Cavo M, Morgan GJ. The impact of intra-clonal heterogeneity on the treatment of multiple myeloma. Br J Haematol. 2014;165(4):441-454.
250. Kumar S, Kimlinger T, Morice W. Immunophenotyping in multiple myeloma and related plasma cell disorders. Best Pract Res Clin Haematol. 2010;23(3):433-451.
251. Robak P, Drozdz I, Szemraj J, Robak T. Drug resistance in multiple myeloma. Cancer Treat Rev. 2018;70:199-208.
252. Paiva B, van Dongen JJ, Orfao A. New criteria for response assessment: role of minimal residual disease in multiple myeloma. Blood. 2015;125(20):3059-3068.
253. Baumeister, S. H., Freeman, G. J., Dranoff, G. & Sharpe, A. H. Coinhibitory Pathways in Immunotherapy for Cancer. Annu Rev Immunol. 2016;34, 539-573.
254. Topalian, S. L., Taube, J. M., Anders, R. A. & Pardoll, D. M. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. Nat Rev Cancer 2016;16, 275-287.
255. Herbst, R. et al. 2013 ASCO Annual Meeting. (Journal of Clinical Oncology (Meeting Abstracts)).
256. Sponaas AM, Moharrami NN, Feyzi E, et al. PDL1 expression on plasma and dendritic cells in myeloma bone marrow suggests benefit of targeted anti PD1-PDL1 therapy. PLoS ONE 2015, 10.