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A Journey Through Myeloma Evolution: From the Normal Plasma Cell to Disease Complexity

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Abstract

The knowledge of cancer origin and the subsequent tracking of disease evolution represent unmet needs that will soon be within clinical reach. This will provide the opportunity to improve patient's stratification and to personalize treatments based on cancer biology along its life history. In this review, we focus on the molecular pathogenesis of multiple myeloma (MM), a hematologic malignancy with a well-known multi-stage disease course, where such approach can sooner translate into a clinical benefit. We describe novel insights into modes and timing of disease initiation. We dissect the biology of the preclinical and pre-malignant phases, elucidating how knowledge of the genomics of the disease and the composition of the microenvironment allow stratification of patients based on risk of disease progression. Then, we explore cell-intrinsic and cell-extrinsic drivers of MM evolution to symptomatic disease. Finally, we discuss how this may relate to the development of refractory disease after treatment. By integrating an evolutionary view of myeloma biology with the recent acquisitions on its clonal heterogeneity, we envision a way to drive the clinical management of the disease based on its detailed biological features more than surrogates of disease burden.

Introduction

Cancer is the second most common cause of death in the entire population.[1] Biologically, a cancer cell survives and proliferates in an uncontrolled fashion, driven by the accumulation of genetic lesions along with a strict cooperation of a "corrupted" microenvironment.[2,3] It is well recognized that most cells in the body withstand random mutagenesis from slight intrinsic infidelity of DNA replication and repair processes, and enzymatic modification of DNA bases.[4] Additionally, exogenous processes may increase this mutation rate, as for example in the case of UV light exposure or tobacco smoke.[5,6] Altogether, this may cause the creation of pre-clinical small clonal proliferations, which in turn may progress to overt cancer by further acquisition, in serial or in parallel fashion, of additional variants and by natural selection acting on the resulting phenotypic diversity.[7,8] This selection will be strongly determined by the complex multicellular niche, where competition for metabolites, oxygen, growth factors, and the necessity for immune escape[9] will dictate which clone is the fittest for that particular environment. Genomic instability will therefore represent an advantage for most cancer cells, since it will facilitate plasticity and ability to adapt in a Darwinian fashion.[8,10] It follows that, in most cancers, tumor heterogeneity is present already at diagnosis.[10–12]

Hematological malignancies represent the 10% of all cancers.[13] In terms of mutation rate, they are classically considered less complex than solid tumors.[4,14] However, within this large family of diseases there are clear differences with a climax of complexity that sees multiple myeloma (MM) above leukemias and lymphomas, sharing similarities with genomic changes described in solid cancers.[15,16]

In this review, we will focus on MM molecular pathogenesis. We will describe the molecular basis of the initiation processes of monoclonal gammopathies, analyzing cell-intrinsic and cell extrinsic factors in the context of the normal B-cell development. Furthermore, we will describe the different patterns of evolution from pre-clinical to overt stages of disease and the impact of MM heterogeneity as a driver for disease development and progression through the stages of monoclonal gammopathy of unknown significance (MGUS), smoldering MM (SMM) and active MM. Given the importance of cell-extrinsic factors in MM pathogenesis, we also will dissect the role of tumor microenvironment in disease initiation and in its progression.

From the cell of origin to pre-malignant stages

Lymphoproliferative diseases are classified based on the cell of origin.[17] MM is an exception to this rule, since the morphological unit of the cancer is represented by bone marrow (BM) plasma cells (PCs), but the transformation is thought to happen in a B-lymphocyte within the germinal center (GC) of lymph nodes.[18] During antigen encounter, naive B cells undergo class-switch-recombination (CSR) and somatic hypermutation (SHM) of the B-cell receptor (BCR), 2 processes aimed at increasing antigen affinity to a peculiar antigen and conferring specific effector

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functions. Of note, these 2 important events are catalyzed by the activation-induced-deaminase (AID) enzyme, which introduces DNA double strand breaks promoting both CSR and SHM.[19] These events are crucial for antibody diversification and adaptive immune responses but pose a risk of off-target mutations and rearrangements, which are thought to be the initiating events of the disease.[18,20] The B-cell, after having acquired these transforming events, is still capable of entering the blood stream and home in the bone marrow, where it would differentiate into an antibody-producing plasma cell and expand clonally also thanks to the microenvironment.[21]

Evidence supporting this hypothesis comes from several studies. Pilarski et al have reported the presence of $CD34^{pos}$ B-cells within the population of peripheral blood mononuclear cells (PBMCs) harboring an hyperdiploid genetic asset and clonotypic rearrangements in MM patients that were absent in the $CD34^{neg}$ population. Based on these results, the authors postulated that the $CD34^{pos}$ B-cell population would represent a source of "MM stem-cells" able to repopulate the clone after the therapy.[22,23] On a similar line, Corradini et al identified pre-CSR B-cells carrying the same IGH clonotypic rearrangement of myeloma cells but expressing μ chains.[24] The presence of a pre-CSR MM precursor would also provide the biological basis for the rare condition of bi-clonal gammopathies, which have been described in several reports.[24–26] Other groups have described memory B-cells recirculating through lymph nodes and peripheral blood with homing/spreading capacity through the BM carrying the MM-specific clonotypic rearrangement.[27,28] Besides confirming a GC origin of MM, these studies highlighted the possibility that the aberrant plasma cell clone in MM is repopulated from phenotypically distinct "stem" cells. Indeed, Matsui et al have described in patients and cell lines a small percentage of $CD138^{neg}$ PCs with MM-specific IGH rearrangements holding both a self-renewal and differentiation potential. These cells have the ability to engraft in mice and give rise to a $CD138^{pos}$ BM plasmocytosis.[29,30] Finally, the possibility that a phenotypically distinct plasma cell clone may harbor differential chemosensitivity and represent the source of relapse has been investigated in vitro and in vivo.[31]

Another question that arises from this proposed mechanism is whether this transformation is an entirely random event, or it is favored by encounter with specific antigens, by some sort of germline predisposition, or by a combination of the two. Indeed, the risk of developing a plasma cell dyscrasia is increased two-fold in relatives of MM patients,[32] and germline transmission of several risk alleles has been described. Among these, *KDM1A*, *ARID1A*, *USP45* and *DIS3* inherited germline mutations have been shown to confer high risk of MM development.[33–37] In some cases, risk alleles have even been linked to specific cytogenetic events, as in the case of the *CCND1* c.870G>A polymorphism being a risk factor for the IGH-CCND1 translocation.[38] However, mechanisms leading to germline transmission of MM susceptibility are still mostly unclear.

Analyses of IGH rearrangements in MM cells have provided evidence of antigen-driven selection, with restricted representation of heavy-chain variable region exons in addition to SHM.[39] However, the antigens underlying the origins of MM clones remain unknown. Bosseboeuf et al have analyzed the pathogen-specific avidity of monoclonal IgG immunoglobulins produced by patients with plasma cell dyscrasias.[40] Of note, in almost 25% of patients the authors were able to determine a specific pathogen: herpes viruses, Epstein-Barr (EBV) and Hepatitis C virus (HCV) were the most represented ones and Helicobacter-Pylori (HP) was the unique bacteria identified by the authors.[40] Several other studies correlated EBV, HCV and HP infections to PCs disorders onset, pointing at a dysregulated and chronic immune response to such pathogens as the trigger for disease development.[40–44] Antigens triggering a MM clone may also be endogenous. Indeed, the incidence of monoclonal gammopathies in Gaucher patients and in obese patients is higher than normal.[45,46] In Gaucher disease, glucocerebrosidase deficiency leads to increased levels of LGL1, which may trigger a specific antibody response. A study focusing on Gaucher patients with monoclonal gammopathies found that in 17 out of 20 of them the monoclonal immunoglobulin was in fact specific for LGL1.[47] Last, chemicals have been implied in MM development through yet unknown mechanisms but likely related to DNA mutagenesis: this is the case for cases of monoclonal gammopathies observed in firefighters after the collapse of the twin towers in New York City,[48] and in Vietnam war veterans exposed to agent orange.[49]

One question that has been impossible to answer for decades is: when does the first hit take place in MM patients? Lately, thanks to the observation that the rate of acquisition of certain mutations is constant over the years, it has been possible to speculate on this. In particular, comparing the number of such mutations in 2 serial samples from the same patients, Rustad et al have extrapolated the time at which the first mutation would have appeared and concluded that the first transforming event may happen as early as the second or third decade of life. This is followed by a slow accumulation of additional events in a timeframe of decades before the transformed cell becomes a clinically evident MM clone.[50] While this fascinating theory has yet to be proven, initial studies on mass spectrometry analysis of MGUS samples have similarly showed the presence of small amounts of monoclonal proteins years before the development of the classical MGUS.[51] Clearly, this also carries the implication of a much needed refinement of the prevalence of such conditions in the general population. It is in fact very likely that these new genomic and proteomic approaches will discover a substantially higher share of the population carrying small plasma cell clones. This will mandate the definition of a new pre-MGUS stage, a "monoclonal plasmacytosis" analogous to monoclonal B-cell lymphocytosis (Fig. 1).

Genomic features driving MGUS development

The possibility of identifying a monoclonal antibody in the serum through protein electrophoresis has led to the identification of a "pre-malignant" PCs clonal disorder called MGUS decades ago.[52] Conversely, similar instances of asymptomatic clonal expansions became apparent for B-lymphocytes through flow cytometry[53] and hematopoietic stem cells through next-generation sequencing[54,55] (NGS) only years later, confirming the multi-step nature of cancer evolution. It is nowadays accepted that almost all MMs are preceded by MGUS,[56] even if in many cases this is not identified at the clinical level.

MGUS is differentiated from MM based on a low to absent burden of clonal BM plasma cells, a low amount of serum monoclonal protein and no signs of end-organ damage, active MM or amyloidosis.[57] As discussed above, MGUS has a GC origin, where chromosome aberrations represent initiating events. From a genomic point of view MGUS therefore shares some common features with more advanced stages: similar to MM in fact, MGUS can be broadly categorized as carrying translocations of recurrent oncogenes into the IGH locus or an hyperdiploid (HD) karyotype. Curiously, the latter consists in multiple trisomies of odd chromosomes (3, 5, 7, 9, 11, 15, 19,

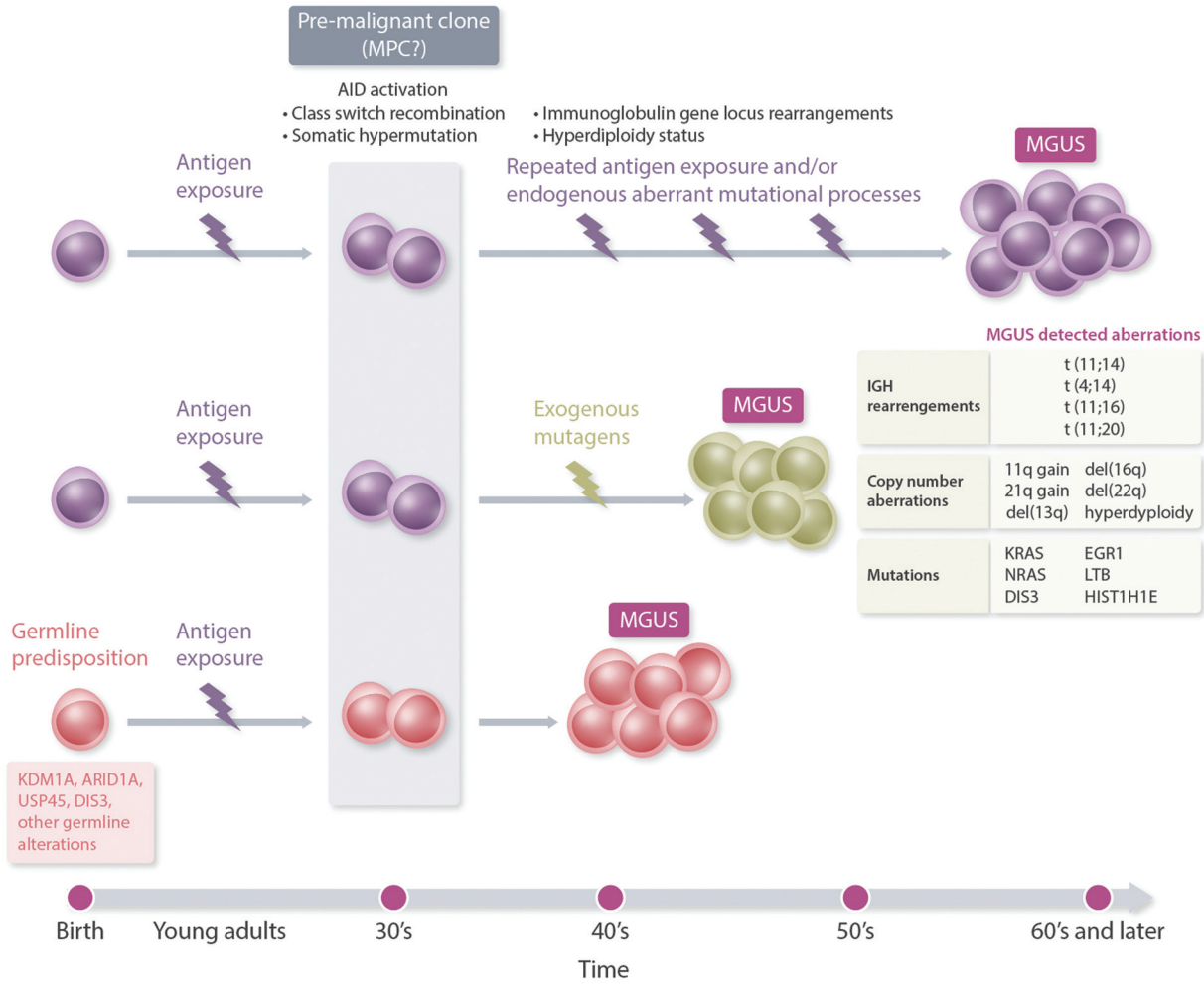

Figure 1. Initiation of monoclonal gammopathies. Timeline of monoclonal gammopathies development. After the antigen encounter a pre-malignant clone starts to grow within the bone marrow. Germline predisposition (red), continuous antigen exposure, aberrant mutational processes or exogenous mutants (green) promote the clone expansion until it could be detected by serum protein electrophoresis due to the abnormal production of the monoclonal protein. Based on the mechanism of transformation, the monoclonal gammopathy may become evident (on average) at different ages in the life of the patient.

21), only sparing chromosomes 13 and 17 which carry important tumor suppressor genes.[58] The onset of IGH translocation can be easily ascribed to aberrant CSR promoted by AID. Evidence for this hypothesis comes from the rearrangement hotspot, close to the canonical CSR breakpoints[59] and from the signature of AID-induced mutations on the translocation partner genes.[60] Promoted by Ig enhancers, overexpression of the recurrent partner genes *CCND1*, *WHSC1*, *MAF*, *MAFB*, *CCND3* in t(11:14), t(4:14), t(14:16), t(14:20) and t(6;14) respectively are thought to mediate transformation. As expected for founder events, IGH translocations are almost always clonal, mutually exclusive with each other and with the HD karyotype.[18,20] The onset of HD and its pathogenetic role in the development of PCs dyscrasias is more mysterious and still debated. Similar to what postulated in hyperdiploid acute lymphoblastic leukemia, one hypothesis is that the HD karyotype derives from a single abnormal cell cycle duplication.[18,61,62] However, preliminary observations by FISH have shown that HD MGUS have fewer overall trisomies than HD MM, suggesting that the acquisition of chromosomal duplications may be a step-wise phenomenon.[63] More recently, thanks to an elegant analysis of mutational signatures, Maura et al drew two important conclusions on the pathogenesis of HD MM. First, gene mutations in HD chromosomes are induced by AID only until the duplication happens, as AID activity is reduced to absent in mutations acquired after the gain. This clearly points to a GC origin of the duplication, similar to IGH translocations.[50] Second, restricting analysis to mutations induced by processes with a constant mutation rate, it was found that the ratio between pre-gain and post-gain mutations was often different from chromosome to chromosome, confirming that different trisomies can be acquired in different time windows. Furthermore, even pre-gain mutations are often acquired in different time-windows, confirming that the HD karyotype is the end result of several rounds of gains, all of which take place in the GC.[64] Concerning the mechanism of transformation of HD cases, this is equally obscure, but an hypothesis is that the upregulation of oncogenes mapping to trisomic chromosomes could be the driver for disease development.[65]

While IGH translocations and HD are transforming events for plasma cells, they are required but not sufficient for myeloma development as many MGUS carry these events without showing evidence of progression for decades. Several studies have therefore investigated the genetic background of MGUS in comparison with MM, as MM-specific events would be those linked to disease progression.[66–68] Within the IgH translocations,

the t(11;14) was more prevalent in MGUS, while all other were more prevalent in MM, suggesting they may confer a different drive towards transformation.[67,68] Indeed, the translocations usually associated with reduced survival t(4;14), t(14;16) and t (14;20), have been detected in MGUS with lower frequency than MM.[68–70] Of note, no translocation involving MYC has been found in MGUS, arguing that MYC rearrangements would be linked to disease evolution.[67,68] Similar to hyperdiploidy, copy number aberrations (CNAs) in MGUS seem to be less represented than in more advanced stages of the disease.[66–68,70,71] Consequently, many CNAs may be found at the subclonal level, that is, only in a fraction of the cancer cells, as is the case for 11q and 21q gains and 16q and 22q deletions.[66] The same was true for chromosome 13 and 17 deletions.[68–70] Copy number gains in chr (1q21) deserve a particular mention, as a trisomies in that locus are a relatively early but rare event in asymptomatic stages, and when present correlate with spontaneous progression. On the contrary, amplifications (>=4 copies) are later events associated with high-risk symptomatic myeloma at diagnosis or relapse,[67,72] again highlighting the multi-step nature of myeloma evolution. Analysis of gene mutations in MGUS has often been hampered by contamination of the tumor DNA by normal plasma cells. However, recurrent mutations were found in the myeloma genes *NRAS*, *BRAF*, *KRAS*, *DIS3*, *EGR1* and *LTB*, and again allelic frequencies were indicative of intraclonal heterogeneity.[67,73] Importantly, no mutations have been detected in tumor suppressor genes such as *TP53*, or in genes involved in DNA repair mechanisms as *ATM* or *ATR* usually enriched in more advanced phases of the disease.[67,74] Not surprisingly given the described heterogeneity, a major advance in our understanding of MGUS biology came from the analysis of single cell transcriptomes (scRNAseq): here, Ledergor et al, showed that within the BM population in MGUS most PCs are normal, but in few cases within the low percentage of total BM PCs there is a high fractional representation of PCs with a malignant transcriptome. This opens the possibility of identifying malignant sub-clones responsible for the eventual disease progression already at preclinical stages.[75]

Altogether, this highlights how the genomic landscape of MGUS may evolve, thus providing the biological bases for clinical progression. It follows that MGUS, despite being a clinically indolent condition for most patients, can show a rather unexpected degree of clonal heterogeneity with some founder lesions and others acquired during disease course. This may serve as a "catalogue" of genomic lesions that provide the seed for further disease progression upon acquisition of a subsequent hit,[20,66–71,73,75,76] recapitulating the "big-bang theory" originally described in solid cancers.[11,50,73]

Disease evolution: From MGUS to smoldering MM and symptomatic phase

The prevalence of MGUS is up to 5% in the general population older than 50 years, with a positive correlation between age and its incidence.[56,77] Because most MGUS patients are not subject to repeated BM examinations during follow-up, it is rare to demonstrate a transition from MGUS to SMM. This hampers the analysis of early events associated with progression to SMM. Rather, what is usually recorded is the rate of progression of MGUS to MM, which is 1% per year and remains constant even after decades.[78] This argues against a slow, constant accumulation of genetic lesions as the cause of transformation, which would cause an increased risk over a long follow-up. Rather, it is plausible that MGUS are truly indolent clonal expansions, and genomic events may ensue at any moment causing transformation. These events would be stochastic, have a very low frequency, and act in bursts rather than in a linear fashion.

Conversely, SMM is an intermediate stage between MGUS and symptomatic MM. It is characterized by a higher disease burden than MGUS, and clonal PCs are usually evident in the BM. End organ-damage, signs of active myeloma and myeloma-defining events are nevertheless absent.[79] Importantly, the rate of progression is not constant: it is 10%/year in the first 5 years, declining to 3% for the next 5 and to 1% after ten year from diagnosis.[80,81] This suggests that a heterogeneous group of patients may fall in the category of SMM: some with incipient MM that does not yet satisfy clinical criteria for diagnosis, and others with a truly indolent, MGUS-like disease despite a large tumor burden. The heterogenous behavior in terms of disease progression is recapitulated also by its biology. Because many SMM may take years to evolve, initial studies have focused on high-risk groups which progressed over a short time. In this context, Bolli et al have described the whole-genome landscape of 11 cases, highlighting the known clonal heterogeneity, and driver events composed by translocations, CNAs, and gene mutations. Structural events, and specifically complex ones, were frequent. Known secondary aberrations were very common, including losses in chromosome 13, 6q, 8p and 16q as well as chromosome 1q amplifications. Overall, the genomic landscape of high-risk SMM resulted very similar to that of MM.[82] Analyzing paired samples, two main patterns of clonal evolution to symptomatic MM were described. The so-called "static progression model", where the malignant population is already present at SMM stage and grows symmetrically into the MM stage.[82,83] And the "spontaneous evolution" model, where the genomic composition of SMM changes at progression to MM owing to loss of one or more subclones and/or acquisition of others that drive progression.[82,83] The biological heterogeneity of these two models is reflected also by the different timeline of their development. If the static model takes on average less than one year to progress, the spontaneous evolution would usually evolve over a longer time due to the need of the acquisition of new genetic lesions.[82,83] Analysis of mutational processes responsible for the catalogue of genomic lesions of SMM was also particularly revealing. Early clones, that is, those associated with disease initiation where enriched for activity of AID and age-related mutational processes. Late clones, that is, those acquired at the time of progression, showed little to no AID activity, and were instead enriched for APOBEC activity and SBS8. While the process responsible for SBS8 is unknown, APOBEC is a family of DNA deaminases that display aberrant activity in a variety of cancers. In MM, they are associated with MAF translocations[84] and generally with a higher mutational burden, complex rearrangements and poor prognosis.[50,60,85,86]

Translating these observations to clinical practice, it is tempting to assume that SMM cases can truly be dichotomized into MGUS-like cases, which need additional genomic events to progress, and MM-like cases, which already show all features of an aggressive neoplasms and only need time to accumulate enough burden/organ damage to satisfy the current clinical criteria (Fig. 2). Recently, a larger study from Bustoros et al confirmed these preliminary observations: the SMM genome was in fact shown to carry most alterations later found in MM.[87] Importantly, this allows the harnessing of genomic data to build predictive models that may be more accurate than the current ones, mostly based on surrogates of tumor burden,[83] and opens

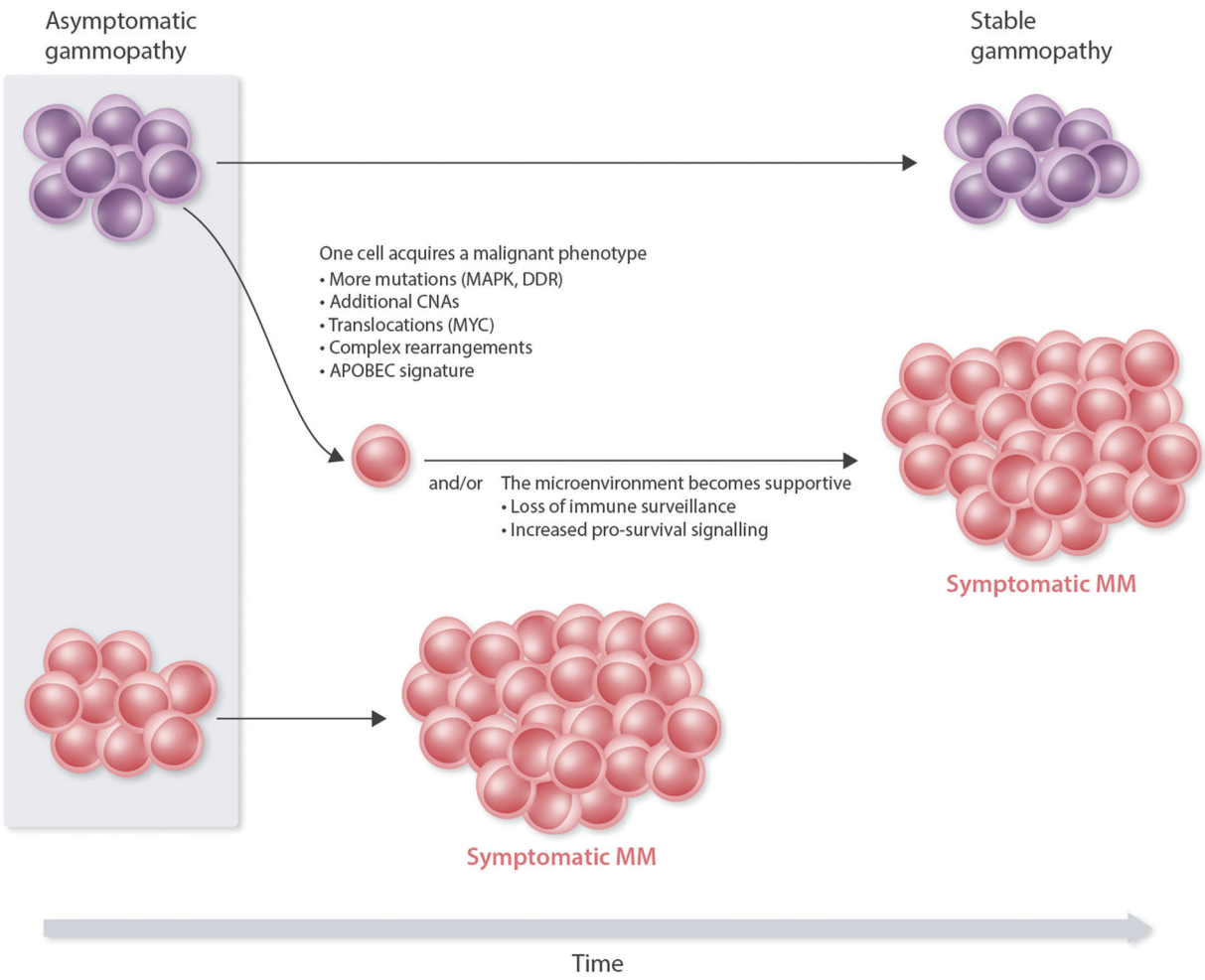

Figure 2. Progression of monoclonal gammopathies. After the onset of a monoclonal gammopathy, the disease proceeds to symptomatic Multiple Myeloma in two possible ways. 1) a MGUS-like pattern (purple), which may remain stable for life or result in the long-term expansion of an indolent disease until the acquisition of a malignant phenotype (red) and the subsequent evolution to MM. 2) an MM-like pattern, characterized by the presence of malignant features already at the onset of the disease (red), which make the clone more aggressive and able to evolve more rapidly.

the field for personalized prediction of risk in SMM based on genomics.[88] Integrating previous findings,[89] the authors indeed showed that the presence of mutations in MAPK genes or DNA repair genes, MYC abnormalities and the t(4;14) translocation were all independent factors predicting progression. Furthermore, a higher contribution of APOBEC-induced mutations was found in samples that progressed, highlighting the role of this process in MM evolution.

Cell-extrinsic factors and their role in disease initiation and evolution

MM PCs are also strictly dependent on the microenvironment, and this is thought to play an important role in disease initiation and progression. The microenvironment of disease initiation is that of the GC, where antigen-presenting cells and T-helper cells start the processes of AID-induced CSR and SHM. Once in the BM, clonal PCs can receive both supportive and suppressive signals from the microenvironment, and since not all patients with MGUS/SMM with a similar genetic makeup eventually progress to MM, this implies that non-genomic alterations may be required. The variable correlation between microenvironment changes and SMM progression has been shown by several studies[90,91] where immune and non-immune cells have been implicated. The immune environment in MGUS and SMM has been studied in depth by single-cell RNA sequencing approaches coupled with functional studies.[92] Already at the MGUS stage, the PC clone was shown to shape a distinct response characterized by a heterogeneous enrichment of T-cells, monocytes and NK cells. CD14+ monocytes in particular showed a phenotypic switch with loss of MHC class II expression, thus acquiring suppressive functions towards T-cell activation. At the SMM stage T-cells showed a considerable loss of CD8+ memory cells, which bear a crucial role in MM immunosurveillance. However, the first direct, *in vivo* evidence for immune surveillance restraining growth of monoclonal gammopathies was provided by a humanized mouse model where xenografts from MGUS and SMM patients were implanted. Here, the xenograft showed unrestricted progression, implicating the patient's immune system as the factor hampering disease evolution. Interestingly, progression in the mouse model was promoted by a minor subclone, underrepresented in the host, showing how the heterogeneous genomic landscape of SMM allows a dynamic clonal escape under a different immune surveillance.[93] Harnessing the immune system to the patient's advantage is therefore an effective option that has proven

clinically valuable. Different compounds able to target the immune-microenvironment and not just the plasma cell have shown remarkable activity. Daratumumab targets the CD38 antigen on the plasma cell and promotes cell death, but is also able to eliminate immune-suppressors cells such as T regulatory or myeloid-derived-suppressor cells, thus enhancing anti-MM response.[94–96] IMiDs may reduce exhaustion markers on T lymphocytes and an enrich the NK population.[97] Moreover, IMiDs may positively interact with Daratumumab potentiating its effect.[96,98]

The non-immune environment also has a role in disease progression and organ damage, showing how the spontaneous, mostly neutral evolution of several subclones in asymptomatic phases provides the required plasticity to drive progression under selection from cell-extrinsic factors. For example, the osteoblastic niche plays a central role in the maintenance of pathological, quiescent MM cells and in the development of bone disease by modulating the expression of hypoxia inducible factor (HIF)-1α and *RUNX2,* master regulators of Notch and Wnt/β-catenin pathways.[99] Moreover, in strict connection with the osteoblastic niche and its drivers, also angiogenesis plays a crucial role in MM development and progression.[100–103] In this particular process, neo-generated endothelial cells may produce pro-angiogenic factors such as HIF-1α[101] or players of the HGF/c-MET axis[104] as well as adhesion molecules, which may foster MM cell survival and resistance to treatment.[105,106] Targeting the non-immune environment may also be a complementary strategy to treat MM. Ulucuplumab, an anti-CXCR4 monoclonal antibody, showed an high rate of response in combination with lenalidomide and an important restriction of PC dissemination.[105,107]

Symptomatic MM, risk stratification and progression to high-risk phenotype

The mutation rate of most cancers is assessed between 10^{-10} and 10^{-7} mutations/bp/division, and MM carries about 1 mutation/Mb of the genome at diagnosis.[4] Several factors other than disease burden dictate the symptomatic status of MM. However, it is conceivable that at the time of MM diagnosis the patient bears billions of clonal PCs. Since mutational processes are ever ongoing, each of these billion cells will keep acquiring additional mutations even after the last full clonal expansion, that is, after the transformation process from a normal plasma cell to a tumor cell is complete. Some of these mutations will be positively selected and result in expansion of subclones bearing them, but most will be neutral, that is, be present uniquely in the cell where they originated.[7] Factoring in the number of clonal PCs at diagnosis and the MM mutation rate, after only one cell division the probability of any possible mutation in the MM genome approaches 1.[108] In other words, even a small tumor is likely to have sampled any possible genomic mutation during clonal expansion before its clinical onset, creating an enormous clonal diversity. Most of the mutations will nevertheless be under neutral evolution pressure, meaning that they will be unique to each cell, and therefore invisible to current bulk sequencing approaches. Depending on the type of pressure though, evolution may change: in asymptomatic stages, this allows escape from immune surveillance[93] and seeding of different anatomical sites, creating a well-known spatial heterogeneity at diagnosis[109]; in symptomatic stages, this diversity will serve as a catalogue of different clones that may survive different selective pressures induced by treatment. Again the complexity of MM genome, its temporal and spatial subclonal evolution, and the mechanism of growth within the BM is more reminiscent of solid cancers than acute leukemias.[16]

When it comes to active MM at diagnosis, among the major questions that recent progress on MM genomics has raised are: can we use this wealth of information to provide a biologically sound and clinically useful MM classification? Can we improve our prognostic models in MM? Can we provide rationale targets for innovative treatments?

Concerning classification, this has not changed substantially from the dichotomy between HD and IGH-translocated cases highlighted by classical cytogenetic studies.[58] Apparently, these initiating events determine both the expression profile of cells,[110,111] and the subsequent genomic abnormalities acquired during progression.[64,112,113] In fact, the non-random distribution of gene mutations and CNAs suggests that the initiating event predetermines which dependency is subsequently built in the genome of that case, at least to an extent. Examples are *KRAS*, *NRAS*, *FAM46C* mutations and *MYC* translocations in HD cases; *CCND1* and *IRF4* mutations in t(11;14) cases; *DIS3* and *FGFR3* mutations in t(4;14) cases. Indeed, this classification seems to really reflect different biological entities. Nevertheless, its clinical utility is questionable since it does not provide prognostically useful information. Despite enormous progress in MM genomics in fact, treatment still follows a "one size fits all" approach. Furthermore, while a robust classification should be stable over time, any prognostic marker may change its value relative to the treatments available. Last, given the dynamic and vast clonal heterogeneity of the disease, prognostic markers can be lost or acquired over time.

An unmet clinical need in MM is therefore a reliable identification of the so call "high-risk" subgroup, defined retrospectively as the 20% to 30% of total cases that do not respond to -or relapse early after- induction treatment even in the context of novel treatments.[114–117] Some genetic categories carry a somehow increased risk due to inherent features, such as *MMSET* and *MAF* overexpression in t(4;14) and t(14;16) respectively. These are captured by the revised international prognostic staging system R-ISS, the first attempt to integrate genetic data in MM prognosis.[118] However, additional events can explain this excess risk: for example, increased APOBEC activity confers worse prognosis. Cases with MAF translocations show hyperactive APOBEC activity,[84] and this may explain their poor response to treatment at least in part. However, the prognostic value of increased APOBEC activity is independent of MAF, as it is observed across all cytogenetic subgroups.[85] Furthermore, different APOBEC isoforms are involved in MAF and non-MAF cases.[50] Future studies will elucidate clinical and biological correlates of this observation. In t(4;14), high-risk features such as amp(1q), del(13), del(17p) are often subsequently acquired. However, secondary abnormalities can be acquired by any cytogenetic subgroup. For example, a fraction of HD cases can acquire IGL-MYC translocations which would significantly worsen the prognosis.[119] It has become increasingly clear how MM shows a vast array of complex rearrangements, including chromothripsis, chromoplexy, and a novel translational mechanism calls "cycles of templated insertions".[64] This and other events, like "jumping translocations"[120] and the formation of isochromosomes can lead to CNAs in recurrent areas of the MM genome such as del(1p), del(6q), del(8p), del(17p), amp(1q), gain (17q). These CNAs are enriched in chemo-refractory cases,[121] and proposed mechanisms of tumor aggressiveness have been extensively reviewed.[115] At the extreme end of the spectrum are extreme aneuploidies like hyperhaploidy, whole genome

duplications and hypodiploidy, all conferring high risk.[64,115] Interestingly, gene mutations seem to play a very little role in the definition of high-risk cases as compared to structural variants, and many of the reported variants in NDMM are often not even expressed.[122] MAPK pathway genes have no prognostic significance, and the same is true for mutations in tumor suppressor genes of the NF-kB pathway and DNA repair pathway, with the exception of *TP53* mutations.[113,117] However, tumor suppressors such as *TP53*, *RB1*, *DIS3*, *CYLD*, *TRAF3*, often show a pattern of bi-allelic inactivation at relapse that is prognostically relevant.[123] Similarly, a combinatorial effect of lesions may confer a specific prognosis that is not evident when any lesion is considered in isolation, as is the case for *PRDM1* deletions along with either t(4;14) or *BIRC3* deletions.[113] Last, increasing numbers of chr(1q) copies confer poor prognosis[124] highlighting how disease prognosis is linked to the complexity of its genome, which is the basis for the definition of "double hit" or "multiple hit" myeloma.

While gene mutations would be easy to diagnose in routine clinical practice, and could be therapeutic targets, their role in dictating disease behavior is still mostly unknown, and therefore the clinical utility of their detection is still limited. Similarly, current treatments with proteasome inhibitors and immunomodulatory agents do not seem to select specific gene mutations, as mediators of treatment response are only rarely mutated.[125–130] However, a somewhat specific sensitivity of t(4;14) cases to bortezomib treatment has been described even if the mechanism remains unknown.[131]

Conversely, rare "druggable" gene mutations such as the BRAF V600E must be approached with caution since they are often subclonal[60] and their inhibition may trigger paradoxical growth of RAS activated PCs.[132–134] Indeed, subclonality of driver lesions is an inherent feature of MM evolution and raises the question as to whether the prognostic value of gene mutations also depends on their clonal fraction. Conflicting data exist on the matter, and some groups advocate a tumor fraction >55% for a subclonal del(17p) before it should be considered prognostic.[135] However, from an evolutionary perspective, high-risk lesions can only be selected positively by treatment, and therefore their simple presence should warn the clinician (Fig. 3). Again on an evolutionary perspective, one could wonder if genomic lesions underlying relapse are already present at diagnosis, just in very few cells and thus "invisible" until selective pressure from treatment is applied, or they are truly acquired during treatment.

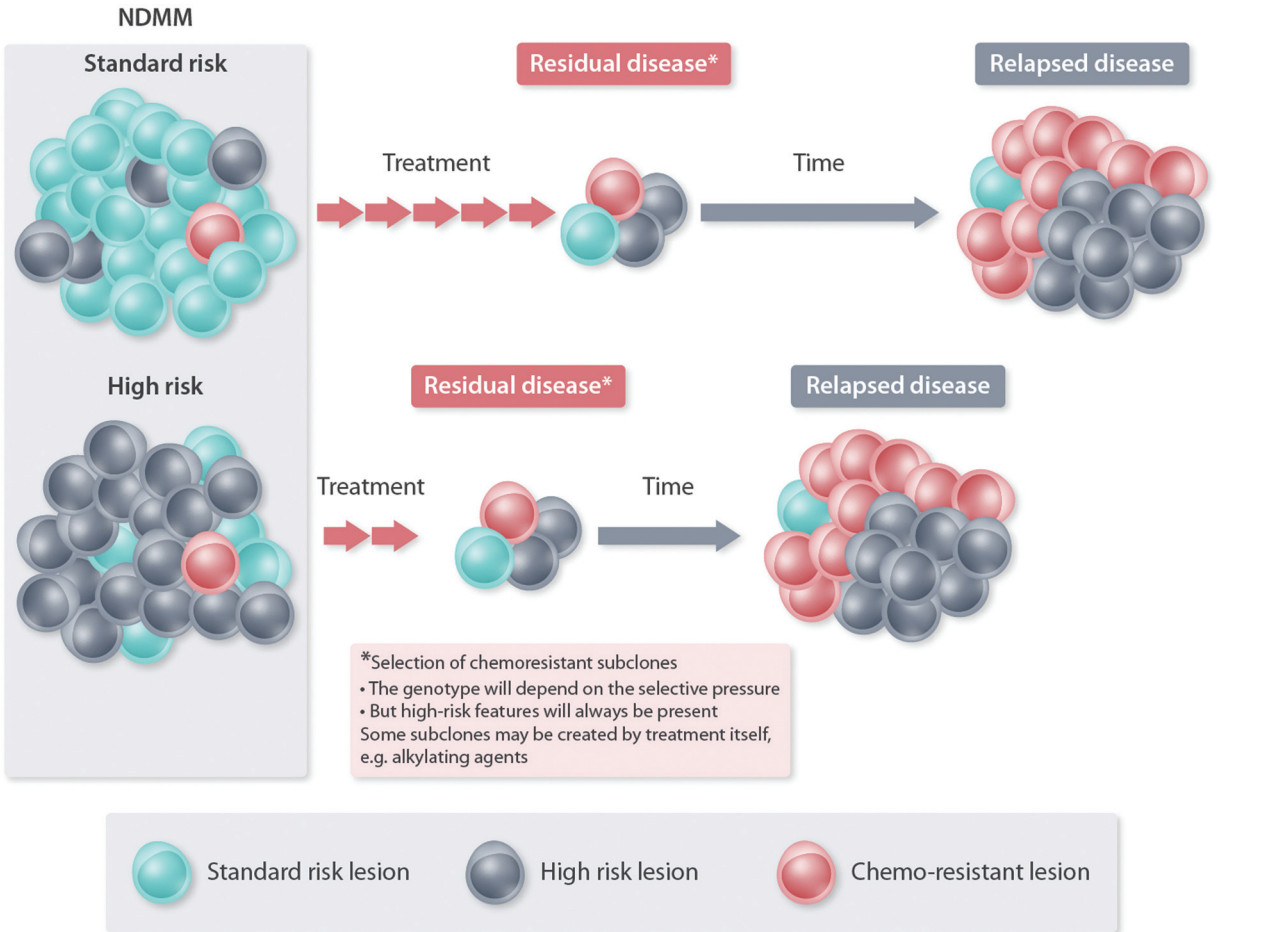

Figure 3. Progression to high-risk myeloma. At diagnosis, MM is composed of a heterogeneous mixture of subclones. High risk features (black) may be absent or poorly represented and most cells would carry standard risk features (green). In high-risk groups, most cells at diagnosis would carry high-risk features. After treatment the disease burden shrinks, but residual cells are likely enriched in high-risk features and possibly pre-existing cells carrying mutations conferring chemo-resistance (red). At relapse, these two features are enriched explaining the lower response rates to subsequent treatments and often lack of response to re-treatment with first line drugs.

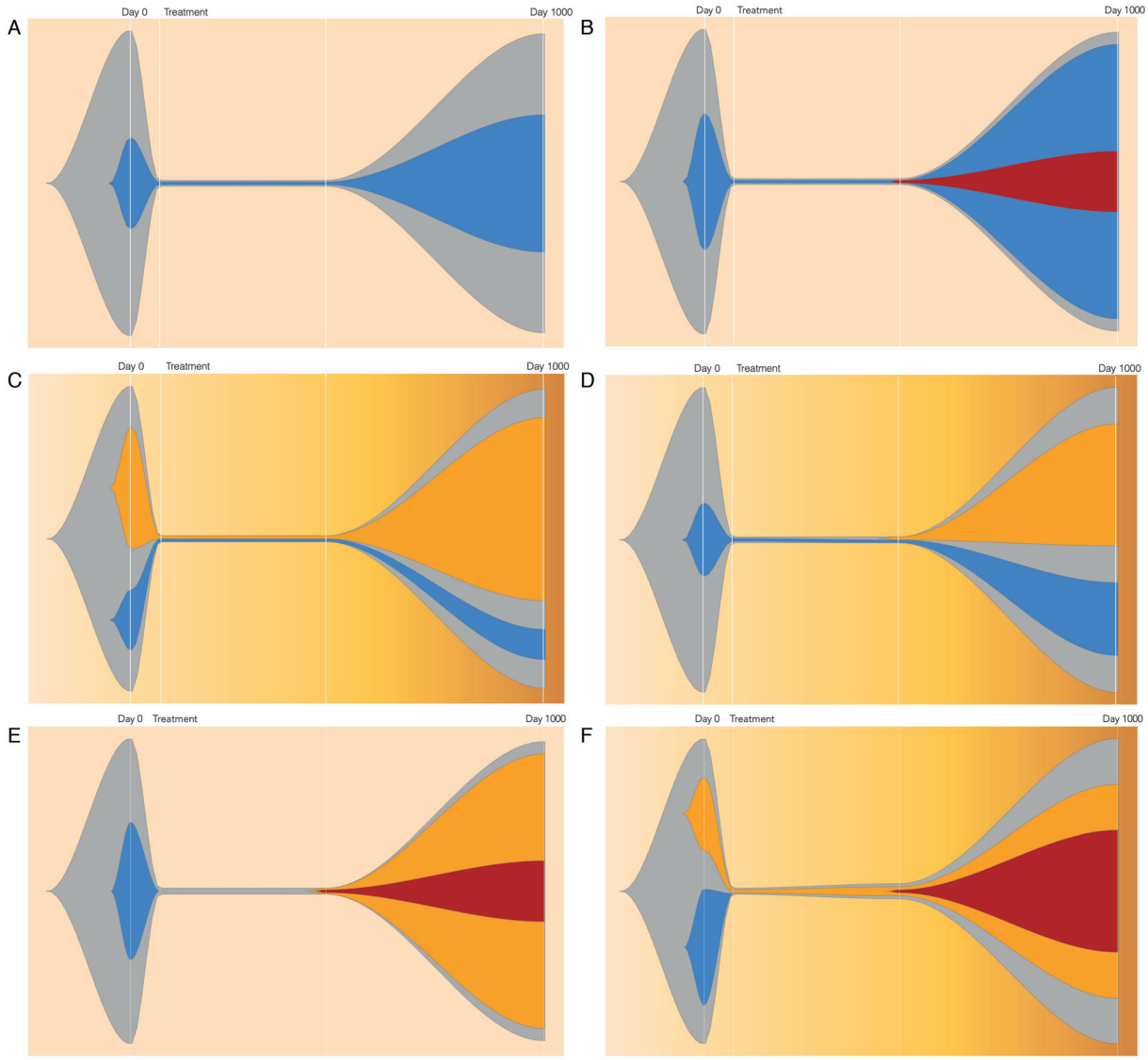

Figure 4. Patterns of clonal evolution from Diagnosis to disease relapse. Treatments that are applied after the symptomatic Multiple Myeloma diagnosis will impose a selective pressure supporting the emergence of peculiar resistant clones. (A) the founding clone (grey) and a minor subclone (blue) both respond to treatment, but both reemerge in different proportions at relapse. (B) At diagnosis, as in A a founding clone (gray) and a minor subclone (blue) constitute the disease burden. At relapse, the minor subclone takes over and from it a second subclone (red) emerges in a linear fashion. (C) Two subclones (yellow and blue) are present at diagnosis but are impacted differentially by treatment. At the time of relapse the yellow subclone becomes predominant owing to supposed intrinsic chemoresistance. (D) the founding clone (grey) and a minor subclone (blue) both respond to treatment, but at relapse a second subclone branches out from the founding clone and contributes to relapse. (E-F) Two examples of branching clonal evolution: a minor subclone (blue) is eradicated by treatment and disappears at time of relapse. In E, at relapse, a new subclone emerges (yellow) from the founding clone and, in a linear fashion, a second emerges from the latter (red); in F, the yellow subclone is already present at diagnosis, reemerges after treatment, again giving rise to a second subclone (red). The fishplots are just examples, and have been generated by the "Fishplot" R package (https://github.com/chrisamiller/fishplot).

A similar question has been addressed in chronic myeloid leukemia, where the cancer cells can overcome tyrosine kinase inhibitor treatment by expanding a pre-existing clone with ABL mutations.[136] In MM, mutational signature analysis has shown that high-dose melphalan can induce mutations in the relapsed clone[50,121] thus supporting the new acquisition of mutations. On the other side, serial sample analysis has highlighted both expansion of a pre-existing small clone and appearance of a new one at relapse[60,133] (Fig. 3). Inherent to MM subclonal heterogeneity is, therefore, the presence of varied patterns of relapse, ranging from no change to complex branching evolution where some subclones are lost and others are gained (Fig. 4). This also applies to relapses analyzed in homogeneously treated cohorts, so that no treatment-specific evolutionary patterns of relapse have been demonstrated so far. More than mutations of drug target genes, which are rare events even under selective pressure (ie, CRBN mutations during lenalidomide maintenance),[137,138] disease relapse was shown to be driven by emerging high-risk subclones carrying high-risk features[121,137] (summary of high-risk and drug resistance genetic features in Table 1). Only deeper sequencing and single cell technologies will be able to resolve this heterogeneity and observe drug-specific patterns of response at the single cell level. Recently, treatment with the BCL-2 inhibitor venetoclax has shown promising activity in MM.

Table 1

Summary of High-risk and Drug Resistance Genomic Alterations in Multiple Myeloma.

Disease stage	High-risk feature	Chemoresistance	References
SMM	Complex rearrangements	not known	#64
SMM	APOBEC signature	not known	#82
SMM	DNA repair pathway mutations	not known	#87
SMM	MYC alterations	not known	#87
SMM	MAPK mutations	not known	#87
SMM	IGH-MYC translocations	not known	#89
MM	APOBEC mutational signatures	Not known	#85
MM	Driver gene mutations	Not known	#112
MM	Mutations, CNAs, translocations	Not known	#113
MM	TP53 and IGLL5 mutations, λ-chain translocations, high-LDH	Not known	#117
MM	t(4:14), t(14;16), del(17p)	Not known	#118
MM	IGL translocations	Not known	#119
MM	TP53 inactivation; amp(1q) + ISS3	Not known	#122
MM	TP53 mutations, amp(1q), t(4:14), t(14;16), del(17p)	Not known	#124
MM	XBP1 mutations/downregulation	Bortezomib	#31
MM	Mutations, Complex rearrangements, CNAs	PIs/IMiDs	#121
MM	Proteasome subunits mutations/downregulation	PIs	#125,126
MM	TJP1 downregulation	Bortezomib	#127
MM	CRBN pathway mutations	IMiDs	#128–130
MM	MAPK mutations	BRAF-MEK inhibitors	#132–134
MM	Antigen gene deletion	BCMA CAR-T cells	#143
MM	CD55/CD59 downregulation	Daratumumab	#144
MM	BCL2-axis gene deregulation	BCL2 inhibitors	#139,146–149

BCMA = B cell maturation antigen, CAR = chimeric antigen receptor, CNAs = copy number aberrations, IMiDs = immunomodulatory drugs, ISS = International Scoring System, LDH = lactate dehydrogenase, PIs = proteasome inhibitors.

Interestingly, venetoclax provided a survival advantage only in patients carrying the t(11;14) or high *BCL2* expression levels and was deleterious otherwise,[139] suggesting this may be the first "personalized" treatment in MM.[140] However it must be emphasized how, while survival is constantly improving in recent years, this is largely thanks to combination treatments and the employment of new drugs and drug classes that show higher activity even in high-risk cases, more than targeting of specific oncogenic pathways.[18,115,141,142]

Pathogenesis-inspired future approaches to diagnosis, prognosis and treatment

An evolutionary perspective of MM development is quite fascinating per se since it highlights an unexpectedly dynamic biology. At the same time, it offers various points of reflection.

The advance of genomic and proteomic analysis is predicted to highlight an increasingly high share of the population carrying a small plasma cell clones, breaking the sensitivity threshold of serum protein electrophoresis. Since most cases of MGUS already pose a burden on healthcare while not being of clinical relevance, it will soon be necessary to categorize monoclonal gammopathies as "biologically evident" vs "clinically relevant", or in other terms define a new stage of pre-MGUS, or monoclonal plasmacytosis.

While the genomics of SMM is still in its infancy, initial data suggest that cases can be segregated based on the number and type of mutations, CNAs, translocations and mutational signatures, with implications for progression. In this respect, it may be time to move from a definition of risk of progression that is based on tumor burden, to one that is based on intrinsic tumor features such as its genomic profile.[83,150] In this respect, the same classical 10% threshold for BM plasmacytosis may be challenged in the future, since it does not correlate with the actual disease biology.[75,151] Taken to its extreme implications, this concept may in the future mandate that fewer patients are classified as SMM altogether, since it may be immediately clear who has an "MGUS-like" profile and can be managed expectantly, and who has an "MM-like" profile and would benefit from early treatment.

In symptomatic MM, extended genotyping seems to offer little advantage to clinical practice as of today. However, knowledge banks built on hundreds to thousands of MM cases with genomic and clinical annotation are highlighting prognostic groups that cannot be captured by FISH alone.[113,124] In the near future, the advent of new drug classes will complicate the treatment landscape of MM, offering potential benefits but also increased costs and risk of toxicity. Among those, cellular or antibody-based immunotherapies[143–145] or *BCL2*-axis inhibitors.[140,146–149] This mandates that novel biomarkers are found to rationalize treatment, implying that genomic analysis will become routine clinical practice at diagnosis and at each relapse.[88] This approach has been proven feasible by several NGS approaches.[150–152] Even more attractively, genomic lesions could be tracked serially over time through mini-invasive procedures such as cell-free DNA testing from peripheral blood,[153–157] paving the way for personalized care in MM.

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