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THE IMPACT OF INTRACLONAL HETEROGENEITY ON OUTCOMES OF MULTIPLE MYELOMA PATIENTS TREATED WITH NEW DRUGS

Presentata da:

Dott.ssa Annamaria Brioli

Coordinatore Dottorato

Chiar.mo Prof.

Lucio Ildebrando Cocco

Relatore

Chiar.mo Prof.

Michele Cavo

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INTRODUCTION

In recent years significant progresses have been made in the understanding of multiple myeloma (MM) biology. These advances have translated into the development of new drugs and in a different approach to treatment, which has ultimately translated into an unprecedented rate of responses and complete remissions (CR). Current strategies for the treatment of MM involve both the concept of sequential blocks of therapy given as an induction followed by consolidation and maintenance and the concept of a continuous treatment. Immunomodulatory drugs (IMiDs) and proteasome inhibitor form the backbone of modern MM treatment, but new and more targeted treatments are under development and are being tested in the contest of clinical trials. It is, therefore, of primary importance to understand the biology of MM and how this biology can guide us in the development of new treatment strategies with the aim of personalising therapy. Cancer is initiated and then progresses through a complex mechanism based on the acquisition of genetic "hits" that change the biology of the target cell from normal to malignant. The "hits" can be broadly divided into "driver" lesions contributing to a selective advantage, and "passenger" lesions that are neutral in their effect [1,2]. It is important to understand the complex genetic landscape of cancer not only because it can inform us on how individual genetic lesions interact leading to disease progression, but also because of its impact on treatment [3-5]. Studies both on solid and haematological tumours have shown that cancer comprises a collection of related but subtly different clones, a feature that has been termed "intra-clonal heterogeneity" [6-9]. This intra-clonal heterogeneity is likely, from a "Darwinian" natural selection perspective, to be the essential substrate for cancer evolution, disease progression and relapse. These ideas derived from evolutionary biology are teaching us that cancer progression is driven via branching evolutionary patterns, rather than following a linear multistep process as was thought previously [4,6,10-12]. In this context the critical mechanism for tumour progression is competition between individual clones (and cancer stem cells) for the same microenvironmental "niche", combined with the process of adaptation and natural selection [4,10]. The Darwinian behavioural characteristics of cancer stem cells are applicable to the plasma cell neoplasm MM. Treatment of myeloma is undergoing an age of new discoveries and improvements. New mutations which are susceptible to targeted therapy are being discovered and tools for better analysing the molecular hallmarks of myeloma, such as high throughput sequencing, single-cell analysis and analysis of paired patient samples are enabling us to understand the evolutionary landscape of MM as well as its natural history in much more detail. The knowledge that intra-clonal heterogeneity is an important feature of MM biology

has changed our way to addressing cancer, now considered as a composite mixture of clones and not as a linear evolving disease. In this variable therapeutic landscape it is important for clinicians and researchers to consider the impact that evolutionary biology and intra-clonal heterogeneity have on the treatment of myeloma and the emergence of treatment resistance. It is clear that if we want to effectively cure myeloma it is of primarily importance to understand disease biology and evolution. Only by doing so will we be able to effectively use all of the new tools we have at our disposable to cure myeloma and to use treatment in the most effective way possible. The aim of the present research project was to investigate at different levels the presence of intra-clonal heterogeneity in MM patients, and to evaluate the impact of treatment on clonal evolution and on patients' outcomes.

BACKGROUND

1. MULTIPLE MYELOMA

Multiple myeloma is a haematological neoplasia characterised by the abnormal proliferation in the bone marrow of clonal plasma cells and B-lymphocytes. Rarely, in 7% to 15% of cases, plasma cells can accumulate in extramedullary tissues, giving rise to extramedullary myeloma [13,14].

MM represents 10-15% of all hematologic malignancies, being second only to Hodgkin lymphoma [14]. It accounts for 1% of all cancer and to 2% of all cancer deaths, with an annual incidence of about 4.6/100000 [15-17]. MM is a disease of old adults, and its frequency increases with aging: median age at diagnosis is 65-70 years and it is rare in people younger than 40 [14,18]. There is a slightly higher incidence in men and Afro-Americans have a probability to develop the disease double the one of Caucasians [14].

The neoplastic clone is characterised by its ability of synthesising, and in most case secreting, monoclonal immunoglobulin, either the complete immunoglobulin or only the light chain portion of it. These monoclonal immunoglobulins, called paraprotein or M component, can be detected in the serum and urine of the vast majority of MM patient; in a small percentage (1 to 2%) of patients, however, the paraprotein is detected neither in the serum nor in the urine, a form known as non-secretory MM (NSMM) [19]. It is believed that in NSMM the neoplastic cells produce a small amount of paraprotein that cannot be detected by serum or urine immunofixation. In this group of patients it is mandatory therefore to perform a test to dose the amount of serum free light chains (FLC) in order to monitor the disease and its response to treatment. The serum free light chain assay is able to evaluate both kappa (normal values 3.3-19.4 mg/L) and lambda (normal values 5.7-26.3 mg/L) light chain. The clonality of the production is than established based on the ratio between kappa and lambda values (FLC ratio, normal values 0.26-1-65): if the ration is below 0.26 there is an excess of lambda light chains production, whilst, a ratio higher that 1.65 indicates an excess of kappa light chains [19,20]. With the use of the serum FLC assay 70% of NSMM were actually shown to have a measurable disease. Furthermore serum FLC assay is not useful only in this subset of patients, but has different uses also in patients with secreting MM and patients with AL amyloidosis [20].

MM aetiology is still nowadays not completely understood; from a number of epidemiologic observations a factor likely to predispose to the disease is the exposure to toxic agents such as radiations, pesticides and products derived from petroleum. Family clusters and genetic loci

associated with an increased risk of developing MM have also been described in the general population, however without clear inheritance [21,22].

1.1 NATURAL HISTORY AND CLINICAL PRESENTATION

The natural history of MM proceeds from a phase characterised by a low tumour burden and the absence of end organ damage (Monoclonal gammopathy of undetermined significance, MGUS, and asymptomatic or smouldering MM, SMM) to a phase of active disease with end organ damage that requires treatment (symptomatic or active MM) [23-27].

Before MM becomes evident, in about one third of the patients, the occasional presence of an MGUS can be detected. MGUS is a premalignant condition, characterised by the presence of a small paraprotein (mainly in the serum) and the absence of any clinical manifestation (Table 1) [26].

Table 1. Definition of MGUS [14,27].

MGUS All three criteria must be fulfilled:
Paraprotein < 3g/dl
Bone marrow plasma cells < 10 %
Absence of organ damage as defined by CRAB criteria

The presence of an MGUS can be detected 3% of people older than 50 years and in 5% of people older than 70 years, and it has a risk of progression of about 1% per year [26,28].

Some patients can also be diagnosed with the intermediate phase of SMM, characterised by high levels of paraprotein and bone marrow plasma cells, but no end organ damage. The risk of progression from SMM to symptomatic MM requiring treatment can be estimated in about 10% per year and is 50% in the first 5 years [25,27]. Criteria for the definition of SMM are summarised in Table 2.

Table 2. Definition of SMM [14,27]

SMOULDERING MM All three criteria must be fulfilled*:
Paraprotein ≥ 3g/dl
Bone marrow plasma cells ≥ 10 %
Absence of organ damage as defined by CRAB criteria

*Any one of the following biomarkers of malignancy upgrades the diagnosis of SMM to MM even if CRAB criteria are absent:

- Clonal bone marrow plasma cell percentage ≥60%
- Involved:uninvolved serum free light chain ratio ≥100
- >1 focal lesions on MRI studies

The diagnosis of active or symptomatic MM is based on the following criteria (all three conditions must be fulfilled)* [14,27]:

Table 3. Definition of MM requiring treatment

Bone marrow plasma cells ≥10% and/or histologically confirmed plasmocytoma
Presence of paraprotein in serum or urine [a]
End organ damage defined as follows (at least one)[b]: [**C**] Elevated serum calcium (calcemia > 10.5 mg/L or higher than normal values) [**R**] Renal impairment (creatinine > 2 mg/dL) **[A]** Anaemia (Hb < 10 g/dL or 2 g < normal value) [**B**] Osteolytic bone lesions or osteoporosis[c]

*These criteria identify stages IB, II e III A/B of Durie and Salmon classification. Stage IA corresponds to SMM.

[a]If no paraprotein can be detected a bone marrow plasma cell infiltration ≥30% or a histologically defined plasmocytoma are mandatory (NSMM)

[b]Organ damages other than the one reported can occasionally be present. In this case it must be clearly demonstrated that organ dysfunction is due to MM.

[c]If only osteoporosis or a single bone lesion or a solitary plasmocytoma are detected it is mandatory a bone marrow plasma cell infiltration ≥30%.

The uncontrolled proliferation of neoplastic plasma cells within the bone marrow, the increased production of immunoglobulin and their peculiar characteristics together with the autocrine and

paracrine production of cytokines are responsible for the main clinical manifestations of the disease [13,29]:

- Bone involvement mainly characterised by osteoporosis and osteolysis: it is the most frequent way of presentation of MM, and it is secondary to the increased osteoclast activity and to the reduced osteoblast action (uncoupled bone reshaping). It is associated with severe bone pain, pathological fractures, spinal cord compression and hypercalcemia.
- Renal involvement: it is due to the increased production and the aberrant nephrotoxicity of light chains that are able to selectively target the renal tubules of the nephron, giving the typical feature called “myeloma kidney” or “myeloma cast nephropathy”. More rarely renal involvement can occur as a tubular and/or glomerular damage, such as Fanconi syndrome, AL amyloidosis, light chain deposition disease or heavy chain deposition disease.
- Bone marrow insufficiency: the most common feature is that of a normochromic and normocytic anaemia, due to heavy plasma cell infiltration within the bone marrow abrogating the normal myelopoiesis.
- Increased infective morbidity: it is linked to a reduced humoral and cellular immunity, due to the suppression of normal immunoglobulin synthesis and antibody response, reduction of the number and activity of $CD4^+$ Th1 T lymphocytes, a reduced cytotoxicity of $CD8^+$ T lymphocytes, and abnormal function of NK and dendritic cells. All these features are linked to an aberrant cytokine production (mainly TGFβ, IL10, IL6, VEGF) from the neoplastic plasma cells and bone marrow stromal cells, and result in an immunoparesis

The natural history of MM is characterised by subsequent phases of remission and relapse, defined by one or more of the above-mentioned clinical features. At each relapse response to treatment is less profound and shorter, with relapses that are more frequent over time until eventually the disease becomes resistant to treatment (relapsed/refractory phase), leading to patient’s death (Figure 1).

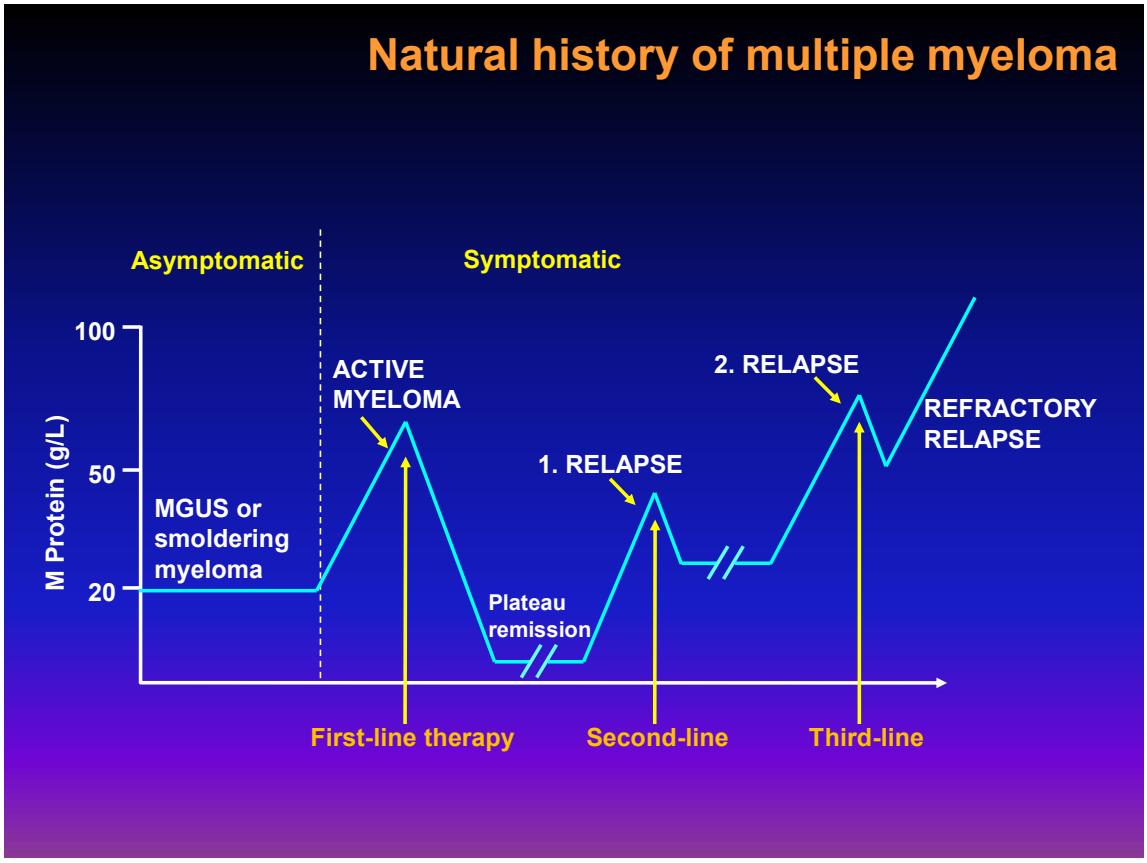

Figure 1. Modified from Hajek [30]

1.2 INTERNATIONAL STAGING SYSTEM AND PROGNOSTIC FACTORS

The high grade of heterogeneity in MM clinical presentation and clinical course has made mandatory the individuation of parameters to distinguish between a more aggressive and a more indolent disease, in order to predict patients' survival and possibly their response to treatment. In 1975 the first staging system, the Durie & Salmon staging system, was introduced. This model identified three different clinical stages (I, II e III), corresponding to an increasingly high tumour burden. The stages are defined based on the levels of haemoglobin, serum calcium, number of skeletal lesions and paraprotein concentration. Each stage can be than divided in two classes (A e B) according if renal function is normal (creatinine < 2mg/dl, stage A) or abnormal (creatinine > 2 mg/dl, stage B) [31].

Table 4. Durie and Salmon staging system [31]

STAGE I All criteria must be fulfilled: Hb > 10 g/dl, normal calcium, ≤1 bone lesion, low levels of paraprotein (IgG < 50 g/l, IgA < 30 g/l, BJ < 4 g/24 h)
STAGE II Not stage I nor III
STAGE III At least one between: Hb < 8,5 g/dl, calcium ≥ 12 mg/dl, ≥ 3 bone lesions, high levels of paraprotein (IgG > 70 g/l, IgA > 50 g/l, BJ > 12 g/24 h)

More recently the International Staging System (ISS) was developed. This model identifies three stages based on the levels of albumin (indirect expression of the secretion of IL6, and therefore of plasma cells malignancy) and β2 microglobulin (corresponding to level of tumour burden, renal function and immune system function) [32].

Table 5. International Staging System (ISS) [32]

STAGE I β2m < 3.5 mg/l and Albumin ≥ 3,5 g/dl
STAGE II Not stage I nor III
STAGE III β2m > 5.5 mg/l

These stages are linked to patients' prognosis, with patients presenting with a stage III MM having a shorter survival compared to patients presenting in stage I.

Other important prognostic factors are patient's age, performance status and the presence or absence of comorbidities.

Disease related prognostic factor are be divided in those linked to the tumour burden and those linked to the intrinsic malignancy of the plasma cells. Factors related to the tumour burden other

than the ISS are: 1) renal function; 2) the degree of bone marrow infiltration; 3) skeletal involvement identified by TC-PET of MRI; 4) platelets value at diagnosis; 5) levels of LDH.

Factor related to the plasma cells themselves are: 1) the levels of C reactive protein (PCR, expression of IL-6 secretion); 2) presence of circulating plasma cells in peripheral blood; 3) lack of response to first line treatment; 4) presence of cytogenetic abnormalities.

Recently the importance of serum FLC levels as a prognostic factor has also been described [27,33,34].

2. MYELOMAGENESIS

Pre-B cells develop in the bone marrow, where the V(D)J rearrangement for the heavy and light chain portion of the immunoglobulin takes place. These cells leave the bone marrow as naïve or mature B cells and migrates in the secondary lymphoid tissues, where they meet the antigens and proliferate differentiating either into plasma blasts or in memory B cells. Plasma blasts can differentiate in short-lived plasma cells (that normally dye within 3 days and produce mainly unmutated IgM) (22) or in long-lived plasma cells, that have undergone somatic hypermutation, isotypic switch and return to the bone marrow, where they can live up to 30 days [35].

MM plasma cells are normally identified as the long-lived plasma cells that reside in the bone marrow.

The study of the immunoglobulin's rearrangement through molecular techniques, such as for example polymerase chain reaction (PCR), demonstrated the presence, in MM patients, of a population of circulating B cells harbouring the same Ig rearrangement of the neoplastic population [36,37]. These data allowed scientists to conclude that the transforming event happens at the level of a lymphoid progenitor and that the germinal centre reaction drives the origin of the disease [35,38-41], however, recent evidence suggests that, at least in a proportion of patient, the transforming event can be attributable at a proB cell stage [35,41].

B cells circulating in the peripheral blood can home specifically in the bone marrow where they establish stringent interactions with bone marrow stromal cells (BMSC), from which they receive signals to differentiate and proliferate in mature plasma cells [42]. In this process this role of cytokines produced both by MM plasma cells and BMSC is crucial.

2.1 BONE MARROW MICROENVIRONMENT AND CYTOKINES[1]

Bone marrow microenvironment is constituted by a cellular compartment, comprising haematopoietic stem cells (HSCs), progenitors and precursor cells, NK cells, platelets, megakaryocytes, erythrocytes, lymphocytes, dendritic cells, fibroblasts, chondrocytes, osteoblasts, osteoclasts and endothelial cells. The extracellular compartment of the bone marrow is made of protein of the extracellular matrix (ECM) like collagens, fribronectin, laminine, proteoglycans, glycosaminoglycan and many soluble factors like cytokines, chemokine and growth factors [44]. The complex network between myeloma plasma cells and the bone marrow microenvironment, acting throughout autocrine and paracrine cytokinic loops and cell-cell interactions, is necessary for the growth, survival and proliferation of plasma cells and the acquisition of drug resistance.

The major families of molecules of adhesion involved are those of the integrin, cadherin, selectin, syndecans and of the superfamily of the immunoglobulin such as: syndecan-1 (also called CD138), VLA4, H-CAM, ICAM1, N-CAM, LFA3, CD56, CD74, VLA5, β2m. The liquid milieu is made of many soluble factors, such as IL6, VEGF, IGF1, TNFα, SDF1α, CD40, TGFβ, bFGF, MIP1α, SCF, HGF, IL3, IL1β, metalloproteasis [44].

Cytokines, growth factors and adhesion molecules are able to activate numerous signal transduction pathways within the plasma cells, the most important being (Figure 2 and 3):

- **Ras/Raf/MEK/MAPK pathway**, activated by IL6, IGF1, VEGF and integrines: it leads to cell proliferation and drug resistance.
- **PI3K/Akt pathway**, activated by IL6, IGF1, VEGF and integrines: inhibits apoptosis.
- **JAK/STAT3 pathway**, activated by IL6: leads to cell proliferation.
- **NFkB pathway,** constitutively activated.
- **Wnt/APC/βcatenin canonical pathway and Wnt/RhoA alternative pathway**, involved in the pathogenesis of bone disease.

The adhesion of MM plasma cells to the BMSCs determines an antiapoptotic and proliferative effect in the plasma cells, and stimulates the production from the BMSCs of IL6. IL-6 is one of the main cytokines involved in MM development, supporting the growth, survival and acquisition of drug resistance in MM cells, thus creating a vicious circle [44].

[1] Contains quotes from "The Impact of Intra-Clonal Heterogeneity on the Treatment of Multiple Myeloma" (43. Brioli A, Melchor L, Cavo M, Morgan GJ. The impact of intra-clonal heterogeneity on the treatment of multiple myeloma. *British journal of haematology*. 2014;165(4):441-454.)

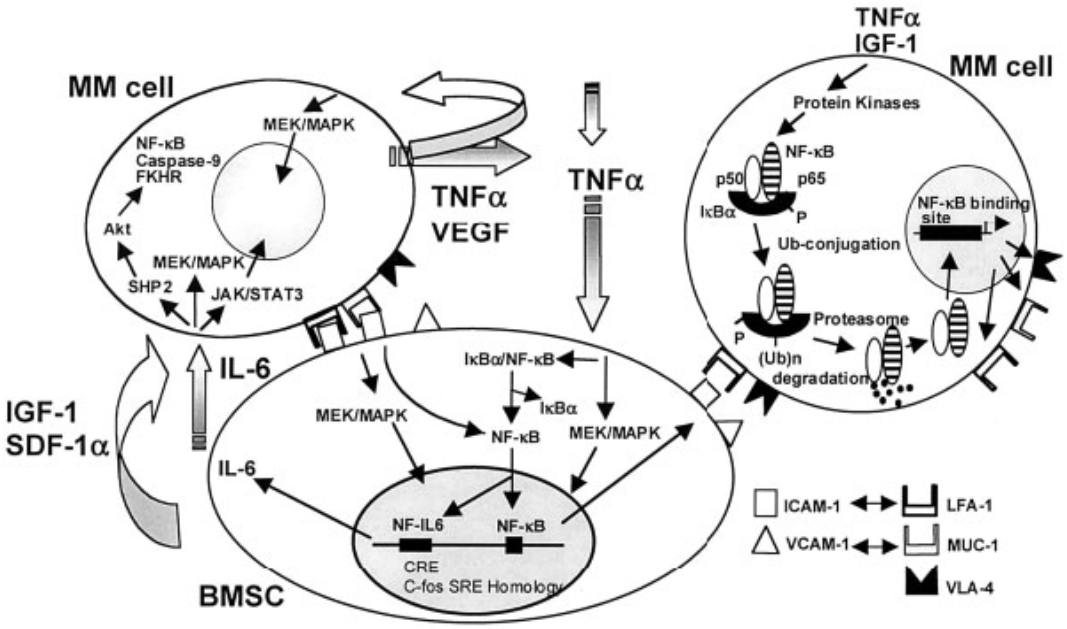

Figure 2. Interactions between cells in the BM microenvironment. Modified from Anderson et al. [45]

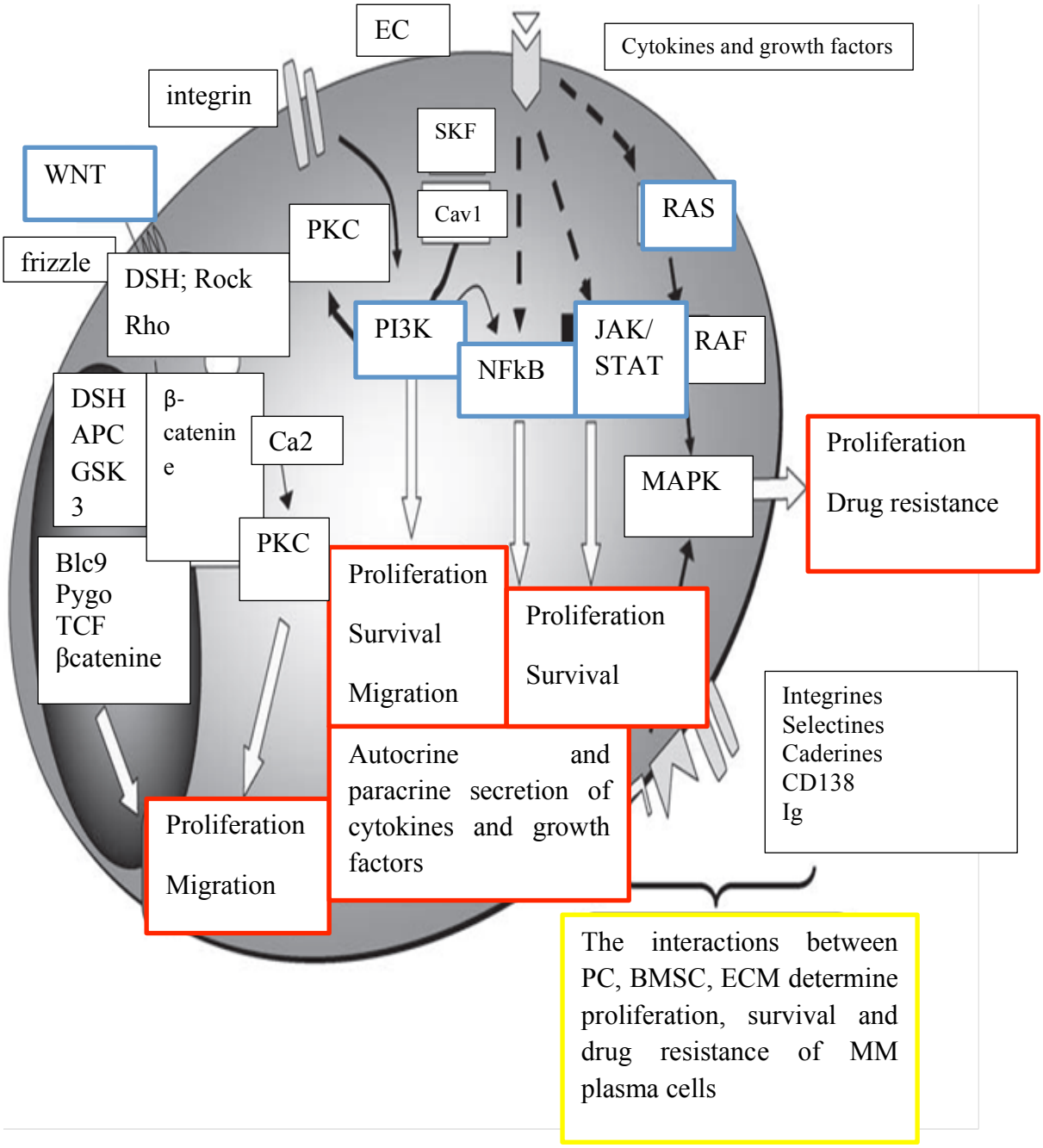

Figure 3. Cross talk in MM plasma cells. Modified from Podar et al. [44]

Recent evidences suggest the existence of a MM stem cell or MM propagating cell (MPC) characterised by self-renewal and proliferation properties [46]. The nature and phenotype of the MPC has still to be fully elucidated, but plasma cells which are $CD38^{high}$ and $CD45^{-}$ can replicate the features of myeloma in SCID-hu mouse models [47,48]. In the same mouse model the MPC were $CD138^{+}$ and cells with this feature could transfer the disease [49]. In contrast to these findings some studies have claimed that the MPC is $CD138^{-}$ [50,51], but this has not been confirmed [52]. In this context more recent flow cytometry studies have suggested that there is equilibrium between the $CD138^{+}$ and the $CD138^{-}$ compartment, in which cells with the biological features of an MPC are

located [53]. It seems, therefore, that $CD138^+$ and $CD138^-$ are not two separate entities, but rather populations with a different phenotype that move from one state to the other [53].

In the BM microenvironment reside also B ant T Lymphocytes, together with NK and dendritic cells. The activity of all these cells is highly down regulated, creating an immune tolerance towards the neoplastic cells that are therefore left free to proliferate.

The efficacy of the so called "novel drugs" (such as immunomodulatory drugs, IMiDs, and proteasome inhibitors, PIs) is linked to their ability to act on the BM microenvironment, disrupting the cross talk between the MM plasma cells and the BM milieu.

2.2 ANGIOGENESIS

Blood vessel can be developed through two different mechanism: vasculogenesis (active in the embryo), during which progenitor cells differentiate in endothelial cells and angiogenesis (active both in embryo and in adults), where starting from blood vessels already present new capillaries are generated.

Angiogenesis, as a source of oxygen and nutrients, is crucial in tumour development. Studies both on animal models and on men have demonstrated the development of new blood vessels in the early stages of tumour development, as well as in the late stages of metastatic dissemination [54,55].

It has long been known that both solid and haematological tumours have a great angiogenic potential, which correlates with their growth and dissemination. The observation that cancer can develop both in tissues rich of blood vessels and in tissues with a low blood density, suggest that tumour cells themselves, or the microenvironment in which they grow, are able to produce factors that stimulates angiogenesis.

Angiogenesis is regulated by the equilibrium of pro and anti angiogenic molecules. When this equilibrium is disrupted happen the so-called angiogenic switch [56]. The decrease of inhibitors concentration and activity, together with the increase in pro-angiogenic factors can occur in different situations, such as hypoxia of the tumour cells (due to an increase of the tumour bulk without a corresponding increase in vessel density), oncogene activation (such as H-RAS or V-SRC) or the inactivation of oncosuppressor genes. Furthermore macrophages (present in high quantity in area poor of blood vessels) are able to release pro-angiogenic factors, such as VEGF, FGF-2, TNFα and nitric oxide [57,58].

During the development of MM, hypoxia and cellular stress are able to induce the angiogenic switch, giving rise to the vascular phase or symptomatic MM. Many studies have shown that the secretion of di VEGF by $CD45^-$ plasma cells is crucial in this step [59]; VEGF is able to stimulate the

chemotaxis and proliferation both of endothelial (through VEGFR2) and of stromal cells (through VEGFR1). Other pro-angiogenic factors that are increased in MM are FGF, HGF, IGF1, IGF-BP3, whilst an antagonist of VEGF, semaphorin 3a, is reduced. This process contributes to the formation of an intricate net of new blood vessels, which sustain the growth of the neoplastic clone. A vicious circle is created in which plasma cells promote angiogenesis, whilst endothelial cells produce VEGF, FGF2, MMP and chemokines that support the growth and migration of neoplastic cells. The microvessel density (MVD) in the BM of MM patients is strictly linked to a higher proliferation index of the PC (plasma cells labelling index: PCLI) and to more advanced disease stages.

The persistence of angiogenesis in the BM of patients that have achieved a complete remission has also been shown, probably linked to a persistent angiogenetic stimulus from a minimal amount of residual disease. This persistent angiogenesis is able to increase the secretion of growth factors important for the malignant clone, thus contributing to disease relapse. Furthermore the aberrant vascularisation of the tumour can be an obstacle to an effective drug delivery in the tumour.

The pivotal importance of angiogenesis in MM development has been confirmed also by the success of IMiDs e PI, whose antitumor action involves an anti-angiogenic effect.

2.3 BONE DISEASE

Bone disease is the most frequent and serious manifestation of MM. During the disease course 70-80% of the patients will develop bone lesions, frequently associated with bone pain and, in 60% of the cases, with pathological fractures.

The area most frequently involved are those rich in BM, such as the spine (49%), the skull (35%), the hips (34%), the ribs (33%), the jaw and the long bones.

MM bone lesions are due to an increased bone resorbtion without a correspondent bone neoformation in areas where plasma cells are infiltrated; these lesions are therefore lytic lesions and do not repair over time, even if a complete remission is achieved.

Bone pain can seriously impair patients' quality of life; bone pain is usually greater in the spine and is increased by movement, being different from metastatic carcinoma pain that is worse at night. In these cases a correct pain therapy is mandatory, following the WHO recommendations; antalgic radiotherapy and orthopaedic surgery (vertebra or kyphoplasty) can also be helpful in selected cases.

A more rare (15%), but also very serious complication of bone disease can be hypercalcemia. Hypercalcemia is due to calcium mobilization from the bones into the blood stream, to reduced bone mineralization and to renal impairment; a prompt recognition and treatment, with the use of hydration and intravenous administration of bisphosphonates, is of uttermost importance.

2.3.1 PATHOGENESIS OF BONE DISEASE

In physiologic condition there is a constant bone remodelling during life. Bone remodelling is sustained both from mechanical solicitation (traction forces given by muscles, tendons and ligaments) and from the metabolic calcium request, regulated by parathormon and vitamin D.

In order to have an optimal function of the skeletal tissue it is necessary that bone remodelling works as a harmonic process (coupled bone remodelling): bone resorbtion from the osteoclast must be balanced by a correspondent bone neoformation by the osteoblasts.

In MM patients bone remodelling is uncoupled, unbalanced in favour of bone resorbtion. The decrease in bone mass is a consequence of the augmented osteoclasts (OC) activity and of their longer survival, with a correspondent inhibition of osteoblast (OB) and decrease in bone neoformation [60,61]. The increased OC activity is evident already in the earlier stages of the disease, whilst the inhibition of OB activity is a later event.

Pathogenesis of OC stimulation

Since the beginning of the 70s it has been hypothesised that the increased OC activity was mediated by local factors (OAF) produced either by the plasma cells (PCs) themselves or by the interaction of the PC and the BMSCs. In the last thirty years a number of cytokines, growth factors and hormones that stimulates OC activity have been found. Among these factors the most important are RANKL (receptor activator of NFkB ligand), MIP1α (macrophage inflammatory protein 1 alfa), IL3, IL6, IL1β. PCs produce cytokines that increase OC differentiation, activity and survival, whilst bone reabsorption induces the release of PCs growth factors, increasing the tumour burden. This further stimulates bone reabsorption in a vicious circle [62,63].

The RANK/RANKL pathway is highly important in bone remodelling both physiological and pathological. RANK is a transmembrane receptor of the family of the tumour necrosis factors, and it is expressed on OC precursors [64]. It's ligand RANKL is expressed on OB precursors and BMSCs as an extramembrane protein, and is secreted by T lymphocytes [65].

Upon binding to RANKL, RANK is able to stimulate OC differentiation and proliferation, inhibiting apoptosis through the NFkB pathway. Osteoprotegerin (OPG), a soluble receptor of RANKL, is able

to inhibit this pathway, limiting osteoclastogenesis. OPG is synthesised by OB, BMSCs and other cell types [66,67].

The equilibrium between the synthesis of RANK and OPG is altered in MM patients, in which there is an overproduction of RANKL and a decrease in OPG levels; these two aspects together stimulate OC activity and bone reabsorption [66,68]. MM PCs are not able to produce RANKL, but are able to induce RANKL production and to inhibit OPG synthesis in BMSCs [69]. Furthermore the neoplastic PCs are able to internalise OPG through syndecan-1, destroying it in the liposomial compartment [70].

Another important chemokine for osteoclastogenesis is MIP1α (Macrophage inflammatory protein-1α), produced by the neoplastic cells in 70% of the patients. MIP1α supports the adhesion between PCs and BMSCs, with a consequent increase in the production of RANKL, VEGF, IL6, TNFα, and in MM cell growth, angiogenesis and bone disruption. Gene expression profiling (GEP) studies have shown that the gene encoding for MIP1α is overexpressed in MM patients; furthermore blood levels of MIP1α correlates with the extension of bone loss and patients' survival [71]. Some studies have also shown a correlation between the levels of MIP1α and patients harbouring t(4;14). In these patients FGFR3 is constitutively activated, resulting into and overexpression of MIP1 α, a more serious bone disease and a worse prognosis [72,73].

Higher levels of IL3 where also seen in MM patients. IL3, *in vitro*, promotes osteoclastogenesis, increases the action of RANKL and MIP1α, supports proliferation of MM PCs and inhibits OB formation [74].

IL6 has a pivotal role both in supporting the tumour clone growth and in increasing OC differentiation. Levels of IL6 are higher in patients with bone lesions than in patients without any skeletal involvement. Many studies have suggested that this cytokine is produced not only by MM PCs, but also by BMSCs, OC and OB [75].

Pathogenesis of osteoblast inhibition

The physical interaction between MM PCs and OB and the elevated levels of cytokines inhibit the maturation of OB precursors and activate apoptotic mechanism within the OB [76]. The development of OB from pluripotent mesenchymal cells involves the activation of Runx2/Cbfa1 and Osterix, a zinc-finger transcription factor of the Runx2 signal cascade [77]. When MM cells are co-cultured with OB progenitors, a significant decrease in OB differentiation can be seen, due to an inhibition of Runx2/Cbfa1 through cell-cell interaction [78]. This also translates in a reduced production of OPG, misbalancing the RANKL/OPG ratio in favour of osteoclastogenesis. OB of MM

patients also show a different GEP compared to their normal counterparts, with an increase in angiopoietin and IL1β and reduced expression of WNT.

The Wnt signalling pathway has a major role in OB maturation and differentiation. The glycoprotein Wnt binds with the co-receptors LRP5 or LRP6 and the complex than binds to the frizzled receptor; the signal transduction phosphorylates βcatenin, which is able to migrate in the nucleus where it up regulates target genes for OB differentiation. In MM patients soluble inhibitors such as Dickkopf-1 (DKK1) and secreted frizzled related proteins (sFRP) block this pathway. DKK1 binds to LRP, blocking the interaction with Wnt, and it has been shown that there is a correlation between the levels of DKK1 and the number of bone lesions [79-81].

DKK1 is also able to increase OC activity, inhibiting the non-canonical pathway of Wnt, reducing the synthesis of OPG and increasing the one of RANKL.

The sFRP act as soluble receptors of Wnt, binding it before it can reach the frizzled membrane receptor; sFRP-2 can inhibit OB differentiation in mice, whilst its role in humans is less clear [82]. Other factors that can reduce OB activity in MM patients are IL3 and IL7.

A better understanding of the mechanism underlining skeletal involvement has been useful in identifying new therapeutics targets:

- RANKL → Human monoclonal antibody anti RANKL (Denosumab), actually in phase I, II and III clinical studies
- MIP1α → Antagonist of MIP1α receptor (CCR1) tested *in vitro* and *in vivo* in preclinical models (MLN 3897)
- DKK1 → Many antibodies targeting DKK1 have been developed; of these BHQ880 is currently undergoing phase I clinical studies.

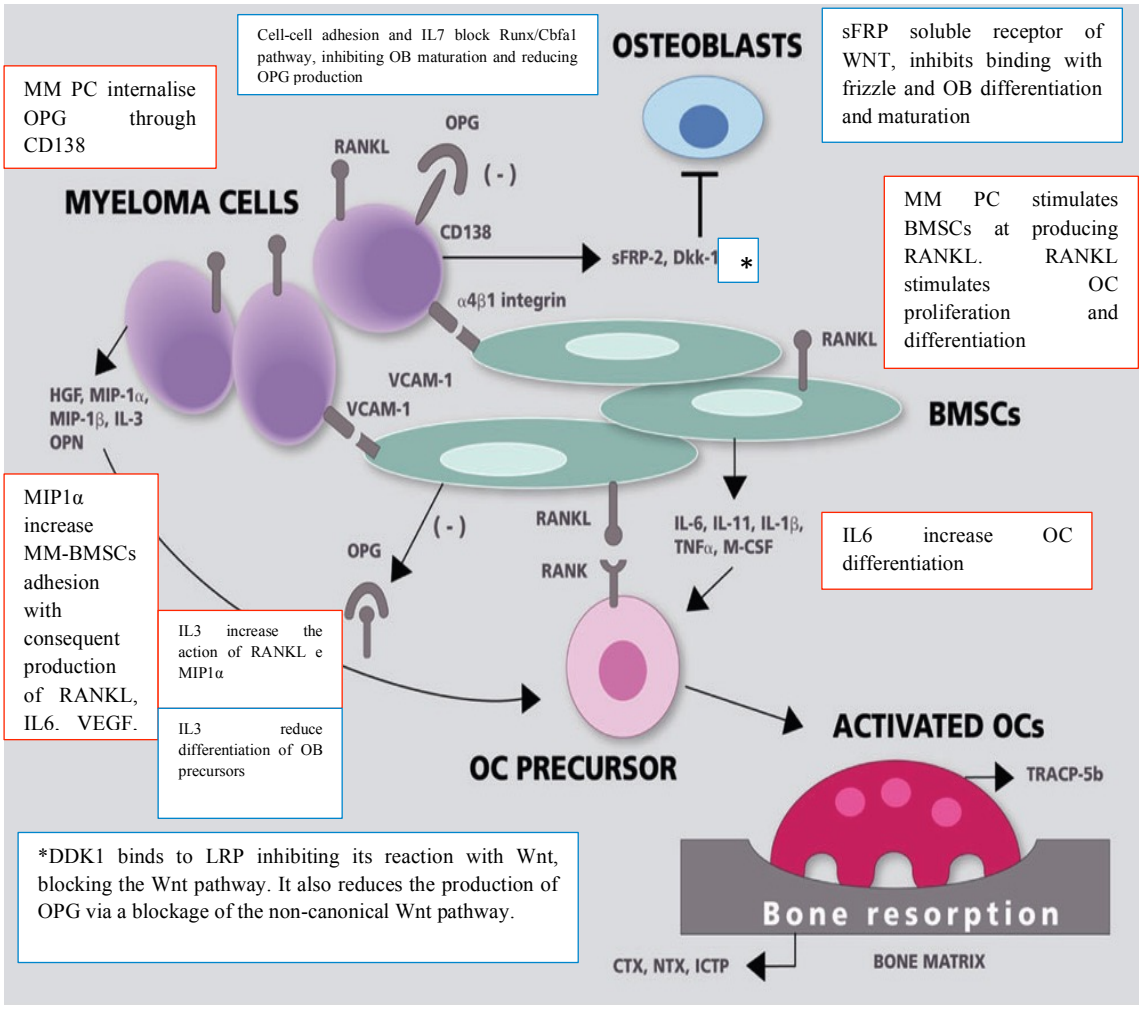

Figure 4. Pathogenesis of bone disease. Modified form Terpos et al. 2009 [83]

2.4 GENETIC, CYTOGENETICS AND MOLECULAR CHARACTERISTICS OF MYELOMA AND THEIR IMPACT ON PROGNOSIS[2]

2.4.1. GENETIC BASIS OF MYELOMA

As already stated, MM can be considered as being initiated via a MPC [46]. Such a cell is thought to be the founding unit of the MM clone, and harbours the biological features of both self-renewal and proliferation. In the classical view MM progression was thought to follow a linear pattern, from the initiating stage of monoclonal gammopathy of undetermined significance (MGUS) to the final stages of extramedullary disease and plasma cell leukaemia. In this model following its initiation, the MPC acquires additional genetic hits that further deregulate its behaviour, giving rise to the clinical and biological feature of symptomatic myeloma.

[2] Contains quotes from "Biology and Treatment of Myeloma" Brioli A. et al, Clinical Lymphoma, Myeloma and Leukemia 2014 (84. Brioli A, Melchor L, Walker BA, Davies FE, Morgan GJ. Biology and Treatment of Myeloma. *Clinical lymphoma, myeloma & leukemia*. 2014;14S.)

Two of the main pathways that are traditionally thought to initiate the transformation of a normal plasma cell into a MPC are translocations into the *IGH* locus and hyperdiploidy [40]. These two distinct lesions, both ultimately leading to the deregulation of Cyclin D genes [40], are mutually exclusive in the majority of patients and are not linked to any specific phenotype. As already stated, molecular archaeology using IgH rearrangements suggests that the germinal centre reaction drives the origin of the disease [35,38-41], however, recent evidence suggests that, at least in a proportion of patient, the transformating event can be attributable at a proB cell stage [41]. In this respect a recently published genome wide association (GWAS) study has identified risk loci for MM at chromosome 3p22.1, 7p15.3 and 2p23.3 accounting for 4% of familial risk in MM, suggesting that alterations of genes mapping in these regions (such as *ULK4*, *DNAH11*, *CDCA7L*, *DNMT3A* and *DTNB*) might precede IgH translocation leading to an increased risk of developing MM [21]. Furthermore it has been shown that memory B cells of MM patients show involvement of genes known to be deregulated by IgH translocations, such as *FGFR3*, *IGH-MMSET* and *CCDN1*, but lack the presence of "later" genetic events like mutation in the *RAS* genes, once more advocating that the transformating events can occur also at a precursor B cell stage, despite probably not being sufficient to maintain the MM clone [85].

2.4.2 CYTOGENETIC ALTERATIONS IN MYELOMA

The study of molecular and genetic alterations has a pivotal role in understanding the biological mechanism that form the basis of MM pathogenesis and progression, and can be useful in clinical practice to stratify patients according to their prognosis.

MM cells have a low proliferative index (PCLI 1-2%) and this leads to a low applicability of conventional karyotyping. With this technique about 30-50% of newly diagnosed MM are shown to have cytogenetic abnormalities (CA) at diagnosis. Fluorescent in situ hybridization (FISH), allowing the study of cells in interphase, is able to identify CA in about 80-90% of M patients, irrespective of disease stage and phase [86].

On the basis of the number and type of CA two major categories of MM have been identified: hyperdiploid and non hyperdiploid MM [87]:

- A **hyperdiploid** karyotype is generally made of 48-75 chromosomes with trisomies of the odd number chromosomes, 3, 5, 7, 9, 11, 15, 19, and 21. Translocations occur rarely. It is associated with a better prognosis and with a disease that is more BMSCs-dependent. It is found in 40-60% of patients.

- A **non-hyperdiploid** karyotype, frequently associated with translocations involving chromosome 14(q32) and monosomy or deletion of chromosome 13(q14). It is found usually in more aggressive and more diffuse diseases.

As in other hematologic neoplasia deriving from the post germinal centre, in MM translocations involving the IgH locus on chromosome 14 or IGL kappa and lambda loci on chromosomes 2 and 22 are very common. These CA are linked to mistakes in DNA modification that physiologically happen when a B cells go through the germinal centre of lymph nodes. These mechanisms are normally switched off in normal and neoplastic plasma cells, and are likely to represent one of the founding events of the MM clone [88].

These translocations normally result in an oncogene being controlled by the strong Ig enhancers, with its consequent over expression. A number of IgH translocations are recurrent in 40% of patients. The genes more commonly involved are: Cyclin D1, D2, D3 (on 11q13, 12p13, 6p21), c-MAF (on 16q23), MAF-B (on 21q12), MAF-A (on 8q24), MMSET e FGFR3 (on 4p16) [87,88].

t(11;14), can be found in 15% of patients, and it leads to an up-regulation of Cyclin D1; it is common in IgM MM and in AL amyloidosis. It is associated with a good prognosis after high dose chemotherapy but with a short survival after relapse [89].

t(14;16), is present in 5-7% of patients. It leads to an overexpression of MAF and it's an unfavourable prognostic factor [90,91].

t(4;14), is detected in 15% of patients, where it leads to an increased expression of MMSET (through the formation of a fusion gene IgH-MMSET on chromosome 4) and fibroblast growth factor receptor 3 (FGFR3). However, in 25% of the patients, the loss of derivative 14 leads to a correspondent loss of FGFR3. t(4;14) is associated with a dismal prognosis after conventional chemotherapy and autologous stem cell transplantation, a short duration of remission and an early relapse [91-93]. GEP analysis showed that patients with t(4;14) have an over expression of 67 genes (including RAS GRP1, CDH2) and a lower expression of 60 genes (including MAPK14, CASP2, HDCA1, DEED) compared to patients negative for this translocation. This partly explains the worst prognosis of t(4;14) patients, although the French group showed that patients with t(4;14), high haemoglobin and low β2microglobuline have a relatively good prognosis [94].

Deletion of chromosome 13 (either monosomy of chromosome 13 or loss of 13(q14)), involving the RB1 gene, is seen in 50% of patients; in 90% of the cases it is associated with t(4;14), and it has been shown that the poor prognosis of patients positive for del(13q) is mainly linked to the co-occurrence of other cytogenetic abnormalities [95-97].

Aberrations of chromosome 1 (gain of chromosome 1(q) or loss of chromosome 1(p)) have a high prevalence in MM. The gain of 1(q21) is associated with an increase in of plasma cells proliferation, due to an overexpression of CKS1B [98]. Loss of 1(p31-32) was found to be associated to a significantly shorter OS in patients treated with autologous stem cell transplantation [99].

Deletion of chromosome 17(p13) is found in less than 5% of patients at diagnosis, but its prevalence increases at the time of relapse. It is associated with the loss of the tumour suppressor gene p53, and it is associated with a dismal prognosis. With the loss of the pro-apoptotic activity of p53 the neoplastic clone becomes independent from the bone marrow microenvironment, with the development of plasma cell leukaemia or extramedullary MM [100].

The **oncogene c-MYC**, is hyperexpressed in the advanced phases of the disease. It is also associated with the independence of the neoplastic clone from the microenvironment and with a higher proliferating capacity, conferring poor prognosis to the disease [87,88,101].

The constitutive activation of the **NFkB pathway** can be due both to mutation inactivating inhibitors of the pathway (TRAF3, TRAF2, CYLD) or to amplification leading to an overexpression of activating proteins (NIK, CD40, TACI, LTBR). This alteration is gaining always more importance in MM taking into account the increasing use in clinical practice of the proteasome inhibitors bortezomib and carfilzomib.

Mutations activating the **RAS pathway** (**N-RAS, K-RAS, BRAF**) are usually associated to an increase in the levels of Cyclin D1 and are though to be early events associated in the shift from MGUS to MM.

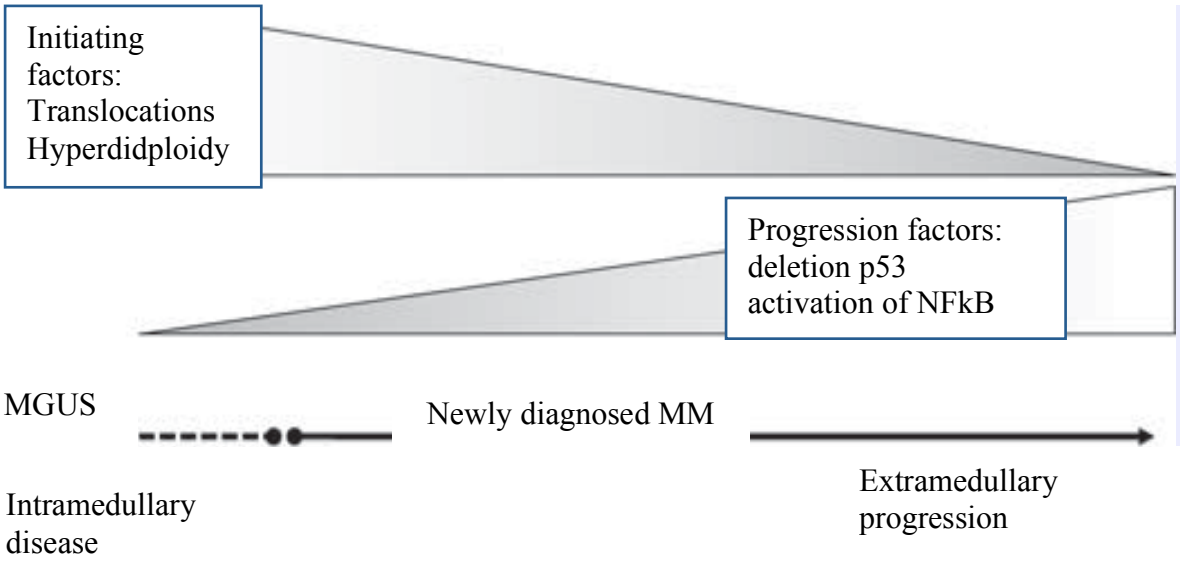

Figure 5. Genetic alterations in MM

Table 6. Cytogenetic-molecular classification proposed by the IMWG [87]:

Type of myeloma	Percentage	Clinical and laboratory characteristics
Hyperdiploidy	45	Favourable, older pts, IgG/k
Non-hyperdiploidy	40	Aggressive, IgA/l, younger pts
Translocations Cyclin D	18	
t(11;14)	16	CCND1, good prognosis, bone lesions
t(6;14)	2	CCND1, good prognosis, bone lesions
t(12;14)	< 1	Extremely rare
Translocation of MMSET t(4;14)	15	Unfavourable prognosis with conventional chemo
Translocations of MAF	8	Aggressive disease
t(14;16)	5	Aggressive disease
t(14;20)	2	Aggressive disease
t(8;14)	1	Aggressive disease
Not classified	15	Various subtypes

2.4.3 MYELOMAS AS A DISEASE OF G1/S PHASE, RAS, MYC, AND NFKB

The overexpression of a D-group cyclin is an early molecular abnormality in MM, leading to a deregulation of the G1/S transition. Overexpression of cyclines of the D-group can occur via different mechanisms, mainly translocations of the IgH genes leading to the deregulation of genes

such as *MAF* [102], *FGFR3* and *MMSET* [40]. It seems, however, that this genetic alteration alone is insufficient to give rise to the clinical hallmarks of MM and additional events are needed to enable the disease to progress [103]. Mutations in the form of single nucleotide variants, chromosomal copy number abnormalities and epigenetic changes are responsible for disease progression [46,104]. Such secondary "hits" drive disease progression activating key oncogenic pathways that may include the RAS/MAPK pathway [87,105,106], *MYC* [107] or the NFkB pathway [105,108].

The prevalence of activating mutations in one of the *RAS* genes (*NRAS* or *KRAS*) is about 50 % in presenting MM [87,106], and is higher in tumours that express CCND1 [108]. There is increasing evidence that MM depends on the continued expression of activated *RAS* [109]; mutations in the *NRAS* gene have been found also in monoclonal gammopathy of undetermined significance (MGUS) patients, although at a significantly lower frequency (7%) [110]. Recently *BRAF* mutations in approximately 4% of MM patients have also been described [105]. It is evident, however, that, even though mutations in the RAS pathway are a driver event in the progression of MM, they are not present in all MM cells, and can be found only in a minor clone. *NRAS* and *KRAS* mutations have similar but non-identical effects and this is strengthened by the finding that both mutations can be present in the same patient [111]. Whether the same cell harbours both the mutations, or they are present in different clones, in a parallel evolution pattern, is a question that still needs to be answered, although recent evidence suggest the possibility of parallel evolution being a feature of MM [111]. Recent biotechnological advances and the possibility of single cell analysis might further elucidate this important question.

The deregulation of *MYC* is a central feature of MM, as is shown by the fact that MM cell lines depend on *MYC* for their survival [112]. The *MYC* gene is located in chromosome 8p14 locus, and abnormalities involving this genome region are frequent in MM patients. In presenting myeloma abnormalities of 8q are generally reported in 15% of cases, while rearrangements at 8q24 have been reported in up to 47% of myeloma patients overall [113-115]. *MYC* is also activated in the transition from MGUS to myeloma, implicating it in disease progression [116]. *MYC* rearrangements result overexpression on MYC due to the co-localization of active super-enhancers in the partner loci; frequently the partner chromosome genes (for example *FAM46C*, *XBP1* or *IGL*) has a known function in myeloma or B cell biology [101].

NFkB is a transcription factor that was found to be important in the development of MM. Both MGUS and MM highly express genes known to be targets of NFkB; this could partly explain the dependency of MM cells on the BM microenvironment and suggests a continued role of extrinsic

signalling in MM [117,118]. BM stromal cells produce extrinsic ligands such as APRIL and BAFF that stimulates TACI, BCMA and BAFF receptors, ultimately activating NFkB pathways and providing critical survival signals to plasma cells [119]. The importance of the NFkB pathway is further highlighted by the finding that both activating and inactivating mutations in positive and negative regulators of the non-canonical NFkB pathway (such as *TRAF2*, *TRAF3*, *CYLD, cIAP1/cIAP2* and *NIK)* have been identified in 20% of patients and in myeloma cell lines [117,118]; mutations in these genes can activate the NFkB pathway without the presence of a ligand [105] and might contribute to the spread of extramedullary disease [108], as well as being related to response to treatment [117,118].
Other lesions that have a greater predisposition to occur late in the natural history of the disease are gain of chromosome 1q, mutation at *TP53* or deletion of chromosome 17p[87,107,108,114,120,121].

2.4.4 IMPORTANCE OF BIOLOGY IN THE PROGNOSTIC STRATIFICATION AND TREATMENT OF MYELOMA

Many attempts have been made to use biology to stratify risk in MM. Myeloma genetic was initially assessed with metaphase karyotyping, however, the procedure is long and frequently infeasible in a terminally differentiated cell such as a plasma cell. FISH assessment has progressively taken the place of conventional karyotyping, and is now the most used and wide spread technique for assessing the biological risk in MM patients. Single nucleotide polymorphism (SNPs) analysis can be used as well to perform a molecular karyotyping in MM, and has been proved to be able to identify biological risk in MM [122]. The classical approach to define biological risk in myeloma is to classify patients as having standard risk if they have no FISH lesion, the presence of t(11;14) or t(6;14). Patients with hyperdiploidy alone are known to have a good prognosis, whilst the presences of translocations like t(4;14) (associated with deregulation of *FGFR3* and *MMSET*) or t(14;20) and t(14;16) (both deregulating *MAF*), have been associated with a poor outcome. Similarly amplification of chromosome 1q and deletion of the short arm of chromosome 17 are known to be negative prognostic factors [108,122,123]. The deletion of chromosome 13, which was thought to be a poor prognostic feature, has largely lost its significance when it was shown that the negative impact on survival was mainly due to the frequent association with the t(4;14) [89]. It has recently been demonstrated that not only the type of genetic lesion is important, but also the number of genetic lesions present in a single patient [124], with the survival of patients being progressively shorter according to the number of adverse FISH lesion co-segregating in a single patient (1, 2, 3, more than 3). This finding led to the definition of new risk stratification based on the number of co-segregating adverse FISH lesions (none, 1, or

more than 1), with patients harbouring more than one adverse FISH lesion being categorized as ultrahigh-risk [124]. However, no consensus still exist regarding the impact that the presence of an adverse FISH lesion can have in patient known to have a good prognostic feature such as hyperdiploid: some groups reported that the presence of hyperdiploidy can abrogate the adverse prognostic impact of a t(4;14) translocation [125], whilst other have shown the opposite [126,127]. Integrating genetic with biological information, such as ISS, further improves risk stratification [124,128].

More recent GEP profiling has also been used to classify genetic risk in myeloma. GEP can be used to classify MM patient into 7 to 10 major categories, characterized not only by different signalling signatures, but also by distinct prognostic impact [129,130]. Interestingly, one of the subgroup identified (corresponding to about 12% of patients) was characterized by a high expression of genes involved in the NFkB pathway, remarking the importance of the NFkB pathway in the pathogenesis and development of MM [130]. The utility of GEP-based risk classification in MM has been independently demonstrated by different groups, which have shown how GEP signatures, comprising 70, 15 or 6 genes, could be associated with different outcomes [123,131-133].

Despite improving our knowledge in the biology of MM and developing tools and algorithms to better stratify the risk of MM patients, we have made significant progress with the outcome of standard and high-risk disease but have had little impact on the outcome of ultrahigh-risk disease [134,135]. Therefore, if we are to improve the outcome of ultrahigh-risk disease we have to understand its biology and use this knowledge to develop targeted treatment strategies. This approach requires us to understand the "actionable mutation" spectrum of myeloma and the agents able to modulate the activity of the mutated genes.

3.THE CONCEPT OF INTRA-CLONAL HETEROGENEITY[3]

Cancer is initiated and then progresses through a complex mechanism based on the acquisition of genetic "hits" that change the biology of the target cell from normal to malignant. The "hits" can be broadly divided into "driver" lesions contributing to a selective advantage, and "passenger" lesions that are neutral in their effect [1,2]. It is important to understand the complex genetic

[3] Contains quotes from "Biology and Treatment of Myeloma" and from "The Impact of Intra-Clonal Heterogeneity on the Treatment of Multiple Myeloma" (43. Brioli A, Melchor L, Cavo M, Morgan GJ. The impact of intra-clonal heterogeneity on the treatment of multiple myeloma. *British journal of haematology*. 2014;165(4):441-454, 84. Brioli A, Melchor L, Walker BA, Davies FE, Morgan GJ. Biology and Treatment of Myeloma. *Clinical lymphoma, myeloma & leukemia*. 2014;14S.)

landscape of cancer not only because it can inform us on how individual genetic lesions interact leading to disease progression, but also because of its impact on treatment [3-5]. Studies both on solid and haematological tumours have shown that cancer comprises a collection of related but subtly different clones, a feature that has been termed “intra-clonal heterogeneity” (see Table 7) [6-9].

Table 7. Intra-clonal heterogeneity in cancer and MM

Author	Disease	N of patients	Disease status	Technique	Major findings
Nik-Zainal, 2012 [7]	Breast cancer	21	Presentation	NGS (WGS)	Prominent sub-clonal diversification was identified with a dominant sub-clonal lineage found in every tumour sample. Existence of long-lived, quiescent cell lineages capable of substantial proliferation upon acquisition of enabling genomic changes is postulated as the necessary substrate for cancer evolution
Gerlinger, 2012 [6]	Renal cancer	4	Relapse	NGS (WES)	Phylogenetic reconstruction revealed branched evolutionary tumour growth. Mutational intra-tumour heterogeneity was seen for multiple genes converging on loss of function, suggesting convergent phenotypic evolution
Anderson, 2011 [8]	ALL	30	Presentation (Presentation/Relapse in 5 cases)	FISH/CNA	Enumeration of CNA in single-cells identified distinctive genetic signatures of sub-clones and their relative frequencies. The more common feature was sub-clonal heterogeneity (up to ten sub-clones) related via a branching tree
Ding, 2012 [9]	AML	8	Presentation/Relapse	NGS (WGS)	AML relapses are characterized by two major clonal evolution patterns: (1) the founding clone in the primary tumour gained mutations and evolved following a linear pathway, or (2) a sub-clone of the founding clone survived initial therapy, gained additional mutations and expanded in a branching way
Keats, 2012 [11]	MM	28	Presentation/relapse	CGH	Three major tumour types identifiable in MM patients: (1) genetically stable, (2) linearly evolving, and (3) heterogeneous clonal mixtures and shifting predominant clones
Walker, 2012 [12]	MM	22	Presentation	NGS (WES)	Intra-tumour heterogeneity identified by the presence of clonal and sub-clonal mutations (e.g. *RAS* mutations). Intra-clonal heterogeneity was confirmed at a single-cell level
Walker, 2013 [136]	MM	36	Presentation (SMM/presentation MM in 4 cases)	NGS (WES/WGS)	Intra-clonal heterogeneity is present at all stages of MM from the MGUS stage. Paired analysis of SMM transformed to MM show that intra-clonal heterogeneity is a typical feature of MM.
Magrangeas, 2013 [137]	MM	24	Presentation/Relapse	SNP array	A branching pattern of relapse is present in 1/3 of MM patients.

This intra-clonal heterogeneity is likely, from a "Darwinian" natural selection perspective, to be the essential substrate for cancer evolution, disease progression and relapse. These ideas derived from evolutionary biology are teaching us that cancer progression is driven via branching evolutionary patterns, rather than following a linear multistep process as was thought previously [4,6,10-12]. In this context the critical mechanism for tumour progression is competition between individual clones for the same micro environmental "niche", combined with the process of adaptation and natural selection [4,10]. According to this hypothesis, following the immortalization of a single cancer stem cell and based on the random acquisition of distinct mutations, different cancer stem cell populations are generated, each with different genotypic and phenotypic characteristics but all sharing the features of self-renewal and proliferation. These different cancer stem cells compete for access to limited resources, and the acquisition of "driver" mutations gives them a survival advantage, leading to clonal dominance [138]. A metastatic site that is spatially different from the original tumour could, therefore, be colonized by a cancer stem cell with a unique set of features, further modifying disease evolution and increasing the genetic complexity of the disease [4,6]. In addition to micro-environmental pressure, therapy itself can act as a selective bottleneck facilitating the death or survival of a different set of tumour clones; distinct clones will therefore dominate at different time points of the natural history of conditions with a relapsing/remitting behaviour, such as the indolent lymphoproliferative diseases [11]. This feature of intra-clonal heterogeneity also has a significant impact on how cancer therapy should be delivered, and its therapeutic effects should now be considered in the context of the ideas implicit in evolutionary biology.

3.1 INTRA-CLONAL HETEROGENEITY AND ITS IMPACT ON MYELOMA TREATMENT

Newer techniques, such as massive parallel sequencing, have significantly changed our understanding of MM biology. It has become evident from recent studies that MM is not composed of a single neoplastic clone, but is rather a collection of multiple sub-clones that compete for the same resources [11,12,136]. This knowledge has also changed our view on the progression of MM from his premalignant condition of MGUS to the final stages of plasma cell leukaemia and extramedullary myeloma. The progression of MM was historically thought to follow a multistep linear process in which "genetic hits" are acquired in a sequential way deregulating the behaviour of a normal plasma cell [46,139]. Next generation sequencing (NGS) techniques have shown that mutations are not homogeneously distributed in all MM cells, but are present at different frequencies within a tumour sample. This finding is consistent with intra-clonal heterogeneity

being a relevant feature of myeloma [136]. In this model disease progression is based on the random acquisition of genetic hits followed by Darwinian selection of the fittest clone(s). On average 3 to 6 different major sub-clones can be detected at presentation in MM [12,111], and serial genomic analyses of samples collected at different points during disease progression have shown that MM can progress via both linear and branching evolution [11,137]. However, clonal heterogeneity is present since the earlier stages of MM development [136], and, if 3 to 6 clones are already present form when the disease becomes symptomatic, than the branching evolutionary patterns represents the best unifying mechanism able to explain the biology and progression of the disease.

A sample from a MM patient has on average 35 mutations [105], a number intermediate between that seen in acute lymphoblastic leukaemia (ALL) [140], a less complex and more homogeneous disease, and the hundreds of mutations reported to be present in solid cancers [141]. If Darwinian evolution theory is to be applied at the biology and evolution of MM, the disease can be considered as being derived from an initially immortalised MPC. As seen in other cancers, the diversity of the MPC supports the evolution of the fittest clone, leading to disease progression [142]; adaptation of the MPC to adverse conditions can further lead to drug resistance and treatment failure [143,144]. If a MM sample is composed of different clones each of which has a different fitness for different environments, then it is likely that sites of disease within a patient will evolve independently, with mutation present in one site but not in another one, a process that will ultimately lead to metastasis [145,146]. In Darwinian terms it is likely that different clones compete for the same stem cell niche, and this is an important concept that has to be taken into account when considering disease progression. Clonal dominance can change over time, and the different clonal tides will lead to differential responses to treatment. It is therefore important to consider how modification of treatment strategies can impact on how clones are selected and progress.

3.2 HOW BIOLOGICAL KNOWLEDGE CAN CHANGE MYELOMA TREATMENT

Considering the rate at which evolution and disease progression is likely to happen, the genomes of standard-risk patients show fewer changes over time compared to high-risk patients who have significantly more variation [11]. Interestingly however, it has been shown that response rates are the same in high- and standard-risk disease [124,135], but, due to their biology, high-risk patients have a significantly shorter progression free survival (PFS) and overall survival (OS) [135]. This notion could allow us to postulate a model in which treatment can be personalised after remission post-

induction has been achieved. The ability to distinguish indolent from more aggressive clones and tailor therapy accordingly is therefore of primary importance in clinical practice going forward.

The introduction of novel agents into upfront therapy has dramatically improved the outcomes of symptomatic MM [134,147-157]. Modern treatment strategy of MM involves the concept of sequential blocks of therapy given as an induction followed by consolidation and maintenance. The induction phase aims at de-bulking the disease, reducing it to the smallest amount possible. Consolidation therapy further reduces tumour bulk, potentially eradicating some sub-clonal populations. Maintenance treatment is given to modify selective pressure and is aimed at keeping tumour cells under control selecting for the most indolent clones and potentially leading to a cure. Even if treatment approaches change according to patients' age and to their eligibility or not to receive high dose treatment followed by autologous stem cell transplantation, the concept of disease de-bulk followed by a continuous treatment is becoming the mainstay of MM treatment protocol irrespective of patients' age [151,157-161].

In the light of a Darwinian evolution theory applied to cancer and based on the ideas implicit in terms of evolutionary biology, it is important to consider the composition of such treatments and how they are delivered. The use of combination of agents with different modes of actions will kill different cell population according to their susceptibility to the different drugs present in the combination, maximising the chances of a cure. It is likely that the more proliferative clones will be totally eradicated by such an approach. Maximising the de-bulk of the disease in the initial phases of treatment, revolving patients to a premalignant disease state, will translate into minimal numbers of cells at risk of transformation, reducing the risk of an aggressive relapse. Alternating chemotherapy with different modes of action will also lead to a cross kill of the different clones, reducing the risk of developing treatment resistance.

However, if we really want to cure MM it is important to take advantage of what we know about its biology. An important concept in the new treatment strategies for MM is the one of maintenance treatment. The aim of a maintenance therapy is to modify residual disease biology, selecting for less aggressive clones thus stabilising the disease. Maintenance acts as a Darwinian selective pressure, adjusting pressure to select for indolent clones. It is important, therefore, to use agents that affect the biology of the stem cell, such as the novel drugs that have the characteristics of selecting for good performing clones. Maintenance treatment should be continued until disease progression, to apply a constant selective pressure to residual clones. It is also important to alternate the use of available agents to prevent emergence of treatment

resistance, alternating the selective pressure on the MPC and to avoid using chemotherapeutic agents that are known to have a mutagenic effect. In the future it might prove important the ability of utilizing the immune system to augment the anti-tumour activity of maintenance. The immune system requires specific and balanced responses to clear pathogens and tumours and yet maintain tolerance. In this respect the use of monoclonal antibodies, bi-specific antibodies or programmed death 1 (PD-1) and its ligands, PD-L1 and PD-L2, can be important. PD-1, for instance deliver inhibitory signals that regulate the balance between T cell activation, tolerance, and immunopathology [162].

Another important new concept in the treatment of MM is that of targeted treatment. Small molecules that inhibit extrinsic NFkB signalling, such as inhibitors of TACI.Fc, IKKb and NIK (MAP3K14) are being developed as potential therapeutic agents [163,164]. There is also some evidence suggesting that some cells can be particularly sensitive to proteasome inhibitors, such as those cells harbouring TRAF3 mutation and that are addicted to constitutive NFkB activation for their survival [118].

As already stated mutations in the RAS pathway are present in 50% of MM samples at presentation, and even if no prognostic relevance for these mutations have been demonstrated, specific inhibitors of this pathway might prove to be important in clinic. An example of this is the *BRAF* mutation V600E that has been demonstrated in 4% of MM patients and that has been shown to be sensitive to the specific inhibitor Vemurafenib [165]. From all this it does not seem unreasonable that in the future the strategy for MM treatment will comprise a mutational screening and detection followed by targeted treatment.

It is also important, however, to consider the potential hazards of chemotherapy delivered with a Darwinian approach. Treatment that modifies the immune system and the biology of the microenvironment might have off-target effects, such as an increased rate of second primary malignancies (SPMs). In fact studies with maintenance lenalidomide reported an increased incidence of developing SPMs in patients receiving the study drugs compared to patients randomised to placebo [151,159,161]. Despite other trials didn't confirm these results [157,166], it is clear that additional research on the biological mechanism that might trigger for the development of SPMs is warranted before any definite conclusion can be drawn, whilst clinicians should carefully inform patients regarding the potential risks and benefit of a maintenance treatment. Applying a selective pressure could also select for more resistant clones, allowing the outgrowth of aggressive clones and effectively decreasing survival post relapse. It is obvious that the negative impact of

these off-target effects needs to be balanced against the positive effect on long-term disease control and the potential for cure.

4. THERAPY

Treatment of MM formally began in the 50s and 60s, with the introduction in clinical practice of alkylating agents (such as melphalan and cyclophosphamide), of nitrosurea, of the vinca alkaloids (such as vincristine) and anthracyclins (such as doxorubicin) [167]. These drugs have proven to be effective in MM when used as single agent, and it was later shown that their effect is increased by the concomitant use of dexamethasone and the combination of melphalan and prednisone (MP) was considered the standard of care for many years. With this combination 50-60% of patients achieved a response, however only 5-10% achieved a complete remission (CR); median duration of response was 1-2 years, and only 5-10% of the patients became long term survivor [168,169].

At the beginning of the 80s the addition to the therapeutic armamentarium of high dose melphalan followed by stem cell rescue opened a new way in the treatment of younger patients [170-172], but it is in the last decade that a real "revolution" began in MM treatment, with the introduction of the so called "novel agents". These "novel agents" are non-antiblastic drugs showing a high activity against the plasma cell clone and its microenvironment. The crucial role of the microenvironment in promoting the growth, progression and development of drug resistance in the MM clone has already been discussed. These drugs were proven to be highly effective in the treatment of advance relapsed/refractory MM, and were subsequently added to the treatment of newly diagnosed patients, both transplant eligible (both as induction pre and consolidation after transplantation) and transplant ineligible. The main "novel agents" that are currently used in clinical practice are: the immunomodulatory drugs (IMiDs), Thalidomide and its analogues (Lenalidomide and Pomalidomide) and proteasome inhibitors (PI), Bortezomib (Velcade) and the second generation PI, such as Carfilzomib and Ixazomib. Other new drugs of different classes and with different mechanisms of action are currently under evaluation in pre-clinical and clinical trials. This drugs, such as histone deacetilase inhibitors, kinesin spindle inhibitors, AKT inhibitors, mTOR inhibitors and monoclonal antibodies are further expanding the treatment landscape.

Currently, to evaluate the efficacy of a treatment the 2006 Uniform Response Criteria of the IMW are used (Table 2) (109).

Table 8. Uniform Response Criteria IMWG 2006, modified from Durie et al and Kyle et al [14,173]:

Complete response (CR) * Negative serum and urine immunofixation, disappearance of soft tissue plasmocytoma and BMPC <5%
Stringent complete response (sCR) CR defined as above plus normal FLC ratio plus absence of clonal plasma cells by IHC or flow cytometry (2-4 colours)
Near Complete Response (nCR) No paraprotein at electrophoresis, but positive immunofixation. This response criteria is absent in the IMWG classification
Very Good Partial Response (VGPR) * Negative paraprotein in serum or urine but immunofixation positive or reduction in paraprotein of ≥ 90% for serum paraprotein and < 100 mg/24h for urine paraprotein
Partial Response (PR) Decrease in serum paraprotein ≥ 50% and reduction in urine paraprotein ≥ 90% or < 200 mg/24h If serum and urine paraprotein are not evaluable a reduction ≥50% in the difference between involved and uninvolved FLC is required If also FLC are not measurable a reduction ≥50% of BMPC is required (proven that at baseline the percentage was ≥30% If soft tissue plasmocytoma, decrease of ≥50%
Stable Disease (SD) Not fulfilled criteria for CR, VGPR, PR or PD
Progressive disease (PD) † Increase ≥25% from nadir of: -serum paraprotein (absolute increase must be ≥ 500 mg/dl) and/or -urine paraprotein (absolute increase must be ≥ 200 mg/24 h) and/or -if paraprotein not measurable, difference between involved and uninvolved (absolute increase must be >10 mg/dL) -if also FLC are not measurable, percentage of BMPC (absolute increase must be ≥10%) Appearance of new bone lesions or soft tissue plasmocytoma or increase of a pre-existing lesions Hypercalcemia (Corrected serum Ca^{2+} >11.5 mg/dL) only due to MM
Additional criteria

Minimal response (MR) (EBMT classification) [174]

Decrease in serum paraprotein ≥ 25% but ≤49% and decrease in urine paraprotein of 50%-89%

If plasmocytoma, reduction of dimension of 25%-49%

Not increase in dimension and number of lytic bone lesions (an increase in a compression fracture does not exclude the response)

All the responses categories require at least 2 consecutive measurements. Additional imaging is not required. Sequential biopsies to confirm responses are not required. VGPR and CR requires both serum and urine measurements, irrespective of the fact that the disease at baseline was measurable only in the serum, only in the urine, in both or in none. For PD assessment an increase in serum paraprotein ≥ 1 g/dL it is sufficient to define a relapse if CM at baseline was ≥ 5 g/dL.

BMPC: bone marrow plasma cells; FLC: free light chains; IHC: immunohistochemistry

*IMWG criteria for CR and VGPR when the disease is measurable only with serum FLC: CR is defined by a FLC ratio between 0.25-1.65. VGPR requires a decrease in the difference between involved and uninvolved FLC ≥90%

† PD: BM criteria are to be used only in patients in whom the disease is not measurable with serum paraprotein, urine paraprotein of FLC levels

EBMT: European Group for Blood and Marrow Transplantation

4.1 THERAPY FOR NEWLY DIAGNOSED PATIENTS

Nowadays MM treatment is started only in the case of symptomatic MM, identified by the presence of the CRAB criteria. Patients with SMM require, at present, only follow-up. It has to be noted, however, that recent evidences suggest a role of early treatment of SMM [175], and that the recent updated criteria for the diagnosis of MM identify as a MM that requires treatment also those cases with a BMPC infiltration higher than 60%, a FLC ratio higher than 100 and the presence of more the one focal lesion at the MRI scan [27].

The choice of the treatment for newly diagnosed MM has to be based on available evidences and on patients' characteristics, such as age, performance status and comorbidities.

Generally patients younger than 65 years and without relevant comorbidities receive a short induction therapy (usually 3 to 4 cycles) with "novel agents" (occasionally associated with chemotherapy), followed by high dose chemotherapy with melphalan and autologous stem cell rescue. A consolidation or maintenance treatment can be added to this therapeutic program. Patients between the age of 65 and 70 can also receive an autologous transplantation, proven that they are fit and with no relevant comorbidities. In these cases a reduced intensity of chemotherapy is usually applied (melphalan 100-140 mg/m^2). The standard for patients older that 70 or not transplant eligible is conventional chemotherapy associated with new drugs, even if new

data are emerging regarding the possibility of treating these patients with chemotherapy free regimens including only new drugs [157].

4.1.1 FIRST LINE TREATMENT FOR TRANSPLANT ELEGIBLE PATIENTS

High dose chemotherapy with autologous stem cell support (autologous transplantation, ASCT) is one of the corner stone of MM treatment for patients ≤65 years. It has a treatment mortality rate of about 1-2% and is able to increase significantly the rates of CR (20-30%) and the duration of PFS and OS (median values 4-5 years) [171,172,176]. High dose melphalan (HDM) is currently regarded as the best conditioning treatment for ASCT (Melphalan 200 mg/m^2; if the patient age is between 65 and 70 years old, or in special situations a reduced dose of 100-140 mg/m^2 can be used).

In order to increase the probability of attainment of a CR and to prolong PFS and OS, at the beginning of the 90s the administration of 2 sequential courses of HDM was introduced in clinical practice (double ASCT) [170,177].

In Table 9 the major randomised trials of conventional chemotherapy vs. ASCT and of double vs. single ASCT are reported.

Table 9. Major studies on ASCT before the advent of new drugs.

Author	Random	N° pts	ORR%	CR%	PFS	OS
Attal IFM90 [171]	ASCT CCT	100 100	81 57	*22 5	28m 18m	57m 44m
Child MRCVII [172]	ASCT CCT	200 201	86 48	44 8	32m 20m	54m 42m
Fermand MAG90 [178]	ASCT CCT	91 94	78 58	57 20	39m 13m	65m 64m
Barlogie S9321 [179]	ASCT CCT	261 255	93 90	17 15	17% 7y 14% 7y	38% 7y 38% 7y (NS)
Barlogie TT1 vs. SWOG [179]	Tandem CCT	152 152	NA	41 NA	37m 16m	79m 43m
Bladé PETHEMA [180]	ASCT CCT	81 83	82 83	30 11	42m 33m (NS)	66m 61m (NS)
Palumbo MMSG [181]	ASCT (mel100x2) CCT	95 99	72 66	25 6	28m 16m	58m 42m
Attal IFM94 [177]	Single Tandem	199 200	84 88	*42 50	25m 30m	48m 58m
Cavo BO96 [170]	Single Tandem	163 158	NA	33 47	23m 35m	65m 71m (NS)
Goldschmid[182]	Single Tandem	Tot 358	NA	NA	23 29 (NS)	NR
Sonneveld [183]	Single Tandem	148 155	86 90	13 32	21m 22m	50 55 (NS)

Notes: pts patients, CCT conventional chemotherapy, ASCT autologous stem cell transplantation, ORR overall response rate, CR complete remission, PFS progression free survival, OS overall survival, TT1 *total therapy I*, IFM *Intergroupe francophone du myèlome*, MRC *medical research council*, PETHEMA *programma para el estudio y tratamiento de las hemopatìas malignas*, SWOG *South west oncology group*, HOVON *Hemato-oncologie voor volwassen nederland*, BO Bologna, MAG *Myèlome autogreffe*, NA not assessed, NR not reached, NS not significant, nCR near CR. *: ≥ nCR, y years, m months

Many studies have identified prognostic factors associated with a longer OS in patients receiving ASCT, such as absence of cytogenetic abnormalities (deletion on chromosome 13 or hypodiploidy), low levels of C reactive protein (CRP), non IgA isotype, ISS stage I-II, low levels of plasma cells infiltration at diagnosis (<30%) and achievement of a good response to induction treatment [184-191]. The quality of response after ASCT is also an important prognostic factor for an extended survival [187,188,192,193].

As previously said, since 2000 the therapeutic armamentarium of MM has been increased by the discovery of new molecules, such as Thalidomide, Lenalidomide and Bortezomib. These drugs have been inserted in the treatment schema of young patients with MM, post as induction therapy pre and as consolidation or maintenance therapy post ASCT, and have significantly increased the rate of at least a VGPR and CR after the first ASCT (rate of VGPR between 54 and 79%) [147,149,160,194], thus increasing PFS. At present however the benefit in terms of long term OS are still not known and a longer follow up is needed [187].

Thalidomide has been one of the first drugs studied in the context of ASCT due to the impressive results obtained in the relapsed/refractory setting. The combination of Thalidomide and Dexamethasone (TD) incorporated into double ASCT resulted in a very good rate of high quality responses (CR/nCR 14% after induction and 36% after ASCT) and an improvement in PFS and OS (median PFS 48 months, OS 65% at 5 years) [195]. The major prognostic negative prognostic factors were del(17p), t(4;14) and high levels of β2microglobulin.

The inclusion of thalidomide in an intensified chemotherapeutic program with an induction followed by ASCT and consolidation chemotherapy (Total Therapy 2: TT2) resulted in a rate of CR of 62% (235) and in a significantly extension of OS (even in patients with unfavourable prognosis due to the presence of cytogenetic abnormalities) [196]; the estimated OS at 10 years was of 50% [197]. Thalidomide associated with cyclophosphamide and dexamethasone was superior to conventional chemotherapy in terms of response rate both after induction and after ASCT [134].

There is a large agreement of data on the superiority, in terms of rates of responses, offered by the combination TD or thalidomide associated with conventional chemotherapy in comparison to conventional chemotherapy such ad VAD or VAD-like [194,195,198,199]. The insertion of thalidomide in the context of ASCT resulted in an increase of PFS after ASCT; more controversy exists regarding a possible improvement of OS.

Similarly to thalidomide, the efficacy of **Bortezomib** in relapsed/refractory patients paved the way for its testing in first line treatment. Results from the studies so far available confirm the high efficacy of bortezomib when administered in combination with other agents in newly diagnosed MM patients. The combination of Bortezomib-Dexamethasone (VD) was shown to be very effective [149], and association with chemotherapeutic drugs such has doxorubicin (PAD) or cyclophosphamide (VCD), further incremented the possibility of achieving a good quality response [160,200-202].

One of the most interesting combinations is that of Bortezomib-Thalidomide-Dexamethasone (VTD). The superiority of VTD was seen both in terms of response rate and of PFS; this three drug combination was also able to improve the poor prognosis given by the presence of a t(4;14) translocation [147,203]. To reduce bortezomib associated side effects (mainly peripheral neuropathy), low doses of bortezomib and thalidomide associated with dexamethasone (vtD) have also been tested, showing good rate of responses with a better toxicity profile [204]. The addition of a fourth drug such as cyclophosphamide results in higher toxicity without a significant increase in responses [205].

In the Total Therapy approach bortezomib was added in the therapeutic protocol TT3, resulting in an EFS and OS of 71% and 78% respectively, with a median follow up of 39 months. The addition of bortezomib to a thalidomide containing approach (TT2 thalidomide arm versus TT3) resulted in a longer duration of CR (2-year sustained CR TT3 92% vs. 81% TT2 thalidomide arm) and in a trend towards a longer OS in patients with a high-risk disease (GEP high risk, t(4;14) positive) [206]. Despite these encouraging results, with a median follow up of 3.9 years the survival post relapse (SPR) was relatively short (14% at 5 years) due to a higher aggressiveness of the disease associated with increased drug resistance [197].

Even more promising than the VTD combination seem the association of Bortezomib, **Lenalidomide** and Dexamethasone (VRD). The VRD combination seems to be well tolerated, with an unprecedented response rate of 100%, and a 40% CR/nCR rate [207]. The same combination with lower doses of dexamethasone (VRd) is now being tested. Similarly to what already seen with thalidomide and bortezomib, several studies have also evaluated the addition of chemotherapy to the VRD regimen. Both doxorubicin and cyclophosphamide have been used in association with VRD, showing a good response rate [208] but at the price of an increased toxicity [209].

The second generation PI **Carfilzomib** is also being tested in the first line treatment of MM patients in association with Cyclophosphamide and IMiDs. The combination of Carfilzomib-thalidomide-dexamethasone (CarTaDex) resulted in an ORR of 91% (CR+VGPR of 18% and 60% pre and post ASCT, respectively) [210]. The association of carfilzomib with lenalidomide and dexamethasone achieved a rate of nCR of 38% after 4 cycles [211].

The unprecedented results seen with the use of new drugs have raised the question whether HDM and ASCT is still needed in the treatment of young patients with MM. A chemotherapeutic regimen with Melphalan, Prednisone and Lenalidomide (MPR) has been compared to HDM

followed by ASCT, showing an advantage of the ASCT arm both in terms of PFS (43 vs. 22 months, p<0.001) and of OS (82% vs. 65% at 4 years, p=0.02) [212].

Therapy after ASCT

Treatment strategy after ASCT is at present a matter of debate. Patients can be offered a consolidation treatment, a maintenance approach or both, with the aim of increasing responses and improving PFS and OS. Consolidation is normally a highly effective regimen given for a short period of time. A second ASCT can also be considered a consolidation strategy, and it is of higher benefit in those patients that have not achieved a CR or VGPR after the first ASCT. By contrast maintenance treatment consists of low dose drugs given fro a prolonged period of time, with the aim of maintaining the response achieved.

Thalidomide was the first of the new drugs used as a consolidation or maintenance therapy. All studies showed a benefit in terms of PFS, whilst an improvement on OS was seldom reported [158,197,199,213,214]. The benefit of thalidomide in terms of OS seems to be abrogated by the use of the same drug in the induction regimen [158,199,215]; furthermore caution is warranted due to the possible emergence of resistant clones and an unfavourable outcome seen in patients with high risk cytogenetic [126,158]. The unfavourable toxicity profile (mainly peripheral neuropathy) further limits the use of thalidomide in the maintenance setting [216].

Bortezomib has been inserted in consolidation and maintenance regimen (usually with as part of a 2 or 3 drugs combination), administered for 2 to 3 cycles after ASCT [160,203,217,218]. Bortezomib consolidation was also compared to observation, showing an improvement in responses for patients receiving treatment (51% vs. 32%, p=0.007) and a significative advantage in terms of PFS (median 27 vs. 20 months for treated and observation patients respectively, p =0.05) [219]. A consolidation treatment with the combination VTD was also shown to significantly improve the outcome (in terms of % of CR+VGPR and of PFS) of patients receiving bortezomib [217,218]. A consolidation treatment with VTD has also a role in inducing molecular remission [220,221]. By contrast data on bortezomib maintenance are currently limited and not conclusive, and further trials are needed to better address the role of bortezomib in this setting [160,203,216].

Lenalidomide seems to be the perfect drug for a maintenance treatment due to its oral administration and its favourable toxicity profile. Many trials are ongoing to better define the role of lenalidomide as a long-term treatment. Patients receiving lenalidomide maintenance (10-15 mg) after ASCT had a significantly longer PFS, with one trial also showing a significant benefit in terms of OS [159,161,212]. Despite these encouraging results concerns have been raised regarding the

possibility that a long-term treatment with lenalidomide after melphalan therapy might increase the risk of developing second primary malignancies [159,161,222]. A longer follow up together with studies designed to address the biological reasons for this increased risks are needed to better understand the exact role and timing of lenalidomide as a maintenance treatment [216].

Table 10. Studies with new drugs in newly diagnosed MM patients receiving ASCT

THALIDOMIDE PRE-ASCT

Author	Random	DVT (%)	N° pts	≥ VGRP (%) preASCT	≥ VGPR (%) postASCT
Rajkumar [198]	®TDvsD	17	200	63vs41 (≥PR)	n.r.
Cavo [194]	TDvsVAD	13	270	30vs15	68vs49
Morgan [134]	®CTDvsC-VAD	n.r.	1111	85vs71	90vs92
Lokhorst [199]	®TADvsVAD	10	402	32vs15	49vs32

THALIDOMIDE MAINTENANCE

Author	Therapy	N° pts	PFS	OS
Morgan [158]	®Induction (CVAD o CTD + ASCT vs. MP o CTDa) + ®maint Thal vs. no maint	818 (induction in 1970)	23% vs. 15%	n.s.

LENALIDOMIDE PRE-ASCT

Author	N° pts	≥ PR %	≥ VGPR %	CR/nCR %	PFS OS
Richardson VRD phase II [207]	35	100	74	57	NR
Palumbo [212]	402				
® MPR	202	n.r.	n.r.	n.r.	OS 65.3%, PFS 22.4 m
vs.					
MEL200	200	n.r.	n.r.	n.r.	OS 81.6%, PFS 43 m
® Maintenance R	198	78	48	23	OS 88% PFS 41.9 m
vs.					
No maintenance	204	77	48	19	OS 79% PFS 21.6 m

LENALIDOMIDE MAINTENANCE

Author	N° pts	PFS	OS
McCarthy [159] Lenalidomide vs. placebo	460	PFS at 3 y	OS at 3 y 88 %vs. 80%
Attal [161] Lenalidomide vs. placebo	614	PFS at 4 y 43% vs. 22%	OS at 4 y 73% vs. 75%

BORTEZOMIB PRE-ASCT AND/OR CONSOLIDATION

Author	Therapy	N° pts	≥ VGPR (%) preASCT	≥ VGPR (%) postASCT	PFS
Harousseau [149]	® VD vs. VAD	482	38 vs. 15	54 vs. 37	36 m vs. 27

					m
Sonneveld [160]	® induction PAD + maint VEL vs. induction VAD + maint Thal	626	n.r.	75 vs. 61	46% vs. 42% at 3 y
Cavo [147]	® VTD vs. TD induction and consolid	480	62 vs. 28	82 vs. 64	68% vs. 56% at 3 y
Rosinol [203]	® VTD vs. TD	202	29vs14 (CR)	59vs40 (CR)	82% at 2 y (OS)
Moreau [204]	® VD vs. vtD	199	49 vs. 39	74 vs. 58	30 m vs. 26 m
Leleu [218]	VTd-ASCT + consolid VTd vs. VTd-ASCT	217	After treatment: 83 vs. 64		TTP: 62% vs. 29% at 4 y
CARFILZOMIB					
Author	**Therapy**	**N° pts**	**PN gr 1-2 (%)**	**ORR (%)**	**DOR**
Jakubowiak [211]	CRd	53	24	100% (≥ VGPR)	n.r.
Sonneveld [210]	CARTHADEX	20	35	68% (≥ VGPR)	n.r.

Notes: RP partial response, VGPR very good partial response, TTP time to progression, PFS progression free survival, n.r. not reported Thal thalidomide, Dex dexamethasone, pts patients, TD thalidomide dexamethasone, VAD vincristine adriamycin dexamethasone, CTD thalidomide cyclophosphamide dexamethasone, TAD thalidomide adriamycin dexamethasone, CARTHADEX Carfilzomib-Thalidomide-Dexamethasone, DVT deep vein thrombosis, MP melphalan prednisone, ⍰ randomisation, y years, m months, ASCT autologous transplantation. *no prophylaxis, consolid consolidation, mant maintenance

4.1.2 FIRST LINE TREATMENT IN PATIENTS NOT SUITABLE FOR ASCT

Many options are nowadays available for elderly patients not suitable for ASCT. In patients younger than 85 years, when possible, experimental protocols are preferred. If that is not possible the preferred treatment should include a Pi or an IMiDs, in association or combined with steroids and low dose chemotherapy. In very old patients (>85 years), or in particularly fragile and unfit patients, the old schema MP (Melphalan + Prednisone) or alternatively Dexamethasone are usually used, in association with new drugs if the performance status is acceptable.

Melphalan-prednisone (MP) has for many years been the standard of care in elderly patients, and is still used as a backbone for combination therapy in this category. The ORR with MP is of about 53%, with only 5% of cases achieving a CR, the PFS is 18 months and OS is 36.5 months [168,169].

The first drug to be added to the classic MP schema was **Thalidomide**, at doses ranging from 100 mg to 400mg). Some studies showed no significant improvement in ORR for MPT compared to MP, but a better PFS and OS [223,224]. Conversely other groups found a better ORR and a higher PFS, but no differences in OS [225,226]. A meta-analysis of six randomised trials comparing MP vs. MPT demonstrated that the addition of Thalidomide the MP significantly increases PFS and OS of about 20%, thus showing a superiority of MPT compared to MP [227].

Lenalidomide, in association with dexamethasone was founds superior to high dose dexamethasone both in terms of a higher response rate (ORR of 78% with 63% ≥ VGPR) and of a longer PFS [228]. Lenalidomide with high dose Dexamethasone (LD) was compared to Lenalidomide with low dose dexamethasone (Ld) showing higher response rate for patients receiving higher doses of dexamethasone, but a better OS for patients treated with Ld due to an increased mortality in patients receiving LD [148]. Results from this study made Ld as the treatment of choice both for young and older patients. Continuous treatment with Ld was found to be superior to Ld for 18 cycles and to MPT, significantly reducing the risk of death (HR=0.78; p=0.02) [157]. For the first time a regimen not containing any chemotherapeutic agent was identified as one of the standard of care for the treatment of MM patients. Similarly to thalidomide, lenalidomide was also associated with MP, with (MPR-R) or without (MPR) lenalidomide maintenance. The association MPR was not superior to MP in terms of PFS (14 months, HR: 0.49, <0.001 vs. 13 months, HR: 0.40, p<0.001), but the addition of lenalidomide maintenance significantly improved the outcomes (PFS 31 months) [151]. Further studies with lenalidomide in elderly patients are summarised in table 11.

Similarly to what has been seen in young patients, one of the most interesting combinations is the association of Lenalidomide-Bortezomib [207,229]. Preliminary results testing this combination show

that results can improve prolonging therapy from 4 to 8 cycles, making this combination interesting also in the non-transplant setting [207]. The addition of doxorubicin the VRD regimen is also under study [208].

The association Ld is currently undergoing evaluation in association with the monoclonal antibody anti CS-1 Elotuzumab.

As already seen with thalidomide and lenalidomide, **Bortezomib** has been added to the MP schema, showing a higher ORR and CR rate and a longer TTP and OS (24 vs. 16.6 months, p<0.001 and 56.4 vs. 43.1 months, p=0.0004, respectively), compared to the standard MP [150]. In consideration of these impressive results, the VMP regimen has become one of the standard of care for patients older than 65 years not suitable for ASCT. The same combination with once weekly bortezomib showed that a weekly administration of bortezomib is better tolerated in the elderly population, with a reduced rate of adverse events, in particularly a reduced risk of developing peripheral neuropathy [230]. VMP has also been compared to VD and VTD: the three regimens showed similar response rates, but a higher toxicity in the VTD arm [231]. Similar results were seen with the combination VMP vs. VTP confirming a better toxicity profile for VMP regimen [232]. The comparison between VCD, VRD and VDCR is also under evaluation in the elderly population [209]. For patients between 65 and 75 years of age a 4 drugs combination of Bortezomib-Melphalan-Prednisone-Thalidomide (VMPT) can also be considered. The VMPT combination has a higher rate of neuropathy, but increases significantly the rate of CR compared to VMP (38% vs. 24%, p <0.001) and improves OS (59.3% vs. 45.9% at 5 years, respectively, p=0.04) [230,233].

The association of Cyclophosphamide, Dexamethasone and **Carfilzomib**, followed by Carfilzomib maintenance has currently been tested, showing an ORR after induction of 96% (76% VGPR, 64% CR/nCR, 24% sCR) and a PFS and OS at 1 year of 86% and 87%, respectively. Six months of Carfilzomib maintenance increased the rate of PR to 100% (68% CR/nCR) [234].

Table 11. Clinical studies in patients not suitable for ASCT

THALIDOMIDE

Author	N° pts	Therapy	≥PR	CR/nCR	PFS OS
Facon [223]	447	MPT vs. MP vs. MEL100+ ASCT	76% vs. 35% vs. 65%	13% vs. 2% vs. 18%	PFS: / OS: 51.6 vs. 33,2 vs. 38,8 m
Hulin [224]	232	MPT vs. MP-placebo	62% vs. 31%	7% vs. 1%	PFS: 24.1 vs. 18.5 m OS: 44.0 vs. 29.1 m
Palumbo [225]	255	MPT vs. MP	76% vs. 47.6%	27.9% vs. 7.2%	EFS: 54% vs. 27% (at 2 y) OS: 80% vs. 64% (at 3 y)
Wijermans [226]	333	MPT-T vs. MP	65% vs. 45%	n.r.	PFS: 15 vs. 11 m OS: 40 vs. 31 m
Fayers [227]	1685	MPT vs. MP	59% vs. 37%	n.r.	PFS: 20.3 vs. 14.9 m OS: 39.3 vs. 32.7 m
Kapoor [152]	1568	MP vs. MPT	66% vs. 45%	3% vs. 13%	n.r.

LENALIDOMIDE

Author	N° pts	≥ PR %	≥ VGPR	CR/nCR %	PFS OS
Rajkumar RD phase II [235]	34	91	56	18	PFS 83% and OS 92% at 2 y
Kumar CRd [236]	53	83	40	2	n.r.
Rajkumar RD vs. Rd [148]	445	79vs68	51vs40	17vs14	OS 87% vs. 96% P=0.0002
Zonder RD vs. Dex [228]	198	78vs48	63vs16	26vs4	OS 79% vs. 73% at 3 y
Niesvizky BiRD [237]	72	90	74	46	n.r.

Kumar EVOLUTION VDCR phase I [238]	25	96	68	CR+sCR 40 (20 sCR)	n.r.
Kumar EVOLUTION phase II [209]	140				OS n.r. PFS at 1 y:
VDRC		n.r.	58	25	86
VDR		n.r.	51	24	83
VDC		n.r.	41	22	93
VDC-MOD		n.r.	53	47	100
ELOQUENT-1 RD±ELO			Trial ongoing		
MM020/FIRST RD until PD (A) vs. RD (B) vs. MPT (C) [157]	1623	75% (A) vs. 62% (C)	n.r.	n.r.	PFS: reduction of risk of death or PD 28% (A vs. C) OS: reduction of risk of death 22% (A vs. C)

LENALIDOMIDE MAINTENANCE

Author	N° pts	PFS	OS
Palumbo MPR-R vs. MPR vs. MP (MM015) [151]	459	31 vs. 14 vs. 13 m	70% vs. 62% vs. 66%

BORTEZOMIB

Author	Therapy	N° pts	ORR	OS	SAEs
San Miguel [150]	® VMP vs. MP	682	>for VMP	46% vs. 34% a 5 y	n.r.
Niesvizky [231]	® VMP vs. VD vs. VTD	502	No differences	88.9% vs. 87.4% vs. 86.1% at 1 y	>for VTD
Mateos [232]	® VMP vs. VTP	260	No differences	n.r.	>for VTP
Richardson [207]	VRD phase II	35	96%	Nr at 1 y	n.r.
Kumar [209]	® VRD vs. VRDC vs.	140	>for VRDC	Nr at 1 y	>for VRDC

	VDC				
Palumbo [230]	® VMPT vs. VMP	511	>for VMPT	No differences	>for VMPT
CARFILZOMIB					
Author	**Therapy**	**N° pts**	**ORR**	**OS**	**SAEs**
Brighen [234]	CFZ-Ciclo-Dex	25	96%	87%	n.r.

M=Melphalan; P=Prednisone; T=Thalidomide; V=Bortezomib; D=Dexamethasone; C=Cyclophosphamide; R=Lenalidomide; ELO=Elotuzumab

®=randomization; nr =not reached; n.r.= not reported

PR=partial response; CR= complete response; nCR= near complete response; VGPR=very good partial response; PFS=progression free survival; OS=overall survival

4.2 THERAPY FOR RELAPSED/REFRACTORY PATIENTS

Despite the advantages in terms of response rate and survival obtained after the advent of the new drugs, the history of MM is still characterised by phases of remission and relapses. At every relapse responses are less profound and shorter, until drug resistance is developed and the disease enter in the so-called relapsed/refractory phase, eventually leading to patient's death. The new drugs such as PI and IMiDs have proven their efficacy also in this subset of patients, significantly increasing OS compared to high dose dexamethasone [239].

A wide variety of options are nowadays available for relapsed patients. If the patient has never received a PI or an IMiD, treatment with these drugs (especially Bortezomib and Lenalidomide) is a good option; an alternative, in case of a long duration of the first remission, is a retreatment or rechallenge with the same molecule or the same class of molecules previously used. In fit patients below 65-70 years of age a salvage treatment with ASCT can be applied, proven that the duration of response after the first ASCT was long enough. Enrolment in clinical trials gives the possibility to use new molecules not yet approved, such as the monoclonal antibody Elotuzumab, Daratumuab (anti CD38) or second generation PI such as MLN.

When choosing the salvage treatment to use, different factors have to be taken into account in order to obtain a good balance between efficacy and side effects. There are disease-related factors: quality and duration of the previous remission, aggressive or indolent relapse, the presence or absence of negative prognostic factors (del(17p), t(4;14), ampl(1q21)), the presence of extramedullary disease or plasma cell leukaemia; and patients-related risk factors: age, performance status, renal function, precedent treatments, residual toxicity, presence of peripheral neuropathy, risk of DVT [240,241].

For very advanced patients, already exposed and resistant to both IMiDs (Lenalidomide) and PI (Bortezomib) (double refractory patients) the prognosis is extremely poor, with an OS and a PFS of 9 and 5 months, respectively [242]; for this group of patients no standard of care is at present available, and several clinical trials are ongoing to find the most effective drug combination. The FDA (Food and Drug Administration) has recently approved Carfilzomib (a second generation PI) for the treatment of patients previously exposed to Bortezomib and IMiDs and refractory to the last line of treatment received. Pomalidomide (a third generation IMiDs) has also been approved for the treatment of patients previously exposed to Bortezomib and Lenalidomide. Other compounds currently under evaluation are the kinesine spindle protein (KSP) inhibitor (ARRY-520). The major studies in relapsed/refractory patients are summarised in table 12. Among the different possibilities for relapsed/refractory patients, the more interesting at present seem to be a retreatment and the new molecules already available Carfilzomib and Pomalidomide.

Table 12. Clinical studies in relapsed/refractory MM patients

THALIDOMIDE ± dexamethasone

Author	Dose Thal	Dex	N° pts	≥PR (%)	TTP/PFS
Barlogie [243]	200-800	/	169	33	20% at 2 y
Tosi [244]	400 (median)	/	65	28	8 m
Schey [245]	300 (median)	/	69	39.5	14 m
Dimopoulos [246]	400	DEX	44	55	≥10 m
Anagnostopoulos [247]	200-800	DEX	47	47	n.a.
Zamagni [248]	100-200	DEX	100	46	22 m

THALIDOMIDE + chemotherapy

Author	Therapy	DVT (%)	N° pts	≥ PR (%)	TTP/PFS
Palumbo [225]	MP+T	0.04	24	42	9 m

LENALIDOMIDE

Author	N° pts	ORR %	TTP	OS
Dimopoulos [249] MM010	351	61% vs. 24%	11.3 vs. 4.7m	p=0.03
Weber [250] MM009	353	61% vs. 20%	11.1 vs. 4.7m	29.6 vs. 20.2
Lonial [251] ELOQUENT-2 RD±ELO	28	82%	NR	NR
Siegel [252] RD+Vorinostat dose escalation	31	47%	6.5 m	n.r.

BORTEZOMIB

Author	Therapy	N° pts	≥ PR (%)	OS (m)	TTP (m)
Richardson [253]	VEL 1.3[1]	202	27	16 (median)	7 in pts with ≥ PR
Jagannath [254]	VEL 1.0[2] vs. VEL 1.3	27 vs. 26	30 vs. 38	n.a.	7 vs. 11
Richardson [255]	® VEL 1.3 vs. DEX[3]	336 vs. 333	38 vs. 18	80% vs. 66% @ 1 y	6.2 vs. 3.5
Ludwig [256]	VEL 1.3-Dex-Bendamustine	79	61	25.6	9.7

Petrucci [257]	VEL 1.0 o 1.3	132	40	n.r.	8.5 in pts with ≥ PR
Richardson [258]	VEL 1.0- Len-Dex	64	64	30	9.5
Berenson [259]	DVD-R	40	49	n.r.	9

CARFILZOMIB

Author	Therapy	N° pts	PN gr 1-2 (%)	ORR (%)	DOR
Siegel [260]	Carfilzomib[1]	266	77	24 (≥ PR)	7.4 m
Vij [261]	Carfilzomib[5]	129	16	52 (≥ PR)	NR
Vij [262]	Carfilzomib	35	14.3	17.1	>10.6 m
Badros [263]	Carfilzomib	50 (RI)	10	26	7.9 m
Niesvizky [264]	Carfilzomib-Len-Dex	40	50	62.5	11.8 m (pts with ≥ PR)
Lendvai [265]	Carfilzomib high-dose ± Dex	34	3	50	n.r.
ASPIRE	®Carfilzomib-Len-Dex vs. Len-Dex	Trial ongoing			
FOCUS	®Carfilzomib vs. best supportive care	Trial ongoing			
ENDEAVOR	®Carfilzomib-Dex vs. VEL-Dex	Trial ongoing			

POMALIDOMIDE

Author	N° pts	Therapy	ORR%	PFS (months)	OS (months)
Lacy [266]	60	Poma: 2mg 1-28 Dex: 40mg/sett	65	13	76% at 2 y
Lacy [267]	35 [b]	®Poma: 2mg 1-28 Dex: 40mg/sett vs. Poma: 4mg 1-28 Dex: 40mg/sett	25 29	6.5 3.2	78% at 6 months 67% at 6 months
Leleu [268]	43 [b]	®Poma: 4mg 1-21 Dex:	35	5.4	14.9

		40mg/sett vs. Poma: 4mg 1-28 Dex: 40mg/sett	34	3.7	14.8
San Miguel [269]	455	®Poma: 4mg 1-21 Dex: 40mg/sett vs.	31	4.0	12.7
		Dex: 40 mg x 4 1-4; 9-12; 17-20	10	1.9	8.1

Thal=Thalidomide; Len=Lenalidomide; Dex=Dexamethasone; VEL=Bortezomib; DVD-R=Dexamethasone Bortezomib Doxorubicin Lenalidomide; NR=not reached; n.r.=not reported; ®=randomisation; *=no prophylaxis; **=no dex; §=with dex; y=years; m=months; [1]pts with more than 2 previous lines of therapy; [2]pts with ≤3 lines of therapy, not refractory to Dex; [3]pts after 1 line of therapy; [4] median 4 lines of therapy; [5]bortezomib naive; [a]Patients refractory to Lenalidomide; [b]Patients refractory to Lenalidomide and Bortezomib; PR=partial response; SD=stable disease; MR=minimal response; PN=polyneuropathy; DVT=deep vein thrombosis; RI= renal impairment; TTP=time to progression; PFS=progression free survival; DOR=duration of response; OS=overall survival; ORR=overall response rate

- **Retreatment with Lenalidomide or Bortezomib**

Many studies are available regarding the retreatment with Bortezomib or Lenalidomide. The combination DVD-R (Doxorubicin-Bortezomib-Dexamethasone-Lenalidomide) has shown the efficacy of Bortezomib retreatment in a group of patients previously exposed to Bortezomib (82%), and Lenalidomide (47.5%). The ORR of the DVD-R combination was of 48.7%, and it was high also in patients previously treated with Bortezomib (≥MR 81.8%) or Lenalidomide (≥MR: 63.1%) or that had failed both (≥MR 60%) [259]. The combination BLD (Bendamustine-Lenalidomide-Dexamethasone) was used in relapsed/refractory MM (RRMM) patients, of which 66% had been previously treated with Bortezomib and 45% with Lenalidomide. The ORR was 76%, with a median PFS of 6.1 months in patients previously exposed to Bortezomib, and not reached in patients previously exposed to Lenalidomide [270]. Bortezomib plus Bendamustine was effective in heavily pre-treated patients who had already received Bortezomib [256]. Retreatment with Bortezomib in patients that had received Bortezomib and had obtained a response for more than 6 months was specifically evaluated showing an ORR of 40% and a TTP of 8.5 months for those patients achieving at least a PR [257].

- **Carfilzomib**

The second-generation proteasome inhibitor Carfilzomib has recently been approved for the treatment of RRMM patients. Carfilzomib was shown to have a prolonged anti-tumour effect in RRMM, irrespective of previous Bortezomib treatment. In patients never exposed to Bortezomib the ORR was of 48%, and it was higher for those patients that received a dose escalation of Carfilzomib (up to 27 mg/m^2) (52% vs. 42% respectively); median DOR was 13.1 months, with a PFS of 8 months. The risk of peripheral neuropathy was relatively small, considering that 50% of patients had pre-existent neuropathy at study entry [262]. In patients previously exposed to Bortezomib, including 14 patients refractory to the most recent line of therapy, the ORR was 17%, with a DOR of 9 months and a TTP of 5.3 months [262].

The major grade ≥3 adverse events, evaluated on 526 patients, were: thrombocytopenia (23%), anaemia (22%), lymphopenia (18%), pneumonia (11%) and neutropenia (10%). Peripheral neuropathy was reported only in 14% of cases, and it was generally mild or moderate, despite 72% of patients having a ≥ grade 2 neuropathy at trial entry [271].

In a different phase II trial 266 patients already exposed to IMiDs e PI were evaluated; 95% were refractory to the last line of treatment, 80% were refractory or intolerant to Bortezomib or Lenalidomide. The ORR with Carfilzomib monotherapy was of 23.7% (28% in patients with adverse cytogenetic); median DOR was 7.8 months and median OS was 15.6 months. The most common adverse events were fatigue (49%), anaemia (46%), nausea (45%), e thrombocytopenia (39%); 12.4% of patients developed a peripheral neuropathy grade 1-2 [260]. A possible activity of single agent Carfilzomib in high-risk patients harbouring adverse cytogenetic abnormalities has also been suggested.

Based on the favourable results of single agent Carfilzomib, its activity in combination has also been explored. Carfilzomib associated with Lenalidomide and low dose Dexamethasone obtained a rate of responses of 62.5% (2.5% sCR, 32.5% VGPR, 27.5% PR); the most frequent adverse events ≥ grade 3 were neutropenia, anaemia and thrombocytopenia [264].

High doses of Carfilzomib (up to 56 mg/m^2), with the optional addition of Dexamethasone in case of unsatisfactory response after 2 cycles, showed an ORR of 47% in patients refractory to Bortezomib (77%), suggesting that a higher dose might be more effective; with a follow up of 18.4 months the median PFS was 4.1 months and the median OS was 20.3 months [265].

Several phase III trials are currently ongoing: the ASPIRE study, comparing Carfilzomib-Lenalidomide-Dexamethasone vs. Lenalidomide-Dexamethasone; the FOCUS study, comparing

Carfilzomib monotherapy vs. best supportive care; the ENDEAVOR study comparing Carfilzomib-Dexamethasone vs. Bortezomib-Dexamethasone.

- **Pomalidomide**

The third generation IMiD Pomalidomide has been evaluated in a large number of phase I and II clinical trials; these trials have identified the dose of 4 mg for 21 days over a 28 days period as the dose to bring forward in the clinic [266-268,272,273].

In patients refractory to Lenalidomide treatment with Pomalidomide (at different dose ranges) in association with weekly Dexamethasone achieved at least a PR in 21-32% [267,272]. Preliminary data suggests that Pomalidomide might have a role in the treatment of high-risk patients. Pomalidomide was evaluated in patients positive for t(4;14) and/or del(17p) showing an ORR of 22%. Interestingly both responses and DOR were higher in patients with del(17p) than in patients with t(4;14) (ORR 32% vs. 15%, DOR 8.3 months vs. 2.4 months, respectively), suggesting that treatment with pomalidomide might improve the outcome of this very high-risk group [274].

Based on the results of a recently published phase III trial, comparing Pomalidomide ad Dexamethasone versus high dose Dexamethasone, Pomalidomide has been approved by FDA and EMA for the treatment of relapsed/refractory patients that have previously received both Bortezomib and Lenalidomide and are refractory to at least one of those. With a median follow up of 10 months the trial showed that, in a heavily pre-treated population (median 5 lines of previous treatment, 75% refractory to Bortezomib and Lenalidomide), the PFS was double in patients receiving Pomalidomide compared to the control arm (4.0 months vs. 1.9 months, HR:0.48; $p<0.0001$). An advantage in OS could also be seen (12.8 vs. 8.1 months, HR=0.74; p=0.028) [269].

Pomalidomide has also been tested in association with proteasome inhibitors and conventional chemotherapy [275,276].

STUDY OBJECTIVES

The presence of intra-clonal heterogeneity has been postulated in a variety of solid and haematological tumour, including Multiple Myeloma. Furthermore the advent of new drugs and the new treatment concept including a maintenance treatment following ASCT for young patients, and a continuous treatment for elderly patients not suitable for ASCT, have raised concerns regarding the possible side effects of new drugs and their long-term impact on the bone marrow of MM patients.

In the light of these concepts, the main objectives of the present research project were to:

1. Confirm the presence of intra-clonal heterogeneity in Multiple Myeloma, both at a general level (with the use of whole exome sequencing) and at a more profound single-cell level in primary samples of patients receiving IMiDs or PI.
2. Assess the presence of intra-clonal heterogeneity at a clinical level, and evaluate its impact on treatment outcomes
3. Evaluate the possible long-term effect of Lenalidomide on the bone marrow of MM patients.

MATHERIALS AND METHODS

1. EVALUATION OF INTRA-CLONAL HETEROGENEITY IN PRIMARY SAMPLES OF MYELOMA PATIENTS

1.1 WHOLE EXOME SEQUENCING

CD138-positive bone marrow plasma cells were selected to a purity >95%, as determined by cytospin, using magnetic-assisted cell sorting (Miltenyi Biotec, Bisley, Surrey, UK). Tumour DNA was extracted using the AllPrep kit (QIAGEN, Manchester, UK); DNA concentration was assessed using Pico-green (Life Technologies, Pailsey, UK). Non-tumour DNA was isolated from white blood cells using the FlexiGene kit (QIAGEN). Approximately 50 to 100 ng of DNA underwent whole-genome sequencing using 120-bp paired-end reads on a GAIIx (Illumina, Saffron Walden, UK) to a median depth of 44x to 59 x according to the study, with 96% to 82% >20x coverage. Paired reads were aligned to the human genome (GRCh37) using Stampy [277] and BWA [278], and duplicate reads were removed using Picard. Sequence recalibration, local realignment and single-nucleotide variant (SNV) calling were all undertaken using the GATK [279] or MuTect [280]. SnpEff was used to functionally annotate all variants. Further filtering of variants and comparisons between samples were performed using code written in R. Variants present in both the peripheral blood and tumour samples were discarded. Only variants sequenced to a minimum depth of 10x in both the tumour and matched normal sample, as well as having a minimum genotype score of greater than 50 (representing a 1 in 100 000 error rate) and no more than one variant read in the normal sample were retained. Variants were determined to be unique to a disease stage or present in both stages by comparing the base calls at the location of the SNV in both samples. Finally, an additional filter was applied for C>A|G>T SNVs to take into account somatic change artefacts reported by Costello et al. Whole-genome copy number was called using a combination of the R package BICseq to perform segmentation of the log ratio of the read depth between matched peripheral blood and tumour samples and the R package CGHcall to produce integer copy calls within the segmented regions. Copy number in exome samples was similarly determined using the R package ExomeCNV. The proportion of cells containing a variant was estimated using the following equation:

$$p = \min(1, \frac{rC}{R})$$

Where p is the proportion of cells containing the variant, C is the integer copy number at that position, r is the number of reads containing the variant and R is the total number of reads. Note

that this method assumes that the tumour cell population is free from normal cell contamination and that detected copy number changes are not sub-clonal.

Owing to the high identity between the human and mouse genomes, two strict precautions were taken to avoid false positives in the xeno-transplanted samples. First, aligned xeno-transplant BAMs were filtered to remove any reads containing more than a single mismatch to the reference genome. Second, private SNVs in xenograft samples were treated as artefacts and removed. As a result of this filtering, the median depth for xenograft samples after deduplication was 31x (range 15-47) with an average of 58.4% of the targeted exome covered at a minimum depth of 20x. All SNVs were manually inspected using IGV to verify that they were both correctly called and correctly annotated as being present or absent in each of the tumour samples.

1.2 SINGLE CELL ANALYSIS

Archival methanol:acetic-acid-fixed single-cells and tumour/normal DNA were obtained. Fixed single-cells from each patient were sorted in a FACSAria cell sorter (BD Biosciences, Oxford, UK) using Propidium Iodide (PI) nuclei staining. Depending on cell availability, we sorted 73-243 single-cells into DNA-lysis buffer in one to three 96-well plates (84 cells/plate). In parallel, lymphocytes from a healthy donor were sorted as wild type and normal copy number controls (10 cells/plate). Finally, 2 wells/plate were left empty to add bulk tumour and peripheral blood DNA from the same patient (positive and negative controls for mutation detection). Additionally, 60 fixed-lymphocytes from a healthy donor were sorted in a separate 96-well plate as reference cells for the definition of copy number thresholds across all interrogated genomic regions. Assay efficiency was calculated using five 2x dilutions for the amplified DNA of six lymphocytes.

A novel approach for single-cell multiplex qPCR analysis was performed (Fluidigm UK, London, UK) [281]. Briefly, single-cells were sorted into lysis buffer followed by specific (DNA) target amplification (STA). This multiplex STA reaction comprises the simultaneous amplification of target regions of interest using TaqMan PreAmp Master Mix and assays (Life Technologies). Mutation-specific genotyping assays were custom-designed following manufacturer's guidelines. Pre-designed genotyping assays for locus known to be in heterozygosis in all tumour samples [114,282] were used as reference (*rs346172* and *rs909895*). Three different TaqMan copy number assays covering each chromosomal region of interest were used for copy number analysis. The STA product was diluted and qPCR was performed using the 96.96 dynamic microfluidic arrays and the BioMark HD (Fluidigm). Genotyping assays were present in replicates (2-4), whereas each multiple copy number assay per region was used in quadruplicates to ensure enough replicates for accuracy in

copy number calling [283]. Translocations, mutations, and copy number aberrations were assessed at the single-cell level. Genotyping and copy number analyses were assessed using Fluidigm Real-Time PCR Analysis v.3.0.2 software (Fluidigm). To estimate copy number values, CopyCaller v.1.0 software (Life Technologies) was used and weighted means of the calculated copy number values for each experimental replicate were determined. Hierarchical clustering was achieved using Pearson correlation and average linkage on the Rock platform[284] and further customized with R. A filtering strategy for wells with low-quality DNA amplification and sub-clones without a minimum cell number was applied. To define tumour sub-clones and the most plausible clonal phylogeny, we used minor modifications to the filtering strategy for wells with low quality DNA amplification and sub-clones without a minimum number of cells, used elsewhere [281]. We removed from further analysis all those cells/rows that failed to amplify in at least one of the reference SNP assays (for mutation call) or in any of the reference copy number assays (for copy number calling). Additionally, Ct values >30 cycles were flagged as not assessable (NA).

For mutation and translocation calling, we transformed Ct results for mutation and translocation assays into binary (1, presence of mutation; 0 wild type) considering the assay replicates per cell (2-4 replicates). Results from all the interrogated mutations per cell were combined and cells with NA values were removed from subsequent analyses. The total number of sorted analysable tumour single-cells was calculated. The threshold to define sub-clonal populations was established in at least 5% of the total of analysable tumour cells. All cells in fractions below such thresholds were removed from further analysis.

For copy number aberrations, cells were initially filtered out if normal reference regions failed to amplify. CopyCaller software was used to estimate the calculated copy number values (cCNV). Nine comparisons were performed in Copycaller, comparing the 3 reference control region assays versus the 3 test region assays. This provided a total of nine cCNVs for each tested region in any of the analysed cell per patient. cCNVs were removed if confidence intervals were below 0.5. Additionally, cCNV>4.5 were not considered, as the number of replicates did not ensure statistical accuracy for copy number estimation [283]. The weighted mean of the cCNVs (weighted copy number values, wCNV) was calculated only when at least 3/9 replicates were available. If less than 3, such region was considered NA in that specific cell. wCNV below 1.5 and above 2.5 were considered as genomic loss (-1) and gain (1) respectively, whereas intermediate values mean no change (0). Then, we plotted histograms with the wCNV for each cell in all plates. Reference array histogram was characterized for the accumulation of cells within the normal parameters, but a

group of cells provided wCNV >2.5 and <1.5 as a consequence of the qPCR technical variation [283,285]. For each region, we calculated the percentage of normal cells misleadingly displaying gains or losses and used the highest frequency as a threshold to set the minimum number of cells required to be considered sub-clone in copy number aberration analyses (sub-clonal thresholds). Qualitative values for each region and cell (loss, no change, gain) were combined and cells with NA value for any region were removed from further analysis. The total number of analysable tumour single-cells for copy number aberrations was then calculated and sub-clonal thresholds for each tested chromosomal region were applied. Hierarchical clustering illustrated the different aberration patterns in clonal populations. Copy number aberrant sub-clonal populations were considered when cells were grouped in numbers higher than each of the sub-clonal thresholds established per chromosomal region. All cells not fulfilling the criteria were filtered out. We then combined both filtered mutation and copy number information for each cell, and followed an additional filtering process with the same criteria for both features. A total number of filtered-analysable tumour cells were estimated and clonal phylogenies and percentages could be accurately established.

1.3 NOD-SCID MOUSE TRANSPLANTATION

Presentantion- and relapse-samples from a PCL-patient were studied by WES. In parallel, female NOD/SCID IL2Rγ^{null} mice approximately 6 weeks old were inoculated with 1x10^6 CD138$^+$cells from the PCL-patient at diagnosis in 20 µl complete RPMI-1640 GlutaMAX^{TM} medium, and monitored for myeloma development over 5 months as before [286]. Mice were then culled and myeloma-xenograft samples were purified for human CD138$^+$ cells.

1.4 GENETIC ALGORITHM IN THE MULTI-SAMPLE EXOME SEQUENCING ANALYSIS

Xenograft-tumour DNA was analysed by WES using a modified pipeline and compared with the tumour exomes from presentation and relapse patient-samples. A genetic algorithm was implemented using the GA package for R [287] to calculate the most parsimonious assignments of mutations to clonal lineages. Custom population generation, fitness, mutation, and crossover functions were written. The population being optimised consisted of a series of vectors, each composed of three parts. The first *n* elements (where *n* is a predicted number of lineages present in the sample) are integers identifying the parent of the lineage n_i, with 0 indicating that this lineage is the root lineage. The next *n* x *m* elements (where *m* is the number of cases observed) are integers, initialized between 0 and 5, but with no upper bound following mutation,

representing the relative abundance of lineage n_i in case m_j. The remaining *l* elements (where *l* is the number of mutations observed) are integers from 1 to *n* representing the earliest lineage in which each mutation l_i occurs. The population generation function produces vectors with exactly one root lineage, where the lineages can be represented as a directed acyclic graph (DAG), and with all remaining elements assigned randomly. The fitness function calculates the predicted proportion of the total sample that should contain each mutation based around the assumption that a mutation should appear in the lineage it first occurs in and all children of that lineage. The proportion of each lineage in case m_j is computed first as the relative abundance of lineage n_i in case m_j divided by the sum of all n_i in case m_j. Then for each mutation the proportions of all lineages containing that mutation are summed. The absolute value of the difference between the predicted proportion and the experimentally observed proportion of each mutation in each case is summed. The fitness function then returns -1 times the sum of these values as a score to be optimized. The mutation function assumes that the predicted lineages, abundances or mutation calls can change with equal probability. In the case that lineages are changed, a completely new DAG for the lineages is produced and replaces the original. In the case that relative abundances are changed, a random relative abundance is incremented or decremented by 1 (in the case that a decrement would reduce abundance below 0, the abundance is incremented instead). In the case that a mutation is changed, a random mutation is assigned a new lineage at random. The crossover function uses single point crossover. However, to ensure that lineages are always representable as a DAG the crossover cannot occur during the first *n* elements of a vector. The remaining parameters required for the GA function were selected as follows: population size: 100, elitism: 2, mutation rate: 0.8, tournament selection, iterations: 1000, type: binary. The function was iterated over n=5:12 predicted lineages, and the best scoring individual was selected.

2. CLINICAL ASSESSMENT OF INTRA-CLONAL HETEROGENEITY IN PATIENTS TREATED WITH NEW DRUGS

2.1 PATIENTS

Patients from the MRC Myeloma XI trial for which complete clinical and laboratory value were available were selected. The MRC Myeloma IX trial (ISRCTN68454111) enrolled 1960 patients and the full design and primary results of the trial have been reported [288,289]. In summary, the trial randomised newly diagnosed multiple myeloma patients to receive thalidomide versus non-thalidomide containing therapy; thalidomide could be given both as an induction and/or as a maintenance regimen. Patients were divided between an intensive and a non-intensive pathway based on their eligibility for ASCT. Primary end-points included PFS, OS and response. Centralised laboratory results were available for 647 patients.

2.2 FLC EVALUATION AND RESPONSE ASSESSMENT

FLC and paraprotein levels were evaluated at diagnosis, post induction, and at relapse in all patients. The International Myeloma Working Group (IMWG) uniform response criteria [173] were used to assess response and relapse in this manuscript based on central laboratory analysis of serial blood and urine samples. Patients were classified as relapsing with free light chain only (FLC) escape if they failed to meet the IMWG criteria for change in paraprotein levels that define relapse but satisfied IMWG criteria for changes in FLC levels (Table 1).

Table 1. Relapse criteria used to define the type of relapse (modified from Durie et al. [173] and Rajkumar et al. [290])

Type of Relapse	Change in Paraprotein Between Maximum Response and Relapse	Change in FLC Levels Between Maximum Response and Relapse
Paraprotein only PO	≥5g/L and an increase ≥25%	Urine FLC <200mg/24hr and iFLC <100mg/L **and** dFLC <100mg/L
Paraprotein and light chain PLC	≥5g/L and an increase ≥25%	iFLC must have increased by ≥ 100mg/L **and** the increase must be ≥25% **and** dFLC≥100mg/L **and** the FLC ratio must be abnormal
Free light chain escape FLC escape	<5g/L	iFLC must have increased by ≥ 100mg/L **and** the increase must be ≥25% **and** dFLC≥100mg/L **and** the FLC ratio must be abnormal
Clinical relapse	<5g/L	iFLC <100mg/L **and** dFLC<100mg/L

iFLC=involved serum free light chain level. dFLC=difference between involved and uninvolved serum free light chain levels

3. IMPACT OF LENALIDOMIDE ON THE BONE MARROW OF MYELOMA PATIENTS

3.1 PATIENTS

A total of 18 patients diagnosed with MM and treated at the Royal Marsden Hospital in London were included. All patients were receiving treatment with lenalidomide, either alone or in association with steroids and had received 0 to 4 prior lines of treatment; 14/18 patients had received ASCT. Patients were categorized according to the time they had been on lenalidomide treatment: eleven patients that had received lenalidomide for less than one year (median 9 months, range 4-12) where categorised as “short-term lenalidomide” (STL), whilst the seven patients that had received lenalidomide for more than one year were defined as “long-term

lenalidomide" (LTL) (median 65 months, range 22-82). Seven additional patients who had received ASCT, but were never treated with lenalidomide, where used as control group.

3.2. FLOW CYTOMETRY ANALYSIS

MPF analysis was performed using the EuroFlow antibody panels [291]; three, 8 colour, combinations (Pacific Blue; Pacific Orange; Fluorescin Isothiocyanate, FITC; Phycoerythrin, PE; Peridinin Chlorophyll Protein Cyanin 5, PerCP-Cy 5.5; Phycoerythrin-cyanin 7, PE-Cy7; Allophycocyanin, APC; Allophycocyanin hilite 7, APC-H7) were used with the aim of investigating relative percentages of the different BM populations (CD3, CD45, MPO7, CD79a, CD34, CD19, CD7, HLADR), B cells (CD20, CD45, TdT, CD10, CD34, CD19, CD123, CD38) and myeloid cells (CD16, CD45, HLADR, CD13, CD34, CD117, CD11b, CD10). EDTA-anticoagulated fresh bone marrow (BM) samples (approximately 0.5 mL) were collected and analysed within 24 hours from collection; a total of 19 samples were analysed (one patient provided samples from both iliac crests at the same time point). Whole bone marrow samples were stained for cell surface markers using the stain-lyse-and-wash direct immunofluorescence technique (myeloid panel), while for those panels including both surface and intracellular markers (BM populations and B panel), staining was performed after cell fixation and permeabilization, using the ADG Fix&Perm® kit (An Der Grub Bio Research GmbH, Austria). Following staining, samples were measured using a BD LSR II flow cytometer (BD Bioscience, San Jose, CA) with the EuroFlow instrument setup data acquisition standard operating procedures [292]. Minimum number of events analysed was 30.000 cells/tube, with a median number of mononuclear cells acquired of 50.000/test. Data were analysed with BD FACSDiva software version 6.1.3 and SPSS version 20.0. Mononuclear and lymphocytic cells were first identified based on light scatter characteristics (sideward scatter, SSC and forward scatter, FSC), then confirmed as $CD45^{+}$ and gated accordingly. For the lymphoid populations both $CD45^{+}$ and $CD45^{dim}$ cells were taken into account.

4. STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS version 20.0 (SPSS Inc., Chicago, USA) and GraphPad Prism 6. Statistical significance of the differences between light chain and paraprotein levels were assessed using Mann-Whitney U tests. Significance between the different BM populations was assessed with the Mann-Whitney U or the Kruskall-Wallis test, as appropriate. Survival curves were plotted using the Kaplan-Meier method. Differences between curves were tested for statistical significance using the log-rank test. Statistical significance was set at p=0.05.

RESULTS

1. EVALUATION OF INTRA-CLONAL HETEROGENEITY IN PRIMARY SAMPLES OF MYELOMA PATIENTS

The evaluation of intra-clonal heterogeneity in primary samples of MM patients followed two different approaches. On one hand we were interested in assessing intra-clonal heterogeneity at a single cell level, while on the other hand the evolution of myeloma with WES was also evaluated. MM evolution was evaluated in two different ways, searching for differences at the different stages of MM (including 4 paired samples SMM-MM) and looking at the clonal dynamics after treatment (one SMM-MM paired sample) and in a xenograft model.

1.1 SINGLE CELL ANALYSIS OF PRIMARY MYELOMA SAMPLES

Using a single cell approach we examined 6 cases of MM of which fixed cells were available at diagnosis, and we were able to detect both a linear and a branching evolution pattern. In the first sample 5–6 tumour sub-clones could be distinguished, wherein each mutation and genomic aberration was acquired in a stepwise linear fashion. Briefly, mutations in 5 genes were analysed in the 89.6% (216/241) of sorted fixed single cells that passed all filtering thresholds (Figure 1a). The most recurrent mutation was ATM c.428A4G (100% of tumour cells) followed by KLK8 c.356A4G (94.4%), GMEB1 c.478A4T and POLE c.776G4A (44.4%), and lastly KRAS c.182A4G (24.5%). The presence of +8q and +21q at sub-clonal levels was also seen with the single-cell analyses. When considering cells with mutations and copy number aberration data, 86.7% (137/158) displayed +8q and 63.9% (101/158) had +21q (Figures 1b and c). Hierarchical clustering defined five clones (Figure 1b) and allowed to delineate the most plausible tumour phylogeny. In this case genetic mutations and genomic aberrations were acquired in a linear sequence, where ATM/KLK8 mutations preceded +8q and +21q, which were followed by GMEB1/POLE and KRAS mutations (Figure 1c).

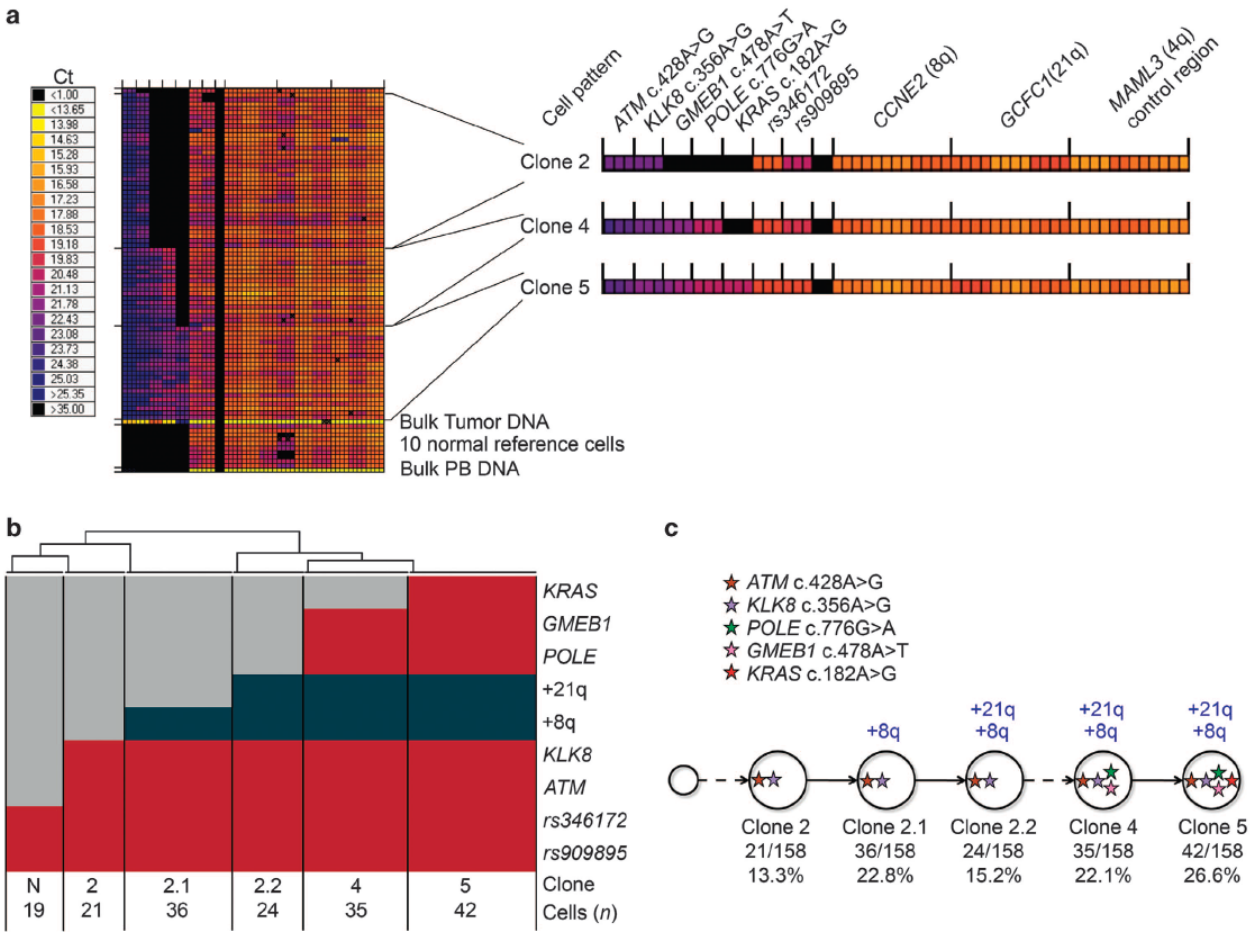

Figure 1. Linear evolutionary pattern. **(a)** Thumbnail heatmap of a Fluidigm array used for the study of this case. Each column shows the interrogated genotyping (left) or copy number (right) assay. Each row represents a sorted single-cell, which DNA is interrogated for mutation presence/absence and copy number values. Rows are sorted to group cells with the same genetic pattern (clones). Bulk tumour and peripheral blood DNA from the same case, and normal donor lymphocytes, are interrogated below. Amplification intensity is colour scale-based, from yellow (high DNA content) to blue (low DNA content). Black means no amplification. Zoomed images to cell patterns are shown on the right. Clone 2 has mutations in *ATM*/*KLK8*, clone 4 further carries mutations in *GMEB1*/*POLE*, and clone 5 has an additional *KRAS* mutation. **(b)** Hierarchical clustering of 158 single-cells which passed quality and thresholds criteria. Grey means no change; red, mutation; blue, gain. Cells are clustered according to their pattern for mutations and genomic aberrations (+8q/+21q) in five clones. N means cells with a normal pattern. **(c)** Clonal phylogeny. Clones evolve via a linear evolutionary pattern where mutations (coloured stars) and copy number aberrations (+8q/+21q) are acquired in a stepwise process. *KRAS* c.182A>G (p.Q61R) is the most recent mutation and generates clone 5. Clonal frequencies are depicted in absolute numbers and percentages.

Two additional cases displayed a similar pattern of linear evolution, although with a lower clonal diversity (2 and 3 clones identified, respectively). Mutations in the RAS pathway were seen to occur later in tumour development in our series. The genetic architecture of the remaining three samples had 4–6 clones detected at presentation, but in these cases clones were related via a

branching phylogeny (Figure 2). In one of the samples analysed del(13q) was detected with FISH. As a mutation in STK4 was present at a sub-clonal level and the STK24 locus is at 13q31.2-q32.3, we aimed to define whether del(13q) preceded or followed STK24 mutation. Using qPCR analysis of the RB1 locus on chr13, del(13q) was shown in 66–87% of the filtered tumour cells (Figure 2a). Combined analysis of both wild-type/mutant STK24 alleles and del(13q) copy number data demonstrated that del(13q) preceded STK24 mutation. It occurred in a sub-clone of clone 1 (clone 1.1), after PCDH15 and TRPA were mutated but before the acquisition of the remaining mutations and the emergence of the most recent clones (Figures 2b and c). Clone 1.1 is the ancestor for both clone 2, which mutated the STK24 allele located in the single copy of chr13, and clone 3, which instead retained the wild-type STK24 allele but acquired ACAD10 and NRAS mutations (Figure 2c). These branching evolutionary patterns are a characteristic feature of myeloma and resemble those described in other cancers [6-8].

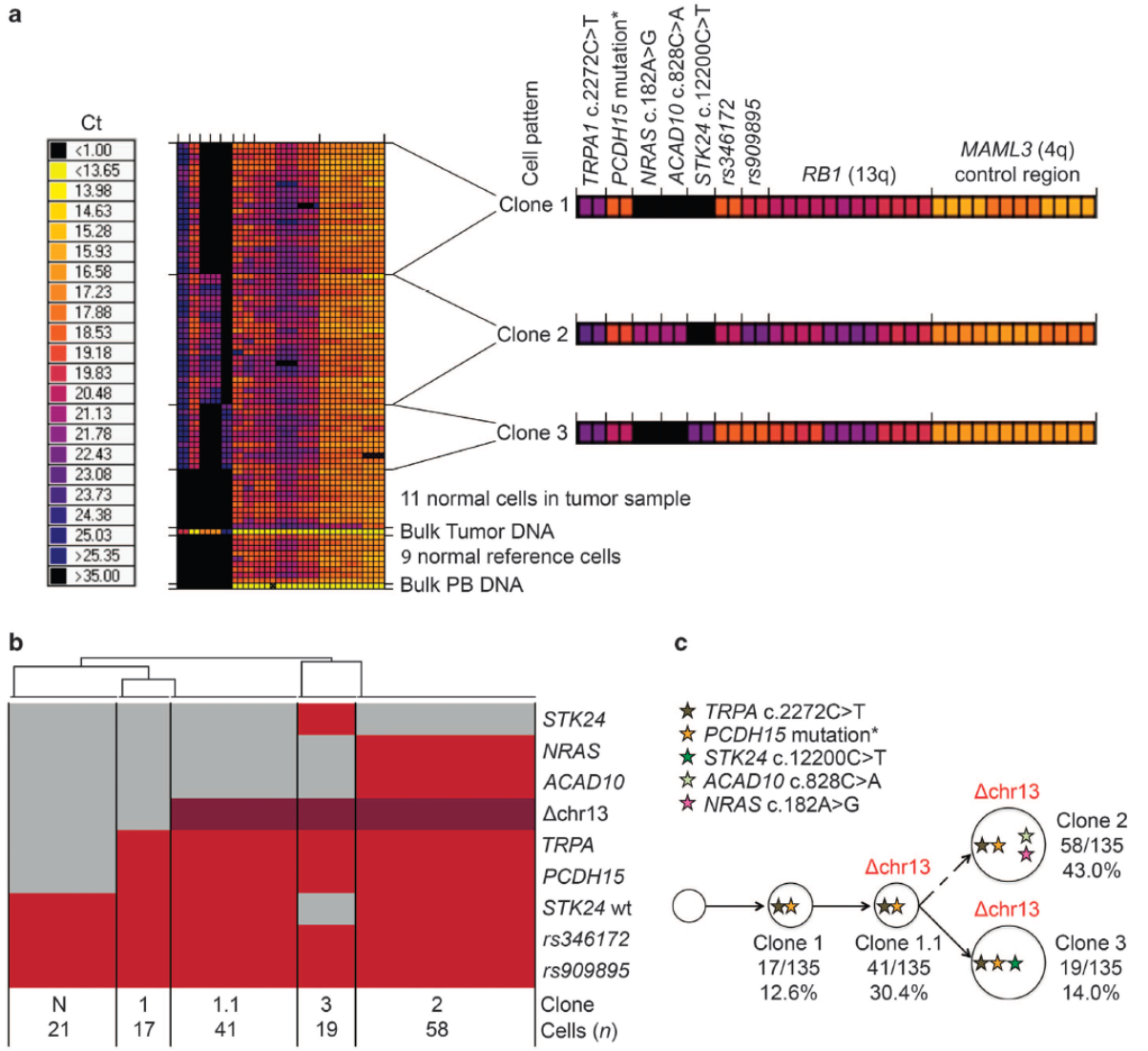

Figure 2. Branching evolutionary pattern. **(a)** Thumbnail heatmap of a Fluidigm array used for this analysis. Zoom image to cell patterns are shown (right). Clone 2 and clone 3 have common mutations in *TRPA*/*PCDH15* to clone 1 but further accumulate independent mutations in *NRAS*/*ACAD10* (clone 2) or *STK24* (clone 3). **(b)** Hierarchical clustering of the 135 filtered single-cells. Grey means no change; red, mutation or wild-type allele (for *STK24* wt); deep red, loss of 13q. Four tumour sub-clones are distinguished, with mutations present in clone 3 (*NRAS* and *ACAD10*) but not in clone 2 (*STK24*). N means cells with a normal and reference pattern. **(c)** Clonal branching phylogeny considering both mutations (coloured stars) and 13q copy number data. Clone 1, which already has *TRPA* and PCDH15 mutations, further acquires del(13q), producing clone 1.1. This clone generates two lineages: clone 2, with mutation in *STK24*; and clone 3, with wild type *STK24* and mutations in *ACAD10* and *NRAS*. *NRAS* c.182A>G (p.Q61R) mutation occurs in an independent branch and relatively recently in myeloma development. Asterisks mean *PCHD15* c.2272_2273delinsTA. Clonal frequencies are depicted in absolute numbers and percentages.

Having shown the myeloma phylogeny, we analysed the putative founder clone as defined by the initiating genetic lesion, the chromosomal translocation t(11:14). All patients analysed had a

t(11;14), which hypothetically served as a marker of the initially immortalized clone. To test this theory, we defined the translocation breakpoint using massively parallel sequencing combined with targeted pull down of the Ig regions and designed translocation-detection PCR-based assays applicable to single-cell assays for three of the six cases. For all three cases, we demonstrated that the t(11;14) was present in 91–100% of tumour cells. Remarkably, we identified an ancestral myeloma clone showing only t(11;14) in two of the three samples with a frequency of 27.5% and 7%, respectively (clone 1, Figures 3a and b). None of the remaining interrogated mutations was present in these sub-clones, suggesting these may be the initiating founders. However, we cannot dismiss the potential presence of other mutations/alterations that we could not detect due to technical issues or that were not included in the analysis. Conversely, the ancestral clone in the remaining case carried the t(11;14) and 4 genetic mutations (clone 1, Figures 3c and d). The exact timeline of the events leading to this clone seems untraceable, as mutations may have followed initial immortalization by t(11;14), occurred simultaneously to translocation or preceded t(11;14). Three cases had double hits in the RAS/MAPK pathway either in the same gene, KRAS (two cases), or in different genes such as KRAS and NRAS (one case). To determine whether these double hits occurred in the same or in independent sub-clones, we interrogated both mutations together with other altered genes at the single-cell level. The first case had KRAS c.183A4C (p.Q61H) and c.199A4C (p.M67L) mutations at similar frequencies (92% and 83%, respectively), and these followed a linear sequence. KRAS p.Q61H occurred earlier than p.M67L, which was acquired soon after clonal expansion, as clone 3 carries both mutations and is present in 83.2% of tumour cells. Although the presence of both mutations seemed to improve clonal fitness due to the predominance of clone 3, the extent to which p.M67L was responsible for this benefit is uncertain. p.M67L is not a known activating mutation and may simply be a passenger mutation in a proliferative clone defined by the activating mutation p.Q61H. Conversely, the double RAS hits in the remaining cases occurred in different clones related via a branching pattern. The second case had 6 sub-clones (Figure 3a) and we confirmed the independent acquisition of 2 KRAS/NRAS mutations. The founding clone that only carried t(11;14) (clone 1, 27.5% frequency) acquired ABCA4 c.3294C4T, and it was this clone 2 (5.0%) that generated two divergent lineages: clone 3 (40.0%) carrying both FAT c.6080T4G and NRAS c.182A4G (p.Q61R) mutations, and clone 4 (5.0%) that had KRAS c.183A4C (p.Q61H). The latter gave rise to a lineage where PCSK6 c.463G4A was accumulated in clone 5 (7.5%), which subsequently originated clone 6 after the IRF4 c.368A4G mutation (15.0%; Figure 3b). In the last case we described five clones and confirmed the co-

occurrence of two KRAS mutations (Figure 3c). Clonal phylogeny identified the earliest clones 1 and 2 (4.0% and 34.0%, respectively) with t(11;14) and a range of mutations, including IRF4 c.368A4G and EGR1 c.1169A4G, the latter being specific of clone 2. Two divergent clonal lineages derived from clone 2, both acquiring KRAS-activating mutations. Clone 3 accumulated KRAS c.182A4G (p.Q61R) and accounted for 26.0% of tumour cells, whereas clones 4 and 5 had KRAS c.34G4C (p.G12R) representing 24.0% and 12.0%, respectively, of tumour cells (Figure 4d). These 2 cases acquired the same convergent phenotype, that is, activation of the RAS/MAPK pathway, in 2 divergent clonal lineages derived from the same clonal ancestor, which subsequently evolved independently.

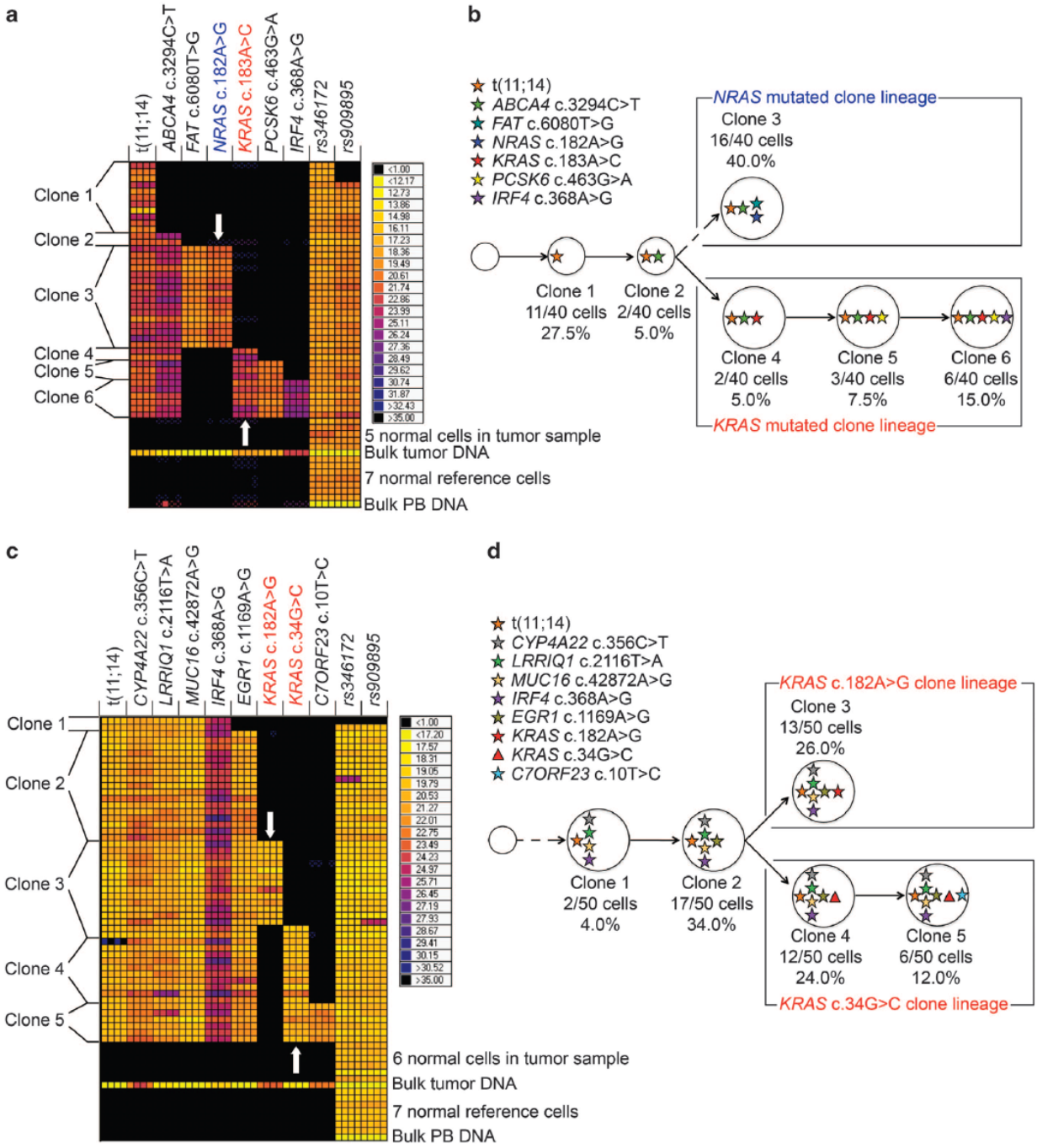

Figure 3. Putative founding role of t(11;14) and parallel evolution in myeloma. Close examination of two cases with branching evolutionary patterns illustrates how independent clones, but originated from the same ancestor, acquire activating mutations in the same gene or in genes from the same pathway. **(a-b)** Single-cell analysis of the first case. **(a)** Results from the used Fluidigm array. Four columns per genotyping assay were interrogated. No copy number assays were used. Clone 1 only shows positivity for t(11;14), representing the most likely founding myeloma clone in this sample. White arrows point out *NRAS* c.182A>G mutations (clone 3) are recognized in different cells that those displaying *KRAS* c.183A>C (clones 4-6). **(b)** Clonal phylogeny considering t(11;14) and mutations. Divergent clonal lineages emerged from clone 2 with activating mutations in *NRAS* (clone 3) or *KRAS* (clone 4). Clone 4 further evolves independently acquiring first *PCSK6* c.463G>A

(clone 5), and later *IRF4* c.368A>G (clone 6) as additional hits. **(c-d)** Single-cell analysis of the second case. **(c)** Array heatmap obtained for this case. White arrows point out cells with *KRAS* c.182A>G (clone 3) do not have *KRAS* c.34G>C (shown in clones 4-5). **(d)** Tumour phylogeny reveals a common ancestor (clone 2) carrying t(11;14) and other mutations (including *IRF4* c.368A>G). Two independent clonal lineages arise from clone 2 and acquire different activating mutations in *KRAS*: clones 3-4. The latter clone further evolves mutating *C7ORF23.*

1.2 INTRA-CLONAL HETEROGENEITY IN MYELOMA IS PRESENT FROM THE EARLY STAGES

Samples from 4 MGUS patients, 26 MM and 2 PCL were analysed by WES. From 4 of the MM samples paired samples at the time of SMM were also available. All the SMM samples met the criteria for high-risk SMM according to the Spanish definition [293]. Median number of non-synonymous (NS)-SNVs in the MGUS exomes was 13 (range 8–18), in SMM 28 (range 20–69), in MM 31 (range 15–46) and in the PCL samples 59 (50–68). The data show that the number of NS-SNVs increases with disease progression from MGUS to PCL. The kernel density plots in Figure 4 show several distinct peaks indicating a similar level of heterogeneity exists at all stages of disease from MGUS to PCL.

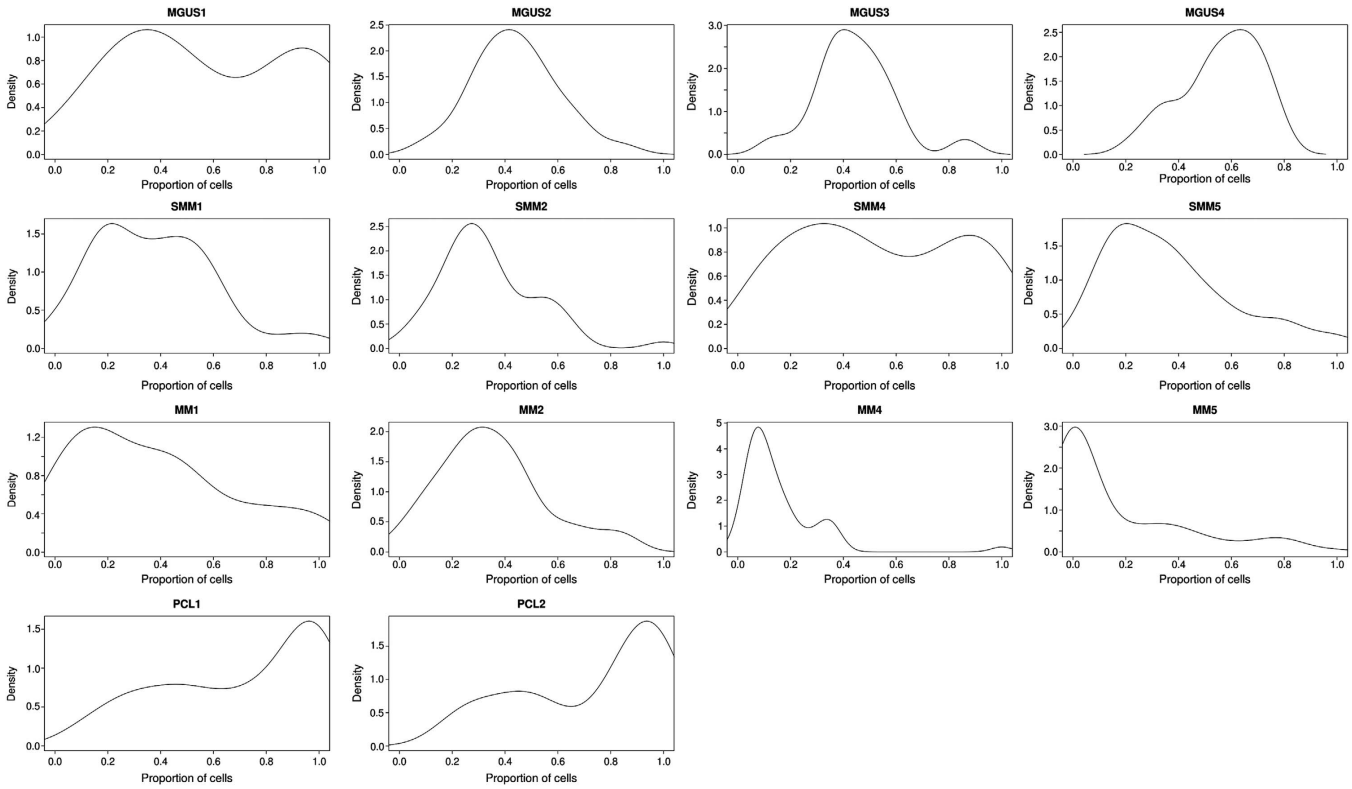

Figure 4. Clonal heterogeneity is present in all disease states. Gaussian kernel density plots indicating the frequency of cells carrying all acquired exonic mutations. Frequency is calculated by adjusting mutant allele burden by copy number of the loci mutated. Top row, MGUS samples; Middle rows, matched SMM/MM samples; bottom row,

PCL samples.

This intra-clonal heterogeneity at the MGUS stage is consistent with clonal diversity arising early on in the process of myelomagenesis and with clonal competition being an essential requirement from the earliest phases of disease. The rate of transformation from MGUS to MM is slow, which is consistent with the requirement for the acquisition of multiple mutations that are able to deregulate driver genes, but these constitute rare events. Having shown the presence of intra-clonal heterogeneity at each stage of the disease, we were keen to understand how the relationship between sub-clones changes with the development of clinical symptoms and whether this relationship followed Darwinian principles. In order to accurately determine the changes occurring in the transition from SMM to MM, we analysed three sets of patients with paired SMM and MM samples taken at least 21 months apart. Of the total acquired SNVs, 93% (range 91–95%) were detected in both the SMM and MM samples, with 81% (range 64–94%) of the variants being present in less than half of the tumour cells at both stages. Only 3.3% (range 0.3–8.1%) of variants were present in greater than 90% of MM cells. This observation suggests that both SMM and MM contain many sub-clones at low frequencies, a feature that would be anticipated if disease progression was the result of clonal competition. After calculating the proportion of cells that contained each variant, by combining base calls and copy number data, it was possible to define the sub-clonal composition of each disease stage. Typically, around six clusters were identified per sample pair, and although these variant clusters do not directly represent clones, they can be used to demonstrate clonal evolution over time.

One paired set of samples was sourced from a patient who was treated with lenalidomide and dexamethasone and clearly demonstrates that chemotherapy results in a reduction in clonal complexity (Figures 5d-f). For example, cluster A was dominant at the SMM stage, with mutations present in 80–100% of cells, but after treatment it has decreased to 0–20% of cells. Some clusters were largely unaffected by the treatment (clusters B, E, F, H and I). Conversely, cluster D has gone from 0 to 20% of cells at SMM and risen to 40–60% at progression to MM, perhaps due to therapy resistance.

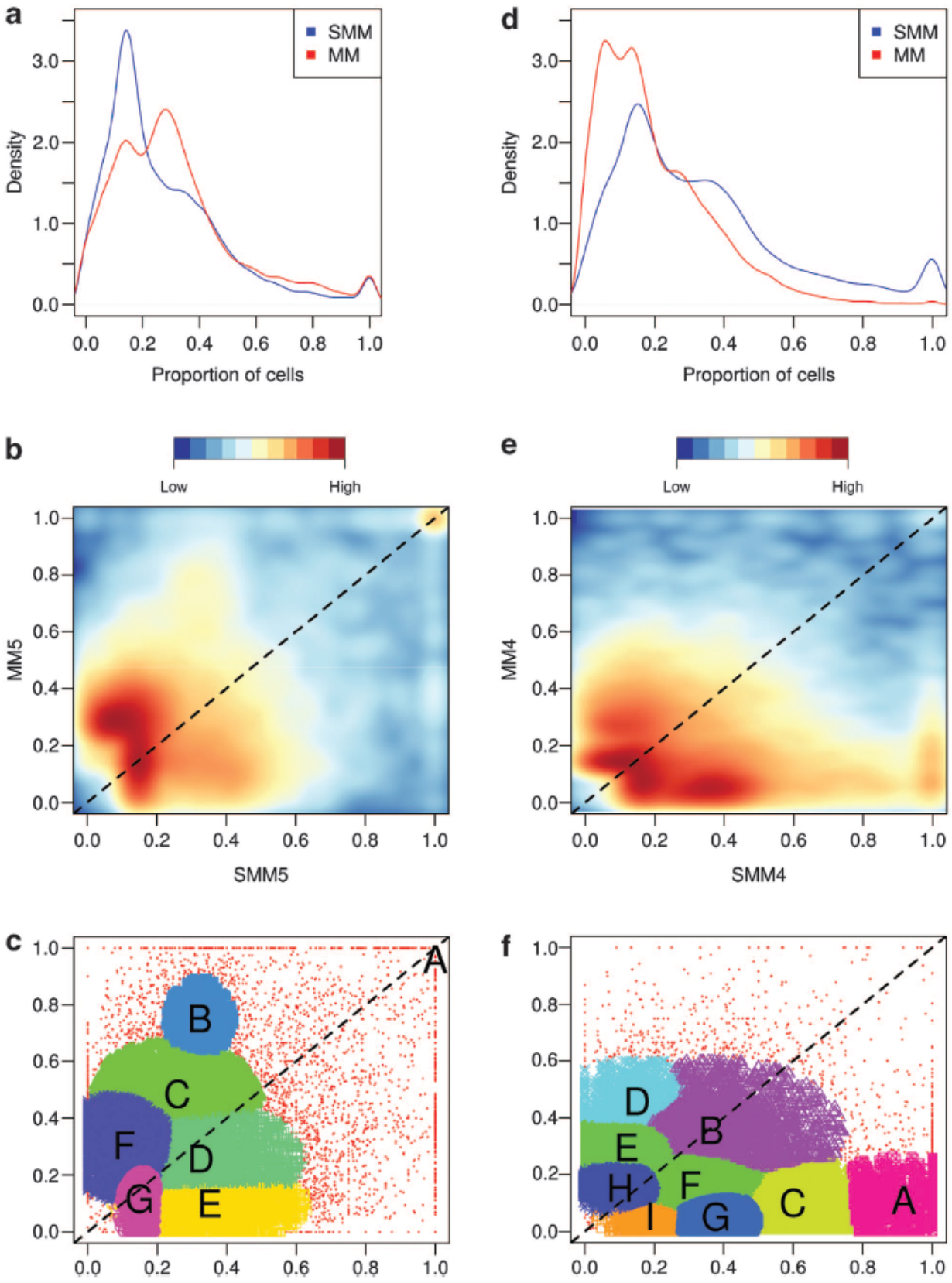

Figure 5. Clonal evolution of paired HR-SMM and MM samples from an untreated **(a–c)** and a treated patient **(d–f)**. **(a, d)** Kernel density plots of the proportion of cells containing each variant from whole-genome sequencing in HR-SMM (blue) and MM (red); **(b, e)** Comparison of the proportion of cells containing each variant in HR-SMM and MM samples. Positive and negative vertical deviations from the main diagonal (marked at a

dashed line) indicate an increase or decrease in the variant from the HR-SMM to MM stage respectively. **(c, f)** After estimating the noise using a nearest-neighbour based classifier, an EM based clustering strategy was used to define clusters of variants. Most of the variants in HR-SMM occur at low frequency, but a distinct increase in frequency for a large set of variants is seen in the MM sample.

There were on average 433 additional novel and unique mutations (range 341–517) gained per sample during the transition from SMM to symptomatic MM, few (mean 2.3) were present within coding regions and only one was NS. Understanding the rate of mutation acquisition is important, and as the time to progression for the studied patients was known the number of variants unique to each SMM and MM sample was used to calculate the rate at which new mutations were gained and existing mutations lost, or at least became undetectable at the level of sensitivity of the test used. The mean across the three cases was 19 mutations gained and 36 mutations lost per month. From a mechanistic perspective it seems that copy number change occurs relatively early in these cases, as using fluorescence in situ hybridization, at a macroscopic level, significant copy number change during the transition could not be seen, a feature that was recapitulated when the sequencing data were used to generate copy number data across the genome. These data indicate that in the three cases untreated paired cases, the acquisition of copy number abnormalities does not facilitate the transformation between the two disease stages and was a feature of earlier stages of the disease.

The limited number of novel NS-SNVs, indels and translocations, which characterize the transformed MM samples indicates that by the time a case has evolved to the SMM stage the majority, if not all, of the exonic genetic diversity necessary to give rise to an aggressive clinical state is already present. We show that 1732 mutations were acquired in the MM samples, of which only one was NS. However, there is a substantial intronic diversity, the significance of which has not previously been demonstrated.

1.3 CLONAL EVOLUTION IN A XENOGRAFT MODEL

To evaluate clonal dynamics over time a xenograft model was also used. In parallel, $1x10^6$ $CD138^+$ cells derived from the diagnostic sample of one patient were injected into the tibia of NOD/SCIDyc(null) mice [286]; presentation and relapse sample from the patient (who had been treated with Bortezomib containing regimen) and two of the engrafted myelomas were analysed by WES sequencing (Figure 6a). The data demonstrated the existence of a complex phylogenetic history with fluctuations in the sub-clonal composition at relapse and in the two engrafted

myelomas when compared with the presentation sample. To prevent any false-positive results due to mouse DNA contamination, only mutations present in the two patient samples (presentation/relapse) were tested in the two engrafted samples, excluding mutations specific to the engrafted myelomas. We identified 152 single nucleotide variants in all 4 samples. Briefly, mutations in 74 genes, including ATM or TP53, were shared across all samples. Although 26 mutations were characteristic of the presentation sample, 42 were specific to the relapse stage. It is noteworthy that three mutations were shared between the xenograft samples and the relapse sample. We postulate that these mutations were present at diagnosis, but at undetectable levels, probably in negligible sub-clones. We used a genetic algorithm to group mutations present in these four samples into sub-clonal populations, and distinguished seven clones (Figure 6b). These were related by a complex branching pattern and all had different number of mutations (Figure 6c). The earliest ancestral clone carried six mutations, two out of which were non-synonymous single nucleotide variants in DNAH14 and FAM47C (clone 1). These mutations were present in all four samples at a 100% frequency, supporting clone 1 as the phylogenetic root (Figures 6b and c). There was a remarkable fluctuation in the clonal proportion in each sample (Figure 6d). Four clones were detected at diagnosis with populations 5 and 7 being the predominant fractions (59% and 23%, respectively). Patient treatment caused a significant population bottleneck in which clones 2, 4 and 7 were extinguished and 41 new mutations were acquired. Consequently, clones 3 and 6 emerged at relapse (17 and 33%, respectively; Figures 6b–d). These clones originated from the earliest ancestral clone 1, which was undetected at diagnosis (Figures 6b–d). These results support the idea that earlier ancestral clones may lead to relapse. The analysis of the engrafted myelomas showed the clonal extinction of clones 2, 4 and 7, and the re-emergence of clone 1, which was present at undetectable levels at diagnosis. Both xenograft samples had similar mutation frequencies, with slight variations between clones 1 and 5 (Figure 6d). Although the genetic architecture of these engrafted myelomas may be even more complex due to the accumulation of additional mutations not tested in this analysis, the results demonstrate that three clones were outcompeted by the remaining ones both during patient treatment and xenotransplant transitions. In addition, we show that earlier clones lead to relapse or engraftment (Figure 6d). Altogether, these findings emphasize the different survival properties of myeloma clones as a consequence of restrictive population bottlenecks such as patient treatment or xenotransplantation.

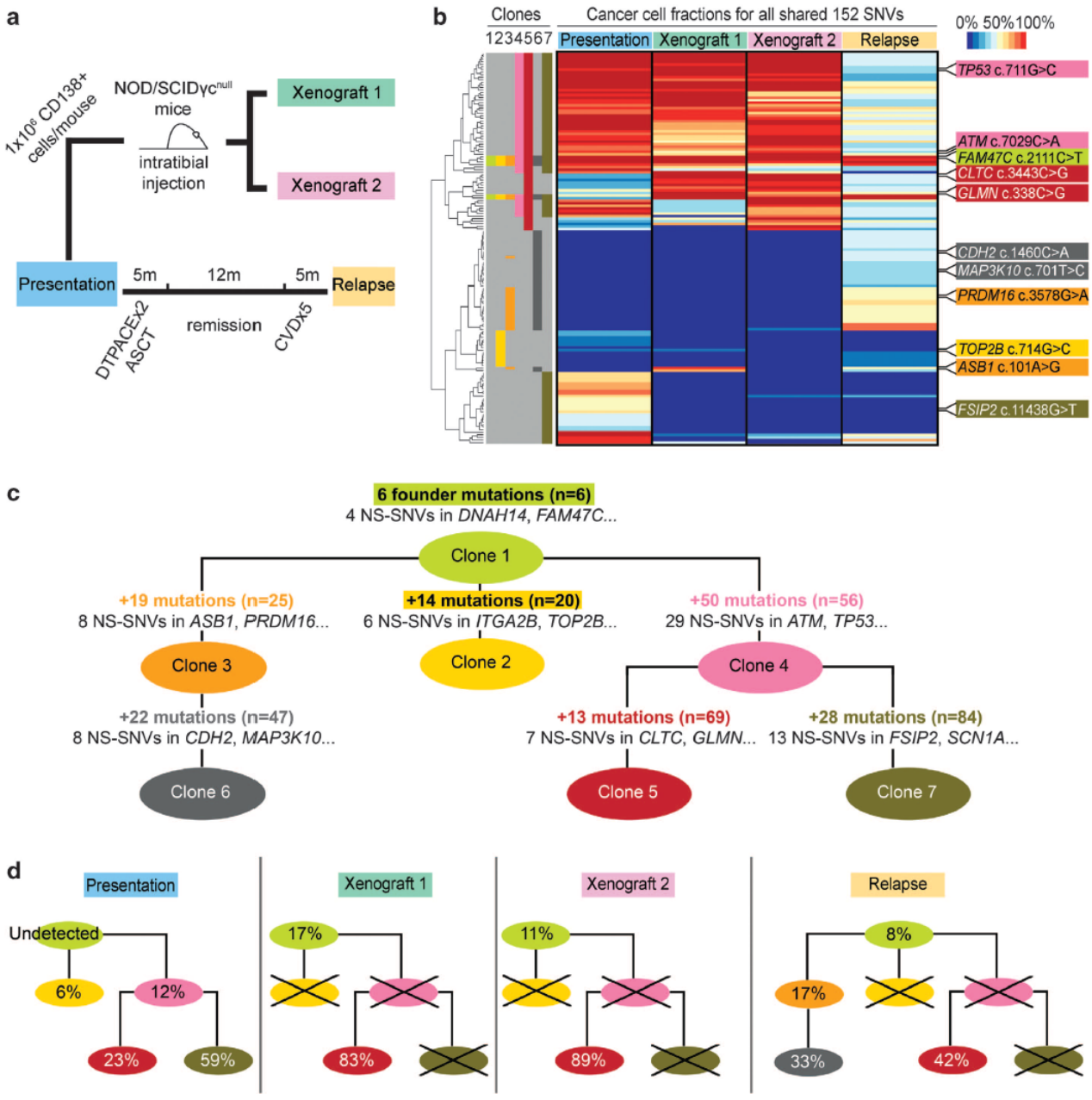

Figure 6. Changes in clonal architecture following patient treatment and *in vivo* NOD/SCID IL2Rγ^{null} transplantation. **(a)** Patient history with xenograft experiments outlined. Isolated DNA from CD138^{+} cells purified from paired-patient samples at presentation [12] and relapse are analysed using exome-sequencing. DNA from CD138+ cells purified from two independent myeloma-xenograft samples is also studied. DTPACE: dexamethasone, thalidomide, cisplatin, adriamycin, cyclophosphamide and etoposide; ASCT: autologous stem cell transplantation; CVD: cyclophosphamide, bortezomib and dexamethasone. **(b)** Cluster of cancer-cell fractions for all 124 SNVs identified in the four samples. Sub-clones are defined on the left using genetic algorithms. A selection of genes is shown with coloured lines indicating the sub-clone in which such genes were firstly mutated (right). **(c)** Phylogenetic natural history of this PCL patient. The seven sub-clones detected by whole-exome sequencing and genetic algorithms are depicted with the same colours as in (b). The number of new mutations and non-synonymous single nucleotide variants (NS-SNVs) together with a selection of key mutated genes are shown in the transition between clones. **(d)** The

proportion of sub-clonal populations, shown as percentages next to each clone, fluctuated in the four analysed samples. There were clones described at presentation that were undetected and, therefore, potentially extinguished at relapse or at the engrafted-myelomas (clone 4 or 5). Conversely, new clones (sub-clones 3 and 7) emerged at the relapse stage as a result of a further accumulation of mutations in sub-clones 1 and 6, respectively.

2. SERUM FLC AS A MARKER OF IMPACT FROM INTRA-CLONAL HETEROGENEITY

To evaluate the impact of intra-clonal heterogeneity from a clinical point of view, we analysed 520 of 647 (80.5%) patients treated within the MM IX trial that had relapsed. A significant increase in both paraprotein and FLC levels (PLC relapse) was observed in 35.2% (183/520), whilst 258/520 (49.6%) relapsed with a significant increase only of their paraprotein levels (PO relapse) and 25 had relapse detected clinically. In 54/520 (10.4%) patients the relapse was characterized by an increase in FLC without a correspondent increase in paraprotein level, a phenomenon termed "serum free light chain escape" [294]; these patients represented 6.5% (24/369) of IgG patients and 19.9% (30/151) of IgA patients, respectively. In 46/54 (85%) patients the increase in involved serum FLC was >200mg/l, that is the level of increase recommended for defining relapse requiring treatment in the absence of clinical symptoms [290]. In these 54 FLC escape patients only 28 (51.8%) had a >200mg/l increase in urine FLC levels.

The paraprotein levels at presentation were significantly lower in both IgG and IgA patients who relapsed with FLC escape (LCO) as compared to patients who relapsed with a PLC or PO (Fig 7a). The FLC levels at presentation and relapse were significantly higher in patients who relapsed with FLC escape compared to those relapsing with PO relapse (Figure 7b). Similarly at relapse FLC levels were significantly higher for patients with IgG FLC escape relapse compared to patients with IgG PLC relapse but there was no significant difference between these groups for patients with IgA myeloma (Figure 7b).

a. b.

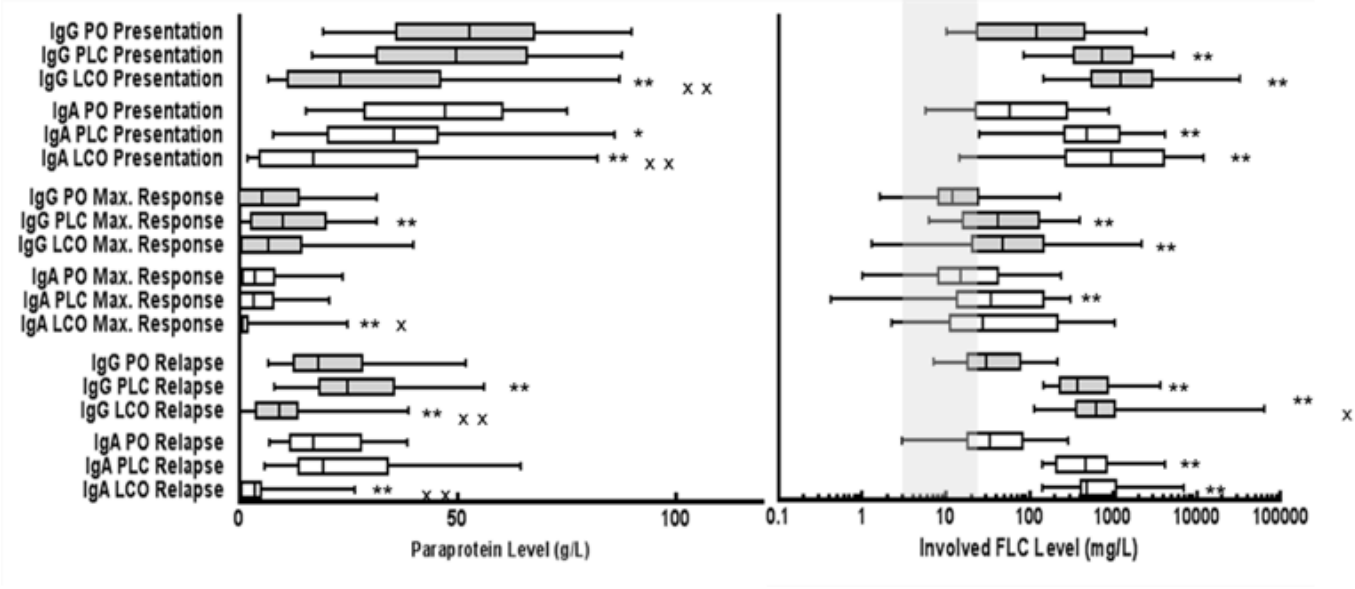

Figure 7. Whisker box plots showing the median, 25th and 75th centiles for **(a)** paraprotein and **(b)** absolute levels of the involved light chain at presentation, maximum response and relapse. Tails represent 5th and 95th centiles. IgG PO n=186, IgG PCL n=138, IgG LCO n=24, IgA PO n=72, IgA PCL n=45 and IgA LCO n=30. Mann-Whitney U tests were performed to assess the statistical significance of the differences between the groups. PO, paraprotein only; PLC, paraprotein and light chain; and LCO, free light chain escape.

* p<0.05 when compared to PO at the same time point

** p<0.01 when compared to PO at the same time point

x p<0.05 LCO versus PLC at the same time point

xx p<0.01 LCO versus PLC at the same time point

Shaded column represents normal range for sFLC.

A difference in survival between IgG and IgA myeloma patients has been previously established, and reflects the short duration of remission in IgA patients [295]. In this study PFS and OS from relapse are 24 vs. 20 months (p=0.003) and 33.6 vs. 28.6 months (p=0.071) for IgG and IgA patients respectively.

PFS was similar between all relapse groups (21.9 vs. 18.0 vs. 20 months for FLC escape, PLC and PO relapse respectively, p=0.766). Conversely, the median OS of patients relapsing with a light chain involvement (either FLC escape or PLC) was approximately 13 months shorter compared to patients relapsing with a whole paraprotein (PO) (Figure 8a, p=0.015); this was mostly attributable to a significantly shorter survival from relapse (27.7 vs. 23.5 vs. 37.4 months, p=0.002, for FLC escape, PLC and PO respectively, Figure 8b).

a. b.

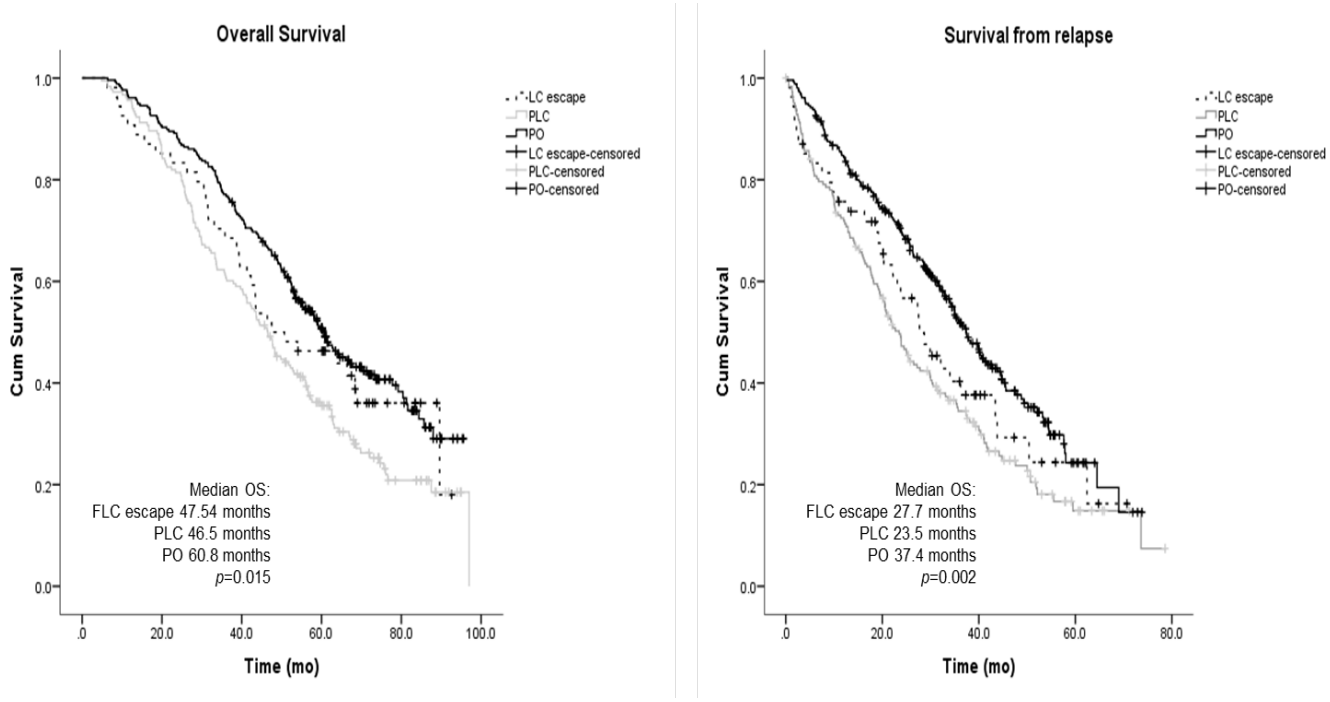

Figure 8. **(a)** Kaplan-Meier curves of overall survival from diagnosis for patients relapsing with whole paraprotein secretion (PO), both paraprotein and light chains (PLC) or patients with FLC escape phenomenon; **(b)** Kaplan-Meier curves of survival from first relapse for patients relapsing with whole paraprotein secretion (PO), both paraprotein and light chains (PLC) or patients with FLC escape phenomenon

Examining the OS from diagnosis and first relapse by paraprotein isotype, IgG patients with PO relapse had a significantly improved OS from diagnosis (64.5 vs. 43.4 and 47.3 months for FLC escape and PLC respectively, *p*=0.007). There was a trend towards increased OS from diagnosis for IgA patients who relapsed with PO, although this failed to reach statistical significance (50.9 vs. 50.7 vs. 40.2 months for PO, FLC escape and PLC respectively, *p*=0.066). Survival from relapse was increased for patients relapsing without FLC involvement in both isotype subgroups (median OS from relapse 40 and 33.2 months for IgG and IgA respectively) compared to patients in which relapse was characterised by an increase in the involved FLC level (median survival from relapse 23.1 and 22.2 months for IgG FLC escape and PLC and 29.9 and 23.8 months for IgA FLC escape and PLC respectively, Figure 9a and 9b).

a. b.

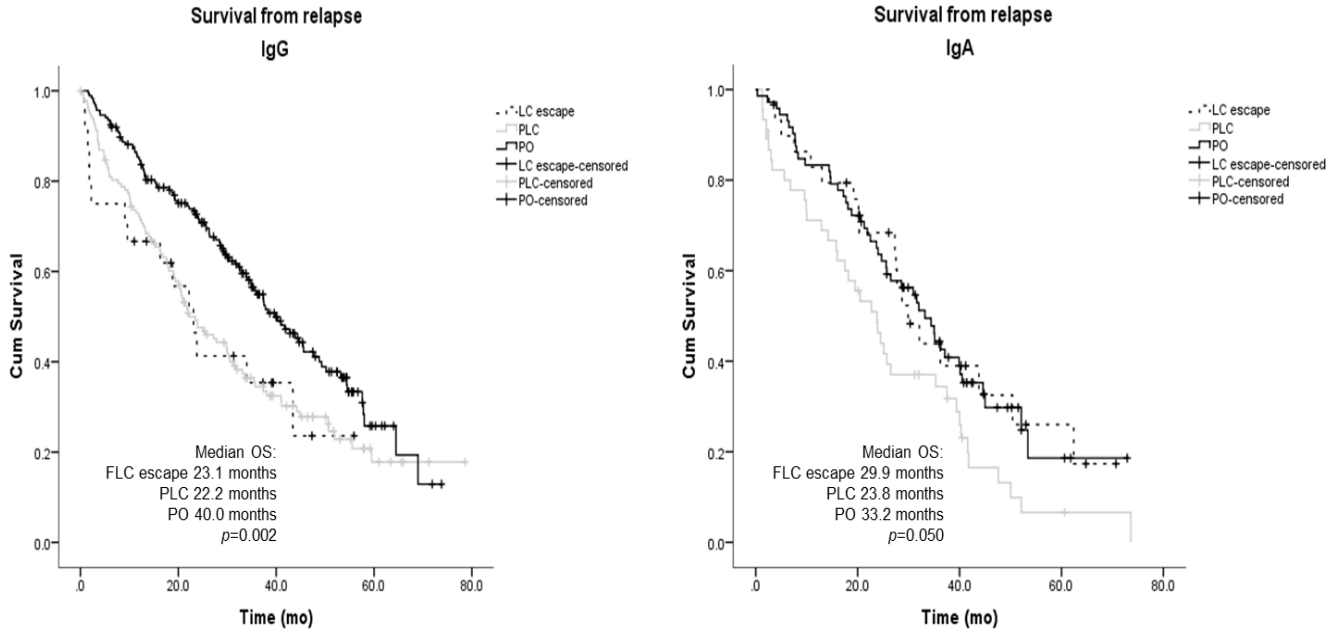

Figure 9. **(a)** Kaplan-Meier curves of survival from first relapse for IgG patients relapsing with whole paraprotein secretion (PO), both paraprotein and light chains (PLC) or patients with FLC escape phenomenon; **(b)** Kaplan-Meier curves of survival from first relapse for IgA patients relapsing with whole paraprotein secretion (PO), both paraprotein and light chains (PLC) or patients with FLC escape phenomenon

A Cox regression analysis including maximum response, age, paraprotein isotype, treatment pathway, thalidomide therapy and the type of relapse identified a maximum response ≥VGPR, an IgG paraprotein, intensive treatment and the type of relapse as variables independently associated with an extended OS; treatment pathway and the type of relapse were also found to be variables associated with a longer survival from first relapse (Table 2).

Table 2. Multivariate analysis of variables associated with an increased overall survival and survival from relapse

	OS		
	HR	CI	p
Max resp ≥VGPR	1.353	1.048-1.746	0.020
IgG	1.353	1.059-1.726	0.016
Intensive pathway	1.677	1.297-2.167	<0.000
Relapse type			0.005
PO vs. PLC	1.480	1.169-1.872	0.001
PO vs. FLC escape	1.130	0.772-1.655	0.528
	Survival from relapse		
Intensive pathway	1.375	1.066-1.775	0.014
Relapse type			0.001
PO vs. PLC	1.560	1.232-1.975	0.000
PO vs. FLC escape	1.284	0.873-1.887	0.204

Max resp: maximum response; VGPR: very good partial response; IgG: IgG isotype myeloma; Intensive pathway: treatment in the intensive pathway of MRC Myeloma IX trial; PO: paraprotein only relapse; PLC: paraprotein and light chain relapse; FLC escape: serum free light chain escape relapse

3. LONG TERM EFFECT OF LENALIDOMIDE EXPOSURE ON THE BONE MARROW

We were than interested in finding out whether a long-term treatment with IMiDs might affect the composition of the bone marrow, thus partly explaining Lenalidomide side effects and the potential increase in second primary malignancies development reported in some studies. Using MPF we were able to observe that the percentage of B cells was lower in lenalidomide treated patients compared with patients not on treatment (median 1% and 2% of total marrow mononuclear cells for LTL and STL respectively versus 5% for the control group). The decrease in B cells was proportional to the time patients had been on lenalidomide treatment, and it reached statistical significance when comparing LTL versus controls (Figure 10a, p=0.04). To be able to identify a possible higher incidence of immature lymphoid precursor forms in lenalidomide treated patients we selected a panel of antibodies that would enable us to detect for precursors as well as naïve B-lymphocytes. We observed that the decrease of B-lymphocytes in patients receiving lenalidomide was due to a reduction of the more mature forms (naïve and mature B cells, Table 3). Higher levels of immature forms (haematogones and pre-B cells) could not be demonstrated

(Table 3), and no aberrant population or phenotype could be identified. The lowest values of B cells were observed in LTL patients. A concomitant relative increase in non-B lymphoid population (Figure 10b) was seen, explained by an increase in T cells (Table 3 and Figure 10a).

When looking at the myeloid fraction, no significant difference could be seen between lenalidomide treated and non-treated patients, irrespectively of the time on treatment (Figure 10c). Looking in more details at the different subset of myeloid cells, similar proportions of myelocytes, metamyelocytes, and neutrophils were observed, both in treated and untreated patients. Time on treatment did not affect the myeloid population (Table 3, Figure 10c) with no increase in immature forms nor the presence of aberrant phenotypes.

Table 3. Mean and median values of different bone marrow population as detected by MPF

		Mean (±SD)			Median (IQR)		
		STL	**LTL**	**No Len**	**STL**	**LTL**	**No Len**
All BM population	Myeloid	54.7 (±13.6)	53.8 (±15.6)	53.6 (±23.6)	52 (46-64)	52 (38.25-69.75)	62 (40-71)
	B cells	3.5 (±3.1)	2 (±2.3)	6.1 (±4.1)	2 (1-6)	1(0-4.75)	5 (2-10)
	T cells	15 (±6.5)	13 (±4.4)	8.4 (±3.5)	16 (8-18)	14 (9.25-15.75)	9(6-10)
		STL	**LTL**	**No Len**	**STL**	**LTL**	**No Len**
Lymphoid population	B cells	9.6 (±9.0)	6.4 (±7.0)	30 (±20.3)	7 (1-20)	2 (1.25-12.5)	28 (13-49)
	Haematogones	3.10 (±4.4)	2.6 (±2.5)	1.0 (±0.8)	2 (0-4.5)	2 (0.25-5.5)	2 (0-2)
	PreB	37.2 (±24.8)	35.1 (±20.1)	24.4 (±17.0)	35 (12.25-63.75)	36.5 (17.25-46.75)	24 (7-42)
	Naïve B	34.3 (±19.6)	29 (±12.1)	48.9 (±14.4)	36 (18.5-45.5)	29.5 (19-36.5)	52 (36-64)
	Mature B	10.5 (±7.7)	12.5 (±5.2)	23.3 (±11.6)	8.5 (6-12)	11 (8.25-18.25)	19 (12-33)
	CD19-	84.7 (±16.7)	93.5 (±6.9)	63 (±22.3)	92 (76.75-96)	97.5 (87.5-98.75)	63 (38-87)
		STL	**LTL**	**No Len**	**STL**	**LTL**	**No Len**
Myeloid population	Myeloid precursor	1.3 (±1.8)	0.9 (±0.6)	0.6 (±0.5)	1 (0-1.25)	1 (0.25-1)	1 (0-1)
	Myelocytes	22.3 (±11.8)	31.1 (±19.1)	29.14 (±12.0)	20 (11.25-36.25)	30 (14.5-48.5)	32 (15-40)
	Metamyelocytes	29.7 (±15.0)	27.4 (±12.2)	35.1 (±13.2)	26 (23-38.25)	26.5 (19-31.75)	35 (32-46)
	Neutrophils	23.5 (±16.4)	25.1 (±12.9)	18.71 (±9.3)	19.5 (15-31)	23 (12.75-34.75)	19 (8-27)

BM: bone marrow; SD: standard deviation; IQR: interquartile range; STL: short-term lenalidomide; LTL: long-term lenalidomide; No Len: never treated with lenalidomide

For All BM populations and Myeloid population values are expressed as percentages of bone marrow mononuclear cells; for Lymphoid population values of B cells and CD19- cells are expressed as percentage of bone marrow lymphocytes and values of B cells subpopulations are expressed as percentage of B cells

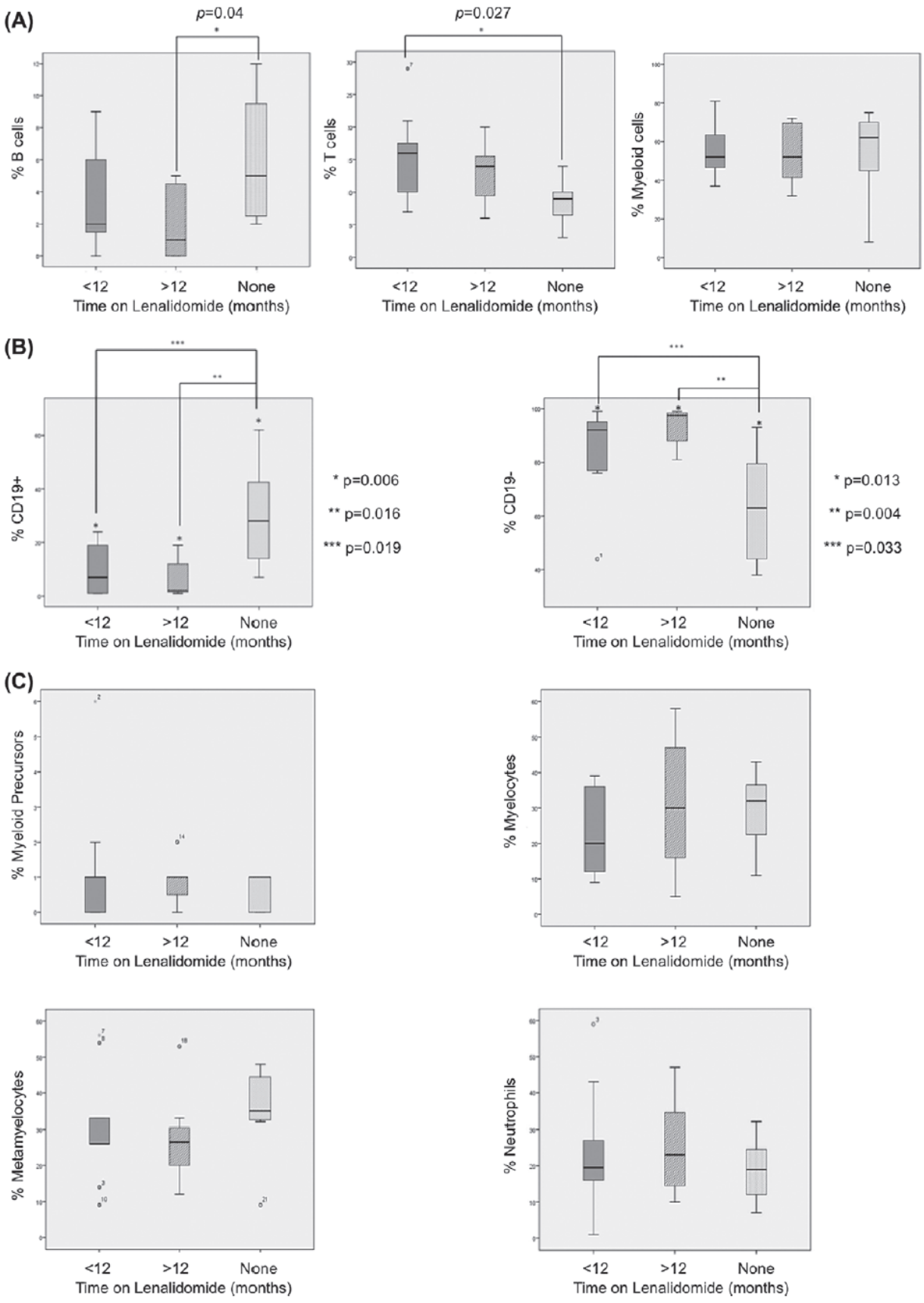

Figure 10. Lenalidomide impact on the different BM populations. The following bone marrow populations were identified: i) myeloid cells $CD45^{+}/MPO^{+}/CD79a^{-}/CD3^{-}/CD19^{-}$; ii) B cells $CD45^{+}/CD19^{+}/CD79a^{+}/MPO^{-}/CD3^{-}/CD7^{-}$; iii) T-cells $CD45^{+}/CD3^{+}/CD7^{+}/CD19^{-}/CD79a^{-}/MPO^{-}$. Specific subpopulations of B and myeloid cells were

identified as follows: a) haematogones: $CD45^{+}/CD10^{+}/TdT^{+}/CD19^{+}/CD20^{-}$; b) pre B lymphocytes $CD45^{dim}/CD10^{+}/CD19^{+}/CD38^{+}$; c) naïve B lymphocytes $CD45^{+}/CD19^{+}/CD10^{-}/TdT^{-}$; d) mature B lymphocytes $CD45^{+}/CD19^{+}/CD20^{+}/CD10^{-}$; myeloid precursors $CD45^{+}/HLADR^{+}/CD117^{+}/CD34^{+}/CD11b^{-}/CD16^{-}$; e) myelocytes $CD45^{+}/CD13^{dim}/CD11b^{+}/CD16^{-}$; f) metamyelocytes $CD45^{+}/CD13^{+}/CD11b^{+}/CD16^{dim/+}$; g) neutrophils $CD45^{+}/CD13^{++}/CD11b^{++}/CD16^{++}$. **(A)** Box plots showing median values of B cells (CD19+), T cells and myeloid cells for patients treated with lenalidomide for less than 12 months (Len < 12 m), more than 12 months (Len > 12 m) or never treated with lenalidomide (No Len). Patients receiving lenalidomide show lower values of B cells and a correspondent increase on T cells. No difference in myeloid cells was observed. Values are expressed as percentages of bone marrow mononuclear cells. **(B)** Box plots of lymphocytes showing a significant decrease of CD19+ lymphocytes in lenalidomide treated patients, proportional to the time on lenalidomide. A concomitant significant increase in CD19- lymphocytes is observed. Values are expressed as percentage of bone marrow lymphocytes (gated based on $CD45^{+}$ positivity and SSC). **(C)** Box plot of myeloid cells showing no differences in the median values of myeloid precursors, myelocytes, metamyelocytes and neutrophils in lenalidomide treated and naïve patients. Values are expressed as percentages of bone marrow mononuclear cells.

Significance was assessed with the Mann-Whitney U or the Kruskall-Wallis test as appropriate. Only significant values are reported

DISCUSSION

The presence of intra-clonal heterogeneity has been has been revealed in a number of solid and haematological tumours, using different approaches [6-9,11,12,145,296,297]. The aim of the present research project was to identify and better characterise intra-clonal heterogeneity in MM, using both a biological and a clinical approach. In the first part of the project WES and single-cell genetic analysis were successfully combined to unravel the complex phylogeny of intra-clonal heterogeneity in MM. Nonetheless these results, though reporting the existence of genetically variegated sub-clones in MM, may have under-estimated such clonal complexity. First, the single-cell analysis was focused on a small list of NS-SNVs, which were selected for mutating putative driver myeloma genes according to previous literature and/or their CCFs. For instance, the putative ancestral clone 1 identified in one patient, carrying only the t(11;14), may likely harbour additional mutations untested in this analysis. Second, clones present at very low levels (<1%) cannot be detected due to the limited number of sorted cells. Despite these pitfalls the study is still able to unravel the main clonal phylogenetic relationships.

In this serie of t(11;14) myelomas, even at first clinical presentation, at a time when clinical symptoms are present and treatment is required, evidence for the persistence of the earliest MPC clone was found. Two cases were characterized by the presence of a sub-clone carrying the t(11;14) as the sole clonal abnormality. This observation together with the detection of the translocation in most of the interrogated cells supports the etiological significance of the t(11;14). This is also in agreement with the translocation being present in MGUS, the earliest phase of myeloma [103] associated with fewer mutations than myeloma [136]. Once the founding clone is established, the acquisition of additional mutations inevitably leads to the genetic and phenotypic variegation within the progeny of the MPC-founder clone. These heterogeneous MPC-descendants are characterized by the ability to self-renew and proliferate, and can be considered as units of selection in terms of a Darwinian basis for tumour evolution [298]. Our results suggest that the clonal remnants of the earliest stages of the disease may persist late in the myeloma natural history.

Clonal phylogenetic relationships have important implications for both chemotherapy and targeted treatment strategies. Although in our analysis both the presence of a linear pattern of myeloma progression and a branching pattern were described, a unifying interpretation of the data supports a branching evolutionary pattern as being the more plausible route for myeloma progression, where contemporaneous clones accumulate independent genetic hits, shaping their variegated mutational and phenotypic profiles. Even in the samples that showed a linear evolution

it is worth considering the potential existence of additional clonal branches, which we could simply not describe. Moreover, as ancestor clones are still present in a tumour with a linear pattern, they may eventually accumulate new mutations, hence sprouting extra phylogenetic branches. Lastly, sampling bias may also confound a linear model, with different sub-clonal branches potentially present at other tumour sites [6,145,297].

Under the same environment and similar selective pressures, independent but not far-related clones may acquire similar mutations conferring important growth or survival advantages, a phenomenon known as parallel evolution. This work demonstrate, for the first time, clear evidence of parallel evolution at the single-cell level in myeloma, as the same genetic pathway (RAS/MAPK) is altered more than once within the same tumour but in divergent clones, which further evolve independently. This finding is similar to data reported in primary tumour and metastatic renal cell carcinoma [6]. *RAS* mutations represent true driver mutations in myeloma, as any sub-clone with activating *KRAS/NRAS* mutations is able to clonally expand and compete under the same selective pressure during myeloma progression. It may represent the Achilles' heel in myeloma based on the use of targeted treatment, as recently seen for BRAF [165]. This opens a new therapeutic window by which pruning aggressive myeloma-clonal branches could positively select the more indolent ancestor clones.

It has also been thought that the acquisition of mutations over time, either in a linear or in a branching fashion, leads to the progression of cancer from an essentially benign to clinically aggressive behavioural states. In this analysis the number of NS mutations defining cases of symptomatic MM is roughly 23, a number intermediate between simple cancers, such as acute myeloid leukaemia [140], and the more complex solid cancers [141]. These data confirm our previous single-cell analysis, showing that approximately six predominant clones can be detected in MM samples, with much greater complexity existing below the sensitivity of the sequencing approach used. MGUS has fewer NS mutations than the later stages of disease, including SMM, MM and PCL. However, looking at the nature and sites of mutation present at each disease stage, they do not differ, an observation that is consistent with the same molecular mechanisms being active throughout the course of the disease. To date, the genomes of the premalignant stages of MM have not been sequenced and compared with those of symptomatic MM or PCL, but the data here reported demonstrate that there is an increase in the number of NS mutations as the disease progresses from MGUS to SMM and MM. This is in line with previous copy number abnormality data, which indicate that the genetic complexity of the disease increases toward MM [299-301]. MGUS

is known to take >25 years to progress to symptomatic MM, whereas SMM with high-risk features takes <5 years to progress. In this context, it is interesting to note that the MGUS samples are very much less complex compared with symptomatic MM, having approximately half the number of NS mutations, whereas the SMM samples have an equal number of mutations compared with symptomatic MM. Clear evidence of sub-clonal heterogeneity in all stages of disease from MGUS to PCL was seen, which is an essential substrate for Darwinian type evolution, once more reinforcing our hypothesis that MM progression is mediated via competition between sub-clones and outgrowth of the fittest. Importantly, it was seen that intra-clonal heterogeneity is present in MGUS, the earliest clinically recognizable stage of MM, and that it is also present in the later stages of the disease, including SMM and PCL. These observations are consistent with intra-clonal heterogeneity being a critical and consistent feature of both the early and late stages of myeloma. It is also consistent with clonal competition being active from the earliest phases of disease until its highly aggressive late stages and provides direct evidence to support the hypothesis that Darwinian evolution mediates progression through the multiple steps of the disease model of MM. The development of PCL via this complex evolutionary process characterized by extreme deregulation of the behaviour of the normal cellular counterpart is consistent with the poor results of treatment associated with this stage of the disease. This situation can be contrasted with the results of the treatment of de-novo acute leukaemia which can be cured probably because of the significantly less number of genetic hits required to deregulate a hematopoietic stem cell. In order to directly demonstrate the sub-clonal relationships underlying the transitions between the disease phases of myeloma, particular attention was paid to the SMM/MM transition using serial samples from the same individuals. This is a critical transition, where an essentially benign state, that it not treated, undergoes a change that results in clinical symptoms consistent with clonally destructive behaviour and the development of end-organ damage. Using kernel plot analysis of these paired samples, changes in clonal composition can be seen, mediated via the expansion of specific sub-clones, associated with the transition, an observation that is consistent with previous observations concerning the clonal expansion of genetically abnormal cells [299]. Looking at the clonal composition of the paired samples, we show that the transformation of SMM to MM is not the result of the outgrowth of a single clone but results from the outgrowth of a number of sub-clones, already present in the SMM sample. It seems that, in MM, up to six clones change during the SMM-MM transition, which is more than that has been suggested previously in paediatric acute lymphoblastic leukaemia [8], reflecting the increased genetic and sub-clonal heterogeneity

seen in MM compared with this rather simple cancer model. The question then arises as to the nature of the molecular mechanisms driving the sub-clonal changes. At the SMM time point, we show that the majority of the NS exonic changes are already present and that only a few additional changes are seen following transformation. Apart from inactivating mutations in RUNX2 and acquired translocations into BRCA2 and UNC5D, we could not identify any truly acquired genetic abnormalities between the paired samples despite thoroughly checking for coding SNVs, indels and copy number abnormalities. Having exhaustively searched for acquired mutational change to explain the transition and having found only limited numbers of potential molecular drivers to explain the development of clinical symptoms and how disease progression could be mediated, alternative explanations should be considered. There are a number of non-mutational changes that could mediate the transition including epigenetic changes, which have been described previously [3,302]. Alternatively, on the basis of the changes in sub-clonal composition, as a consequence of an increase in tumour bulk there is a threshold effect above which the MM cells modify the microenvironment, such that they favour increased proliferation and expansion of a sub-clone best suited to that environment, the net consequence of which is sub-clonal expansion and the development of end-organ damage. When these observations based on the experimental characterization of the multiple disease phases of myeloma and the transition of SMM to MM in particular are considered, it seems likely that SMM is not a distinct disease entity but is rather a transition state between MGUS and MM, where the sub-clonal structure is evolving. MGUS is a benign clinical state, which is stable with no clinical symptoms, that contrasts with presenting myeloma when tumour bulk has reached a critical threshold and clinical damage has developed. The in-between state, SMM, seems to represent a phase characterized by ongoing competition between sub-clones with the rate of transformation depending upon the nature and rate of mutations that drive the process. We present a coherent hypothesis based on the data we find in myeloma tumour sequencing data. In this hypothesis, adaptation is driven by genetic variation in the tumour cells. At presentation the only selective pressures are those that occur naturally and would involve competition for the myeloma cell niche in the BM and the avoidance of selective pressures exerted by the immune system. Once treatment has been initiated, the selective pressures change and possibly the evolution of the tumour are enhanced by exposure to treatment.

Remnant ancestor clones, although not necessarily the primordial founding clone, have been shown to lead to relapse [4,9,11,136,137]. These clones are postulated to represent MPC units of

selection, critical for progression through the clinical stages of myeloma, and for relapse. Using a xenograft model we could see that early, and likely founding, MPC-clones remain present at diagnosis, and that different clones exhibit distinct survival and propagating abilities following patient treatment and xeno-transplantation. Early clones present at low, or even undetectable, levels at diagnosis were able to survive treatment and lead patient relapse generating new prevalent clones. This finding was partially reproduced in xenograft-samples. It seems clear that sub-clonal populations are involved in selection and competition during myeloma progression and that their fluctuation is conditioned under changes in their cell micro-environments as produced by patient treatment or xeno-transplantation.

Since the finding that intra-clonal heterogeneity is an important feature in MM, a number of studies have biologically evaluated this issue using different molecular and genetic approaches [11,12,111,136,303,304]. However the clinical impact that intra-clonal heterogeneity may have on patients' outcome is still not completely understood. Furthermore it would be important for clinicians to have a quick and easy tool that allows them to assess the presence of intra-clonal heterogeneity from a clinical perspective. Monitoring for the type of immunoglobulin produced and secreted at relapse (either a whole immunoglobulin or just a light chain) provides an excellent tool to study the global impact of intra-clonal heterogeneity. The basic premise in this model is that patients harbour different clones with different secretory behaviour [305] i.e. one clone is able to produce a complete antibody, while the other secretes only a FLC and this can be used as a marker for sub-clonal progression. There is great heterogeneity in the patterns of monoclonal immunoglobulin types and levels secreted by the myeloma clones of different patients at presentation, before therapy has been administered. This was clearly shown by the observation that patients in the different groups, despite having similar proportions of kappa (61-67%) and lambda subtypes, had very different levels of FLCs and paraprotein since presentation. As expected, the groups with higher FLC levels at presentation (FLC escape and PCL) had a higher proportion of patients in which light chains were detectable in the urine at presentation. Within individual patients these patterns and levels may change as the patient enters remission and relapse, reflecting changes in numbers and proportions of sub-clones.

We describe that patients relapsing with increasing levels of free light chain have a worse outcome from this time point than patients who relapse with increasing levels of only the whole paraprotein. Importantly we describe for patients relapsing after presentation with an IgG or IgA paraprotein 10.4% relapse with FLC escape, 49.6% with an increase only in whole paraprotein and

35.2% with an increase in both FLC and paraprotein levels. In order to explain these observations we postulate that chemotherapy is differentially active against the clonal cells producing the intact paraprotein or the FLC only secreting clone. In such a model the type of relapse is a marker of a heterogeneous disease, in which different clones predominate at different time point (Figure 11).

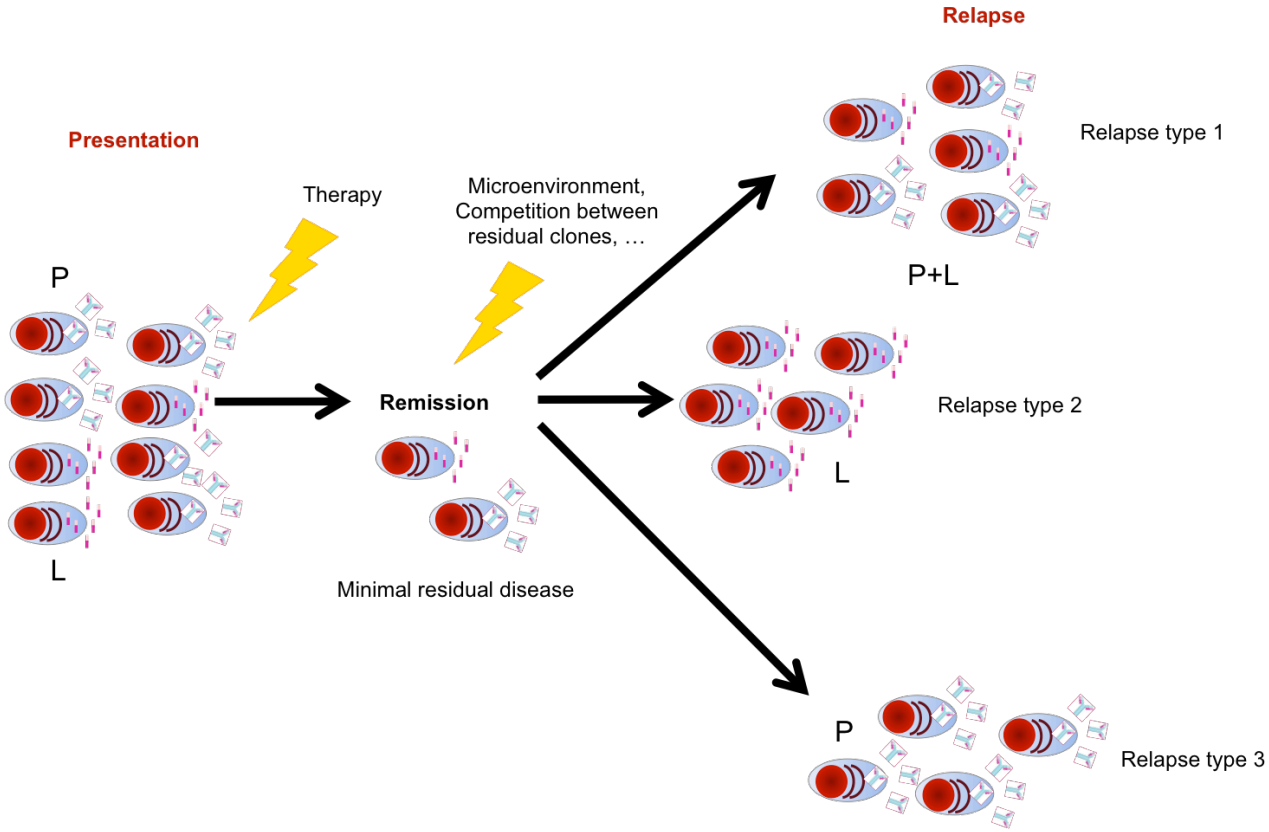

Figure 11. Model of Darwinian evolution in MM assessed by the type of paraprotein secreted. One clone is able to produce a complete antibody, while the other secretes only a FLC. Chemotherapy is differentially active against the different clones, as different is the impact of other evolutionary bottlenecks such as microenvironment or competition for the stem cell niche. The different selective pressures applied will determine which of the clone(s) will survive and give rise to the relapse. The different clonal composition at relapse will ultimately impact on the different sensitivities to subsequent treatments and therefore on survival.

P Intact immunoglobulin secreting plasma cell

L Light chain only secreting plasma cells

Relapse type 1 relapse characterised by both free light chains and intact immunoglobulin secreting plasma cells

Relapse type 2 relapse characterised only by free light chains secreting plasma cells

Relapse type 3 relapse characterised only by intact immunoglobulin secreting plasma cells

The better outcome from relapse for patients relapsing without increasing levels of FLC might reflect the presence of higher sensitivity to treatment in the clone characterised by whole

paraprotein production only. This hypothesis is strengthened by the fact that having a relapse characterised only by an increase in paraprotein levels (PO) retained its prognostic value in a multivariate analysis that included treatment received at diagnosis (thalidomide vs. non thalidomide based), treatment pathway and response achieved.

These results provide further evidence to support the idea that intra-clonal heterogeneity and clonal evolution are a general feature associated with disease progression and treatment resistance in myeloma.

To evaluate the impact of a long-term treatment on the BM of MM patients we performed a flow cytometric analysis of patients treated with lenalidomide for different lengths of time. Our results show that lenalidomide has a minimal impact on bone marrow cellular compartment, but does have an impact on the B cells with a significant decrease in the number of mature B cells. An increase in T cells was seen but no discernible impact on the myeloid compartment was noted. None of these features seem to correlate with a mechanism that could explain an excess risk of SPMs reported in some studies with the use of lenalidomide maintenance [151,159,161].

We demonstrate a significant decrease in the percentage of B cells between lenalidomide treated and lenalidomide untreated patients. These results confirm data previously reported [306], however, we were able to show, for the first time, that this decrease is proportional to the time on lenalidomide treatment, and it is more pronounced in patients that have been on lenalidomide for more than one year. Our data provide further evidence to support the immunomodulatory effect of lenalidomide, which is proportional to the time on treatment.

Lenalidomide is known to potentiate the immune response via the stimulation of T and NK cells [153,307-311], and indeed we found a higher number of T cells in lenalidomide treated patients.

In vitro studies have shown that lenalidomide does not affect the myeloid stem cell compartment, but it may impair terminal maturation [312]. Our analysis showed that there was no long-term impact on neutrophil maturation in lenalidomide treated patients. The impact on the different myeloid subpopulations was comparable between all the three groups analysed. These data are in line with lenalidomide being non-toxic for the bone marrow myeloid compartment. Consistent with this and in contrast to previous reports [312], we did not observe a decrease in the neutrophil percentage in lenalidomide treated patients, which is likely explained by the fact that all the patients had the bone marrow performed at the end of the resting week, before starting a new treatment cycle, and therefore when neutrophils' count had recovered. *In vitro* studies have already shown that IMiDs reversibly down-regulates PU.1 [312], a transcription factor required for

the terminal differentiation of myeloid precursors into competent neutrophils and PU.1 deficient hematopoietic progenitors have reduced neutrophils maturation [313].

Importantly we did not identify any cellular indication that lenalidomide treated patients are more likely to develop hematologic SPMs, such as a significantly higher percentage of immature forms or the co-expression of aberrant markers. These results were confirmed with morphological and histological assessments, and are in line with data previously reported that did not find any significant difference between the number of dysplastic alterations, before and after lenalidomide, as assessed by morphology and by flow [306]. Although we only examined a small number of patients, we did not find any indication that long-term treatment with lenalidomide significantly modifies the myeloid compartment and our observations confirm the reversible effect of lenalidomide on the bone marrow of MM patients.

CONCLUSIONS

Based on our results, we conclude that MM is a heterogeneous disease characterised by the accumulation of mutations and genomic aberrations at clonal and sub-clonal levels [11,12,105,136,137,303]. We successfully combine two state-of-the-art techniques such as WES and single-cell genetic analyses to better define the phylogenetic relationships between clonal populations in myeloma at clinical presentation and relapse. We conclude that the most plausible scenario for myeloma development is through a branching evolutionary pattern, and we also describe parallel-branching evolution where two divergent clones independently acquired the same convergent phenotype (RAS/MAPK pathway activation). We provide evidence of the utility of monitoring clinically for the presence of intra-clonal heterogeneity, and we suggest the free light chain assay as a useful tool to follow this heterogeneity from a clinical point of view. We also show that long-term treatment with IMiDs, although having an effect on the bone marrow, does not significantly modify the myeloid compartment and does not increase the percentage of immature cells or the development of aberrant phenotypes.

Taken all together these findings support the idea that clonal diversity is the elementary foundation for Darwinian selection and underlies disease progression of the disease and the development of treatment resistance. These findings have substantial implications for biopsy-based prognosis and targeted-therapy strategies.

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