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# Cutting edge genomics reveal new insights into tumour development, disease progression and therapeutic impacts in multiple myeloma

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## Summary

Multiple Myeloma (MM) is a haematological malignancy characterised by the clonal expansion of plasma cells (PCs) within the bone marrow. Despite advances in therapy, MM remains a largely incurable disease with a median survival of 6 years. In almost all cases, the development of MM is preceded by the benign PC condition Monoclonal Gammopathy of Undetermined Significance (MGUS). Recent studies show that the transformation of MGUS to MM is associated with complex genetic changes. Understanding how these changes contribute to evolution will present targets for clinical intervention. We discuss three models of MM evolution; the linear, the expansionist and the intraclonal heterogeneity models. Of particular interest is the intraclonal heterogeneity model. Here, distinct populations of MM PCs carry differing combinations of genetic mutations. Acquisition of additional mutations can contribute to subclonal lineages where "driver" mutations may influence selective pressure and dominance, and "passenger" mutations are neutral in their effects. Furthermore, studies show that clinical intervention introduces additional selective pressure on tumour cells and can influence subclone survival, leading to therapy resistance. This review discusses how Next Generation Sequencing approaches are revealing critical insights into the genetics of MM development, disease progression and treatment. MM disease progression will illuminate possible mechanisms underlying the tumour.

**Keywords:** multiple myeloma, genomics, tumour evolution, intraclonal heterogeneity, clinical impacts.

## Biology of multiple myeloma

Multiple Myeloma (MM) is a haematological malignancy resulting from the uncontrolled proliferation of malignant plasma cells (PCs) within the bone marrow (BM). MM is an age-dependent malignancy and the second most common haematological cancer (Singhal & Mehta, 2006). Despite advances in therapy, MM remains a largely incurable disease with a median survival of 6 years. Notably, MM accounts for 20% of all deaths from haematological cancers and 2·1% of deaths from all cancers (https://seer.cancer.gov/statfacts/html/mulmy.html, Zweegman *et al*, 2014). Strategies such as chemotherapy with combinations of newly developed drugs and autologous stem cell transplantation (ASCT) are used to manage the disease following diagnosis (Palumbo & Anderson, 2011). Over the last 10 years, the increase in the spectrum of available treatment options has seen a two-fold increase in patient survival (Palumbo & Anderson, 2011).

The initiating oncogenic events that lead to the development of MM are thought to originate with the establishment of a founder precursor PC clone within the germinal centre of a peripheral lymphoid organ (Morgan *et al*, 2012). Healthy PCs are derived from B lymphocytes, which undergo rearrangement of their immunoglobulin (Ig) genes within the BM to generate precursor cells that express functional B cell receptors (surface immunoglobulins) (Shapiro-Shelef & Calame, 2005). Following this, immature B cells migrate from the BM to the germinal centre of a peripheral lymphoid organ where they undergo affinity maturation in response antigen exposure, specific for the B cell receptor. Somatic hypermutation (SHM) initiates point mutations in the hypervariable regions of immunoglobulin heavy chain locus (*IGH*), resulting in the generation of highly specific Ig (Klein *et al*, 1998). Furthermore, class switch recombination (CSR) initiates antibody class switching, through deletional recombination of the Ig locus switch region, producing functional Ig of different isotype (Stavnezer, 1996). Both molecular mechanisms of SHM and CSR are initiated by the expression of the enzyme activation-induced cytidine deaminase (AID, also termed AIDCA), which generates double stranded DNA breaks in the Ig loci (Morgan *et al*, 2012). Maturation of B cells in the germinal centre leads to the development of memory B cells and plasmablasts, which are rapidly

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produced and short-lived effector cells of the early antibody response (Nutt *et al*, 2015). Terminal differentiation of plasmablasts leads to the development of long-lived antibody-producing PCs. The PCs subsequently migrate to the bone marrow and/or lymph nodes and are involved in the body's immune response, producing Igs which serve to specifically bind to and destroy foreign antigens present in the body (Shapiro-Shelef & Calame, 2005).

In MM however, the neoplastic PC clone, having sustained primary and potentially secondary mutations, migrates to the BM, where it expands in number and produces abundant, intact clonal Ig, known as Monoclonal (or "M") protein or paraprotein (Wang & Young, 2001). Abnormal PCs can also migrate to, and settle at other sites within the BM, where they expand in number to form myelomatous tumours at multiple sites (Ghobrial, 2012). The myelomatous tumours disrupt normal homeostatic bone turnover, blood cell production and organ function, leading to the clinical hallmarks of MM, including elevated paraprotein in conjunction with suppressed immunoglobulin production and organ or tissue impairment. These clinical determinants of MM are known as the CRAB criteria (hypercalcaemia, renal insufficiency, anaemia and bone lesions) features (Kyle *et al*, 2011).

## Development of multiple myeloma

The development of MM is thought to involve a multistage transformational process, as a result of the acquisition of multiple genetic mutations that deregulate normal PC activity (Fig 1) (Walker *et al*, 2014). Common initiating events in PCs include Immunoglobulin Heavy Chain (*IGH*) translocations and hyperdiploidy, which result in the proliferation of monoclonal PCs, leading to development of a pre-cancerous, asymptomatic disease stage known as Monoclonal Gammopathy of Undetermined Clinical Significance (MGUS) (Bergsagel *et al*, 2005). MGUS is a slowly proliferative and relatively stable pre-myeloma stage in which paraprotein levels in the serum are <30 g/l and BM PC numbers account for <10% of the nucleated cell count within the BM (International Myeloma Working Group 2003, Landgren *et al*, 2009). MGUS affects approximately 3% of the population aged over 50 years and 5% of people aged over 70 years (Kyle *et al*, 2006). Each year, MGUS patients have a 0·5–1% risk of progressing to MM (Rajkumar *et al*, 2014). For this reason, MGUS patients are currently monitored but remain untreated until their condition progresses to a symptomatic disease stage. However the risk of progression is not uniform in all cases, influenced by disease biology factors, such as the type and concentration of M protein, serum free light chain ratio, bone marrow plasmacytosis, proportion of clonal plasma cells and presence of immunoparesis (Rajkumar *et al*, 2014). Risk of progression can be stratified by the Mayo Clinic model based on clonal PC burden with M protein values and skewed free light-chain ratios (Rajkumar *et al*, 2005) or the Spanish study group multiparametric flow cytometry model (Perez-Persona *et al*, 2007). For MGUS, the Mayo Clinic identifies 3 important risk factors of progression: non-IgG isotype, serum M-protein concentration >15 g/l and a skewed free light-chain ratio (<0·26 or >1·65) (Rajkumar *et al*, 2005), whereas the Spanish study group assesses the ratio of aberrant PC (aPC) to normal BM PCs, where MGUS risk factors are an aPC/BM PC ratio >95% and DNA aneuploidy (Perez-Persona *et al*, 2007). MGUS is followed by an intermediate asymptomatic Smouldering Multiple Myeloma (SMM) stage, where patients do not show evidence of Myeloma-Defining Events (MDEs) or amyloidosis (Rajkumar *et al*, 2014). The SMM stage is known to have an annual risk of transition to MM of 10% in the first 5 years following diagnosis, 3% in the next 5 years and 1·5% in the subsequent years thereafter (Rajkumar, 2016). For SMM, the Mayo Clinic identifies risk factors for progression as BMPCs >10%, M-protein concentration >30 g/l and a skewed free light-chain ratio (<0·125 or >8) (Dispenzieri *et al*, 2008), while the Spanish study group identifies risk factors of an aPC/BM PC ratio >95% and immunoparesis (Perez-Persona *et al*, 2007). The transition to MM is accompanied by an increased plasmacytosis with the presence of ≥10% clonal BMPCs or the presence of biopsy-proven bony or extramedullary plasmacytomas, and the presence of at least one of the MDEs (Rajkumar *et al*, 2014). The MDEs include evidence of CRAB features or at least one of the biomarkers of malignancy; including either the presence of clonal BM PCs percentage ≥60% or a serum free light chain ratio ≥100 or >1 focal lesion (from magnetic resonance imaging studies) (Rajkumar *et al*, 2014). In the final stage of the transformation process, malignant PC clones may gain independence from the BM, enter the peripheral circulation, leading to Plasma Cell Leukaemia (PCL), or form tumours in soft tissue or organs, leading to Extramedullary Myeloma (Walker *et al*, 2014).

Using karyotyping and molecular cytogenetic techniques MM PCs have been classified under three key subtypes; hyperdiploid, non-hyperdiploid or unclassified (Fonseca *et al*, 2009). Hyperdiploid MM cases are characterised by trisomies and account for 50–60% of patients with MM (Sawyer *et al*, 2016). Non-hyperdiploid cases exhibit chromosomal translocations of the *IGH* locus, which are present in 45% of patients with MM (Mikhael *et al*, 2013). Interestingly, *IGH* translocations arise through the normal process of B cell development. As previously described, during late B cell development, AID-induced CSR leads to double stranded breaks in the *IGH* locus, most of which are repaired locally. However, error in reassembly can lead to illegitimate recombination of the *IGH* locus with double stranded breaks elsewhere in the genome, resulting in aberrant chromosomal translocations of the *IGH* locus, where partner oncogenes are put into proximity of strong *IGH* enhancers leading to the hallmark event in MM (Gonzalez *et al*, 2007; Morgan *et al*, 2012). The three most common *IGH* abnormalities include the t(4;14) (chromosome bands p16q32), t(11;14) (chromosome bands q13q32) and t(14;16) (chromosome bands

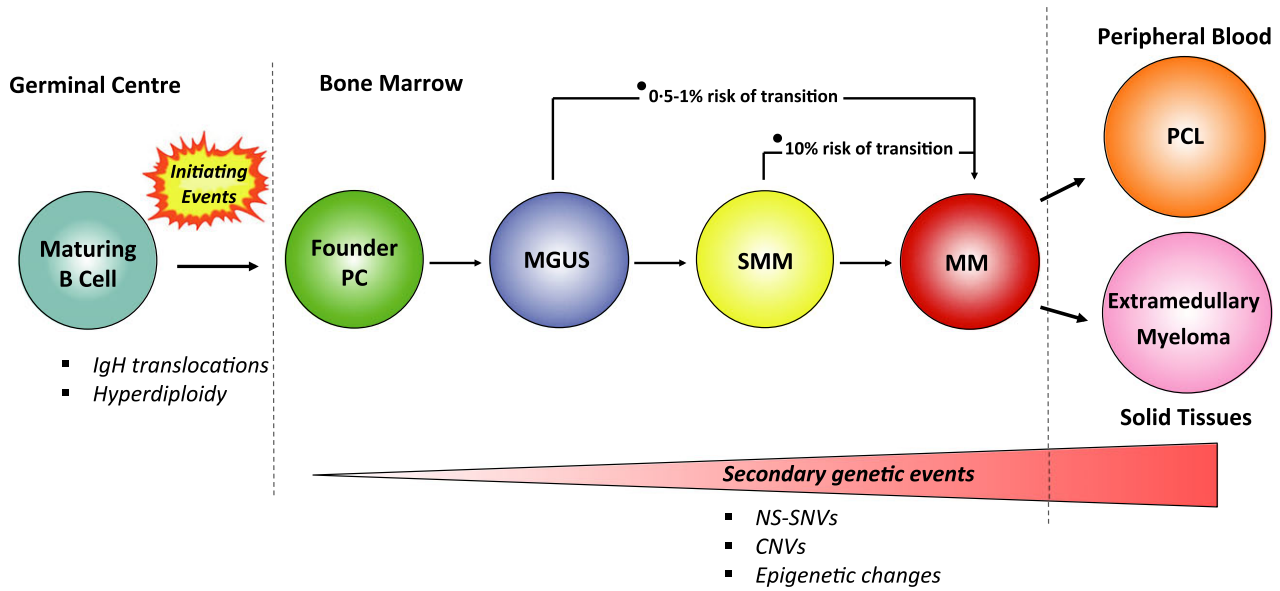


**Fig 1.** The development of MM is a multistage transformational process. Common initiating events of *IGH* translocations and hyperdiploidy during B cell development deregulate normal PC behaviour, leading to asymptomatic MGUS. Further mutational load leads to the intermediate stage of SMM before final transformation to symptomatic MM. MGUS is known to take >25 years to progress, with patients having an annual 0·5–1% risk of transition to MM. Whereas SMM takes <5 years to progress, with an annual 10% risk of progressing to MM in the first 5 years. However, the risk of progression is not uniform and is dependant on disease biology factors, as discussed above. MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PC, plasma cell; PCL, plasma cell leukaemia; SMM, smouldering multiple myeloma.

q32q23) (Fonseca *et al*, 2009) translocations. Non-hyperdiploid cases represent a more aggressive form of MM and are associated with poor prognosis (Bergsagel & Chesi, 2013). Other cytogenetic abnormalities, including monosomies of chromosome 14, and unaltered chromosome structure, are also present at a lower frequency of MM patients (Mikhael *et al*, 2013; Rajkumar, 2016). Further studies have identified that this may represent a novel hyperhaploid subtype of high risk MM disease (Sawyer *et al*, 2016). Risk type of active MM can be stratified based on the specific genetic lesions harboured by a patient into high risk (t(14;16), t(14;20) and del 17p), intermediate risk (t(4;14, gain 1q, hypodiploidy and del 13) and standard risk (t(11;14), t(6;14) and trisomies) (Mikhael *et al*, 2013; Rajkumar, 2016). However, the early common primary pathogenic events of hyperdiploidy and *IGH* translocations are believed to be insufficient to cause active symptomatic MM, as asymptomatic MGUS patients can harbour these abnormalities and show no clinical symptoms (Fonseca *et al*, 2002; Chapman *et al*, 2011). The acquisition of secondary genetic events of non-synonymous single nucleotide variations (NS-SNVs), copy number variations (CNVs) and epigenetic changes are also required to initiate MGUS and PC malignancy leading to MM disease progression (Chesi & Bergsagel, 2011; Morgan *et al*, 2012). In addition to the oncogeneic mutations occurring in the PC, there has been significant advancement in the understanding of an important role played by the BM microenvironment in supporting MM disease progression through the proliferation, survival and drug resistance of MM PCs (Mitsiades *et al*, 2006; Abe, 2011; Manier *et al*, 2012; Noll *et al*, 2012; Lawson *et al*, 2015).

## A new paradigm in multiple myeloma development

New high-resolution Next Generation Sequencing (NGS) techniques represent an important advance in genomics, providing researchers with powerful tools for genetic analysis at single nucleotide resolution, enabling the identification of critical disease mutations and disease vulnerability. To date, no single gene mutation or combination of mutations have been identified as being common to all MMs at presentation (Weston-Bell *et al*, 2013). These findings suggest that multiple diverse genetic aberrations, and molecular pathways, are responsible for the onset of disease. Furthermore, the genetic abnormalities that are characteristic of each transformational stage of MM (MGUS, SMM and MM) have not been fully identified. This has been attributed to the low-resolution cytogenetic techniques previously used, which possess relatively low sensitivity of identification.

Of particular interest is the elucidation of how these key genetic aberrations contribute to tumour evolution in MM. Defining these critical disease mutations will provide insight into the possible mechanisms underlying disease progression, and identify key biomarkers of disease risk and provide novel therapeutic targets. Evaluation of the available cytogenetic and NGS studies reveals evidence for the existence of

multiple tumour evolution models within MM. As a result three models of tumour evolution in MM are postulated:

1 Linear model
2 Expansionist model
3 Intraclonal heterogeneity model

### *Linear model of tumour evolution*

Classical cancer biology theory proposes a linear model of evolution, where tumours are derived from a unicellular origin with clonal growth pattern as a result of sequential accumulation of genetic mutations (Bahlis, 2012).

As discussed earlier, the development of MM is considered to be a multistage transformational process where patients with MGUS progress through an intermediate SMM transition stage before developing symptomatic MM. Initial genome analyses by Chapman *et al* (2011) identified MM tumour-specific mutations by comparing corresponding tumour to normal PCs using Whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES) (Weston-Bell *et al*, 2013) techniques. An average of 35 NS-SNVs and 21 chromosomal rearrangements that disrupted protein coding regions were identified in MM (Chapman *et al*, 2011). Following this, Walker *et al* (2014) carried out the first WGS study, comparing a small number of premalignant MM stages (MGUS $n = 4$, SMM $n = 4$) and symptomatic MM stages (MM $n = 26$, PCL $n = 2$) to reveal tumour acquired NS-SNVs as a function of disease progression, where genetic complexity increases through the stages of MM. MGUS PCs were found to harbour a median of 13 NS-SNVs, which increased to 28, 31 and 59 through SMM, MM and PCL, respectively. These findings are consistent with a linear model of tumour evolution in MM, where the sequential acquisition of NS-SNVs reaches a mutational threshold in the SMM PC, resulting in uncontrolled clonal proliferation leading to MM disease progression (Fig 2).

### *Expansionist model of tumour evolution*

The expansionist model of tumour evolution infers that all necessary genetic mutations are present in a subset of PCs at the MGUS disease stage, and it is their subsequent expansion that leads to MM disease progression.

Studies using low-resolution molecular cytogenetic techniques have detected the same genetic mutations throughout all stages of MM transformation. Fluorescence *in situ* hybridisation (FISH) analysis has identified *IGH* translocations, chromosome 13q and 17p deletions and gain of 1q throughout all stages of MGUS, SMM and MM (Chiecchio *et al*, 2009; Lopez-Corral *et al*, 2011). Interestingly, the number of PCs harbouring these specific genetic abnormalities increased with disease progression, suggesting that clonal PC expansion was due to selective advantages (Avet-Loiseau *et al*, 1999; Chiecchio *et al*, 2009; Lopez-Corral *et al*, 2011). Notably also, sequential analysis of 5 patients (2 MGUS-MM, and 3 SMM-MM) revealed a higher proportion of PC harbouring the genetic abnormality that was observed at diagnosis upon progression to MM (Lopez-Corral *et al*, 2011). Further high resolution CNV analysis using high density single nucleotide polymorphism (SNP) arrays have indicated an increasing genetic complexity as disease progresses towards MM, with a progressive increase in the median number of CNVs through MGUS, SMM and MM from 5 to 7·5 to 12, respectively (Lopez-Corral *et al*, 2012). Frequent abnormalities observed in MM include gains on chromosome 1q, 3p, 6p, 9p, 11q, 19p, 19q and 21q together with deletions on chromosome 1p, 6q, 8p, 12p, 13q, 14q, 16q, 17p, 17q and 22q (Lopez-Corral *et al*, 2012; Walker *et al*, 2012). Alterations of 11q and 21q gains, and 16q and 22q deletions were previously viewed to be MM-specific, however, it has been shown that these alterations are present within minor subclones at the MGUS stage (Lopez-Corral *et al*, 2012). Furthermore, WGS of sequential SMM-MM has demonstrated little difference in the median number of NS-SNVs present at both stages, with 28–31 respectively, reported (Walker *et al*, 2014). These findings suggest that the predominant PC clone may already be present at the SMM stage, and it is the outgrowth that leads to the initiation of MM disease progression. Interestingly, recent WES and genotyping study of paired random bone marrow-focal lesion samples has revealed insights into spatial heterogeneity, where both similarities and differences of site-specific SNVs and CNVs were observed to contribute to disease progression (Weinhold

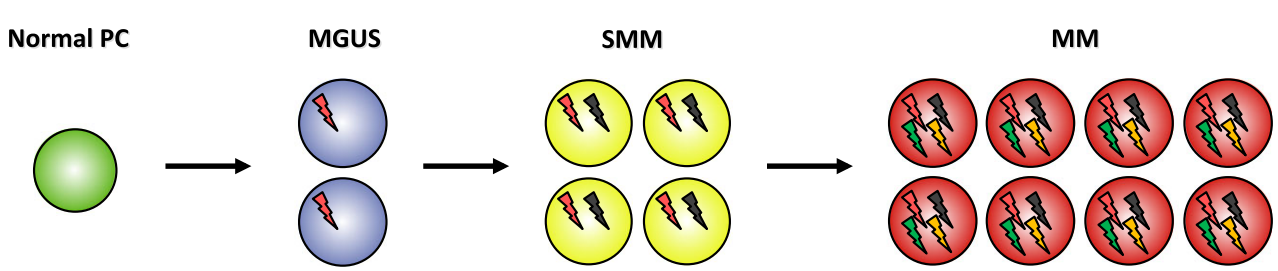


**Fig 2.** The linear model of tumour evolution: The sequential acquisition and accumulation of multiple genetic mutations [represented by distinct bolts increasing through MGUS (red), SMM (red + black) and MM (red + black + green + yellow)] in PCs leads to MM disease progression. MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PC, plasma cell; SMM, smouldering multiple myeloma.

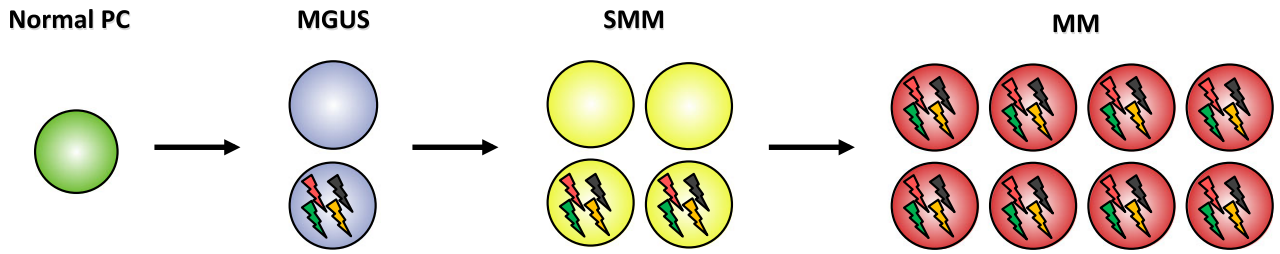


**Fig 3.** The expansionist model of tumour evolution: All necessary genetic mutations are present in a subpopulation of MGUS PCs (represented by all the distinct bolts (red + black + green + yellow) present in one), and it is their eventual expansion that leads to MM disease progression. MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PC, plasma cell; SMM, smouldering multiple myeloma.

*et al*, 2015). In some paired samples up to 90% variants were shared in both site samples, and a prominent pattern of the outgrowth of subclonal bone marrow sample CNVs as clonal events in focal lesions samples was observed (Weinhold *et al*, 2015). Taken together, these findings support an expansionist model of MM evolution, where a subpopulation of MGUS PCs harbour all the required genetic mutations, and their subsequent expansion results in MM disease progression (Fig 3).

### *Intraclonal heterogeneity model of tumour evolution*

The intraclonal heterogeneity model of tumour evolution posits a genetically heterogeneous clonal structure at the asymptomatic MGUS disease stage, where "Darwinian" competition occurs between distinct PC subclones in response to selective pressures, leading to the outgrowth of dominant PC subclones and subsequent MM disease progression (Nowell, 1976; Greaves & Maley, 2012).

Initial cytogenetic studies have indicated a genomic complexity where only a proportion of the clonal PC population carried any specific abnormalities at each stage of MM disease (Chiecchio *et al*, 2009; Lopez-Corral *et al*, 2011, 2012). The advent of NGS techniques has allowed genetic analysis at single nucleotide resolution, resulting in a higher power in detection of clonal architecture. A specific mutation being clonal or subclonal within a tumour sample can be determined by the proportion (of mutated reads) of total tumour cells that harbour the specific mutation, adjusted to any normal (non-tumour) contamination and the copy number of the locus. From this a phylogenetic tree of clonal/subclonal fractions can then be constructed to estimate the intraclonal evolution that may be occurring within the tumour. Initial sequencing studies on MM patients using WGS and WES have led to the discovery of frequent significantly mutated genes ("driver" genes), and the existence of a heterogeneous genetic landscape, where coexisting clones of differing genetic architecture arise during the evolution of MM (Egan *et al*, 2012; Walker *et al*, 2012; Bolli *et al*, 2014; Lohr *et al*, 2014). These "driver" genes are thought to influence clonal fitness (selective advantage and dominance) of malignant PC clones harbouring these mutants, driving MM disease progression in a branching manner.

While MGUS is known to be a benign disease stage that is far less genetically complex than MM, intraclonal heterogeneity has been demonstrated through all stages of MM, suggesting that disease progression is mediated via competition between subclones and outgrowth of the fittest of these subclones from the earliest stage of MM (Egan *et al*, 2012; Walker *et al*, 2012; Bolli *et al*, 2014; Lohr *et al*, 2014). Large cohort sequencing studies in MM have identified recurrently mutated genes associated with disease pathogenesis. The initial study of 38 MM tumours with matched normal genomes identified significant frequent somatic mutations occurring in MM, involving the *KRAS, NRAS, TP53, BRAF* and *IRF4* genes and six newly discovered cancer-associated genes; *CCND1, FAM46C, DIS3, PNRC1, ALOX12B, HLA-A and MAGED1* (Chapman *et al*, 2011). Further large cohort NGS studies performed by Lohr *et al* (2014) ($n$ = 203 patients), Bolli *et al* (2014) ($n$ = 67 patients) and Walker *et al* (2015) ($n$ = 463 patients) have also identified the presence of mutations in *KRAS, NRAS, TP53, BRAF, FAM46C, DIS3, IRF4, TRAF3* and *CYLD*, of which *KRAS, NRAS, TP53, BRAF, FAM46C* and *DIS3* are now believed to be "driver" genes in MM disease progression. The RAS/MAPK pathway is frequently observed to be deregulated in MM, with recurrent mutations occurring in *KRAS, NRAS* and *BRAF* (Bolli *et al*, 2014; Lohr *et al*, 2014; Walker *et al*, 2015). Mutations in *KRAS* and *NRAS* tend to mainly occur with mutual exclusivity, however have been observed to co-exist in 2% of patients (Walker *et al*, 2015). Interestingly, these "driver" mutations have been identified as being present in clonal fractions in some patients and subclonal fractions in other patients, suggesting "driver" events may also arise during later stages of MM tumour evolution (Bolli *et al*, 2014; Lohr *et al*, 2014). Furthermore, although affecting the same pathway, they have been identified to occur subclonally or in a nested fashion, where one mutation clone is identified as a subclone of another (Lohr *et al*, 2014). While it would be expected these subclonal population would exhibit improved survival advantage, owing to the presence of mutations in multiple "driver" genes, they do not appear to have sufficient selective advantage for outgrowth to clonality (Lohr *et al*, 2014). This advancement in our understanding of the intraclonal heterogeneity in MM illustrates the consideration required towards therapeutic choices, where suboptimal outcome would be

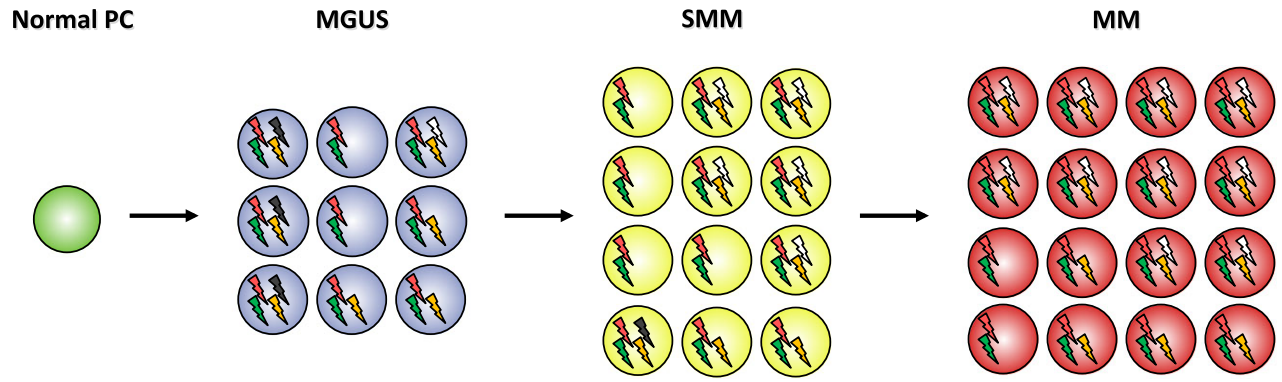


**Fig 4.** The intraclonal heterogeneity model of tumour evolution: Distinct PC subclones carry different combinations of genetic mutations (represented by MGUS subpopulations harbouring distinct combinations of coloured bolts), with the dominance of subclones changing with MM disease progression. Predominant subclones harbouring "driver" mutations, conferring increased fitness potential, are able to survive the microenvironment pressures and contribute to MM disease progression (represented by the PC subpopulation harbouring distinct bolts red + green + yellow + white). MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PC, plasma cell; SMM, smouldering multiple myeloma.

observed if a patient is treated for a "driver" mutation that exists only subclonally. These unique findings support an intraclonal heterogeneity model of MM evolution, where subclones harbour differing combinations of mutations, with the genetic landscape of subclones changing as MM disease progresses (Fig 4).

## Single cell analysis of multiple myeloma

In more recent years, novel single cell analysis techniques have provided a sophisticated method for unravelling tumour genomics and evolution at a more detailed level. By unravelling clonal diversity and frequency of genetic mutations, single cell analysis is able to provide a greater understanding of the genomic complexity and clonal architecture that is present at the individual cell level within a bulk tumour. Current platforms available for single cell capture and subsequent genomic interrogation include Fluorescence-Activated Cell Sorting (FACS)-based automated single cell deposition and the Fluidigm C1 integrated fluidic chip (IFC) system (Fluidigm Corporation, South San Francisco, CA, USA).

The existence of clonal heterogeneity presents a complex situation when analysing tumours. Distinct clonal populations of cells cannot be identified by conventional tissue average data analysis (Gundry *et al*, 2012). Furthermore, random low abundance mutations are currently inaccessible by standard high throughput sequencing approaches because they cannot be distinguished from sequencing errors (Gundry *et al*, 2012). At the genomic level, it is difficult to identify whether mutations are present in the same tumour cells or arise from distinct clonal populations of cells present within the bulk tumour (Yadav & De, 2014). Consequently, isolated detection of dominant clonal populations of tumour cells could bias classification, prognosis and treatment of disease (Yadav & De, 2014).

Application of single cell analysis techniques in MM supports the existence of multiple independent subclones within tumours. Single cell studies of t(11;14) MM have described the existence of 2–6 different clones, which are related by linear and branching phylogenies, highlighting the presence of intraclonal heterogeneity at MM diagnosis (Melchor *et al*, 2014). Melchor *et al* (2014) demonstrated the existence of subclonal heterogeneity with parallel evolution of the RAS/MAPK pathway in distinct single cells, giving rise to divergent subclonal populations of mutant *NRAS* clone lineage and mutant *KRAS* clone lineage. Furthermore, single cell genotyping has revealed a subclonal substructure in MM with 3 main clones harbouring *ATM* mutations where 2 subclones exhibited other mutually exclusive mutations (Walker *et al*, 2012). The same study also identified intraclonal heterogeneity of mutations in "driver" genes *NRAS* (present in 32% and 96% of tumour cells) and *KRAS* (20% and 72% of tumour cells) (Walker *et al*, 2012). Interestingly, activating *KRAS* mutations were present as minor subclones in one case, observed in 20% and 48% of tumour cells (Walker *et al*, 2012). This suggests that subclones are continually at risk of developing "driver" mutations that can confer a growth and survival advantage leading to clonal dominance over time (Walker *et al*, 2012). This advancement in the understanding of the intratumour heterogeneity in MM illustrates the consideration required towards therapy and its clinical implications. A recent study using *in vitro* modelling of MM cell lines that are bortezomib-sensitive or -resistant has generated a novel gene expression profiling (GEP) signature that can predict drug response to proteasome inhibitors (Stessman *et al*, 2013). The analysis of this GEP at the single cell level in pre-treated MM cell lines and drug naïve patient samples was able to identify pre-existing single cell subpopulations that were resistant to proteasome inhibitors, demonstrating the possible requirement of therapies tailored towards subclonal populations within bulk tumours (Mitra

*et al*, 2016). This has also been developed into the computational software package SCATTome (single-cell analysis of targeted transcriptome) that can predict probability of drug response of single cells based on the MM expression signature (Mitra *et al*, 2016).

Collectively, these NGS and single cell studies support clonal heterogeneity as a model of MM evolution where "Darwinian" competition between heterogeneous PC subclones initiates non-linear accumulation of mutations and outgrowth of dominant subclones driving MM disease progression.

## Limitations of published studies in multiple myeloma

To date, a limited number of NGS studies of MM have been performed, with the first "Initial genome sequencing and analysis of multiple myeloma" carried out by Chapman *et al* (2011). The majority of these studies involved single timepoint studies of MM PC samples or have relied on the use of unpaired MGUS and MM PC samples. Consequently, these studies are only able to provide a detailed indication of the genetic landscape at the MM disease stage and an indirect indication of genetic mutations associated with MM disease progression.

WES analysis of serial samples ($n = 15$), collected at disease progression or relapse post-treatment with later timepoint samples collected at relapse/progression after further lines of treatment has revealed major patterns of tumour evolution associated with MM progression: (i) No change in clonal composition, (ii) Differential clonal response, with proportions of subclones changing over time, (iii) Linear evolution, with a new subclone emerging over time, and (iv) Branching evolution, with the emergence of new clones and decline of other clones (Bolli *et al*, 2014). Only one study has investigated paired SMM-MM samples ($n = 4$), finding that intraclonal heterogeneity is a typical feature in MM, whereas SMM is a transition state where subclonal structure is evolving (Walker *et al*, 2014). Interestingly, only one unique NS-SNV was identified in MM, demonstrating that most of the required mutations for transition to symptomatic MM are already present. Additionally, comprehensive analysis of paired presentation and relapse/progression samples after combination high dose therapy ($n = 33$), using WES as well as gene expression and copy number profiles revealed that a majority of patients ($n = 22$) relapse through a branching tumour evolution pattern, with others showing linear evolution and differential response (Weinhold *et al*, 2016). Furthermore an increase in bi-allelic inactivation of tumour suppressor genes (mainly *TP53* and *FAM46C*) was associated with relapse, with double-hit events in *TP53*: del (17p)/*TP53*$^{mut}$ or del(17p)/*TP53*$^{del}$ characterising a subgroup of patients with worse outcome after relapse (Weinhold *et al*, 2016). Further serial sample studies of this nature and/or sampling of different sites from the same patient are required for a greater understanding of genetic heterogeneity in MM disease progression.

A comprehensive approach would be to perform NGS studies of sequential paired MGUS-MM samples from the same patient. At present, no longitudinal progression studies of paired MGUS-MM PC samples have been performed. This is due, in large part, to the difficulty in establishing a cohort of patient samples from individuals when first diagnosed with MGUS, who subsequently develop symptomatic MM. Additionally, due to the nature of premalignant disease, MGUS samples contain a low number of tumour PCs compared to normal healthy PCs, which results in high contamination and low yield of tumour PCs on isolation. The thorough genomic analysis of both bulk tumours and single cells on paired MGUS-MM patients represents a unique approach to identify key "driver" genes that are mutated and/or aberrantly expressed during disease progression. This approach would derive gene signatures indicative of pathways that are deregulated during the MGUS to MM transition. Furthermore, genomic data derived from such a study may allow for the identification of biomarkers that can predict which MGUS patient will progress to MM.

## Epigenetics in multiple myeloma development

Extensive studies of MM have been performed using cytogenetic and genomic approaches, however, relatively little is known about the role of epigenetics in driving MM disease progression. The rate of epigenetic change in cancers has been estimated to be orders of magnitude higher than that of genetic change occurring, and could be a major determinant of clonal evolution (Greaves & Maley, 2012). The key epigenetic mechanisms known to alter and regulate gene expression are DNA methylation and histone modifications.

Changes in DNA methylation status, such as hypermethylation leading to gene inactivation and hypomethylation inducing genomic instability, have been observed in many cancer types (Kulis & Esteller, 2010). Methylome studies, comparing MM and its transition stages (MGUS, SMM and PCL) with normal PCs, have shown an increase in the number of differentially methylated gene loci associated with disease progression (Salhia *et al*, 2010; Walker *et al*, 2011; Heuck *et al*, 2013). The presence of genetic hypomethylation has been implicated as an important and early mechanism which drives MM disease progression (Salhia *et al*, 2010). Hypomethylation is associated with genomic instability and often coupled with altered chromatin structure, changes in DNA methyltransferase activity, loss of imprinting and an increased frequency of CNVs (Walker *et al*, 2011). Further studies have identified distinct profiles of epigenetic modifications linked with MM disease transition stages, with global hypomethylation occurring at MGUS-MM transition and hypermethylation occurring at MM-PCL transition (Walker *et al*, 2011). Specifically, the DNA methyltransferase, DNMT3A, was observed to be underexpressed in MM due to

the actions of hypermethylation, providing insight into the possible mechanism of hypomethylation observed in premalignant stages of MM (Heuck *et al*, 2013). Gene ontology enrichment analysis has revealed that hypomethylation in MM may favour bone invasion by increasing interactions with the bone marrow extracellular matrix, initiating adhesive interactions and the formation of lytic bone lesions (Salhia *et al*, 2010). Interestingly, highlighting the heterogeneity also observed at the methylation level, gene-specific hypermethylation has also been associated with MGUS-MM transition, with 77 affected genes having roles in developmental, cell cycle and transcriptional regulatory pathways identified (Walker *et al*, 2011). Further gene-specific hypermethylation has been identified at MM-PCL transition, with 1802 affected genes that are associated with cell signalling and cell adhesion pathways (Walker *et al*, 2011). Hypermethylation is proposed to deregulate adhesion of MM PCs to the bone marrow, facilitating independence of malignant PCs from the bone marrow niche, leading to PC egress from the marrow and entry into the peripheral circulation and development of PCL (Walker *et al*, 2011). Furthermore, a recent study has also identified hypermethylation of developmentally regulated B cell enhancers as a new type of epigenetic modification associated with the pathogenesis of MM (Agirre *et al*, 2015).

Initial methylome analysis had revealed that methylation status is not associated with specific genetic alterations (Salhia *et al*, 2010). In contrast, other studies have identified specific MM cytogenetic subgroups that exhibit individual methylation profiles, with a t(4;14) group, two separate t(11;14) groups and two separate hyperdiploid groups described (Walker *et al*, 2011). The t(4;14) cytogenetic subgroup displays frequent hypermethylation, akin to that observed in PCL, signifying that the methylation status may influence the aggressive clinical phenotype usually observed in both cases (Walker *et al*, 2011). However, the mechanisms that cause abnormal DNA methylation patterns in MM are yet to be determined (Dimopoulos *et al*, 2014).

Complex epigenetic mechanisms involving histone modifications are also reported to contribute to the pathogenesis of cancer (Plass *et al*, 2013). While DNA methylation is relatively constant, histone modifications are dynamic in nature. The main regulatory mechanism of the epigenome is histone acetylation, which is maintained by the interplay of two enzymes: histone acetyl transferases (HATs) catalysing the addition and histone deacetylases (HDACs) catalysing the removal of acetyl groups on lysine residues of histones.

In MM, HDAC inhibitors have been reported to have potent anti-myeloma activity both *in vitro* and *in vivo* (Smith *et al*, 2010). However, a clear understanding of which HDACs are expressed by MM PCs is lacking (Smith *et al*, 2010). Alternatively, histone methylation has been implicated to play an important role in MM development. For example, the high risk cytogenetic t(4;14) subgroup exhibits dysregulated expression of Fibroblast Growth Factor Receptor 3 (FGFR3) and Multiple Myeloma Set Domain (MMSET, also termed NSD2), a histone methyl transferase (Kalff & Spencer, 2012). Universal expression of MMSET in t(4;14) MM suggests that MMSET is critical for myeloma pathogenesis and/or progression (Marango *et al*, 2008). Interestingly, further to its histone methyl transferase activity, MMSET has been identified to enhance the activity of HDAC1 and HDAC2, and therefore plays a role in altering histone acetylation. MMSET has been shown to be beneficial to the survival of MM PCs as *in vitro* MMSET knockdown affects genes involved in key survival processes such as cell cycle, apoptosis and adhesion (Brito *et al*, 2009). These findings reveal insights into new epigenetic therapeutic strategies in MM. However, further laboratory and clinical studies are required in this emerging area.

## Current therapies and impacts in multiple myeloma

Although MM remains a largely incurable disease, advances in clinical research have produced effective treatment methods for disease control. Different strategies are employed for asymptomatic and symptomatic disease stages where MGUS/SMM stages require clinical monitoring while active MM is treated immediately and, in most cases, aggressively to induce disease remission (Palumbo & Anderson, 2011). In the past decade, the survival of MM patients has more than doubled due to the introduction of new effective drug classes, including immunomodulatory drugs (lenalidomide, thalidomide, pomalidomide, daratumumab and elotuzumab), histone deacetylase inhibitors (panobinostat) and proteasome inhibitors (bortezomib, carfilzomib and ixazomib), and the increased use of ASCT (Kumar & Russell, 2014; Palumbo & Anderson, 2011; https://www.themmrf.org/multiple-myeloma-knowledge-center/myeloma-drugs-guide/). Patient risk status is evaluated based on the factors of age/performance, renal function and presence or absence of high-risk genetic abnormalities, which all plays an important role in the treatment selection (Kumar & Russell, 2014). Treatment comprises three phases: induction, consolidation and maintenance. Current induction treatments for newly diagnosed patients who are eligible for ASCT, include two-drug combination therapy of dexamethasone with lenalidomide (Rd), thalidomide or bortezomib. Three-drug combination treatments for newly diagnosed patients include bortezomib-cyclophosphamide-dexamethasone (VCD), bortezomib-thalidomide-dexamethasone (VTD) or bortezomib-lenalidomide-dexamethasone (VRD) (Rajkumar, 2016). These combination treatment strategies are used to induce a complete response in patients before ASCT, followed by maintenance treatment with thalidomide or lenalidomide (Palumbo & Anderson, 2011). For patients ineligible for transplantation, preferred treatments include the melphalan-based combinations of melphalan-prednisone-thalidomide (MPT), Rd (for elderly patients) or the bortezomib-based combinations VRD, VCD or VTD (Rajkumar, 2016). In more recent years the

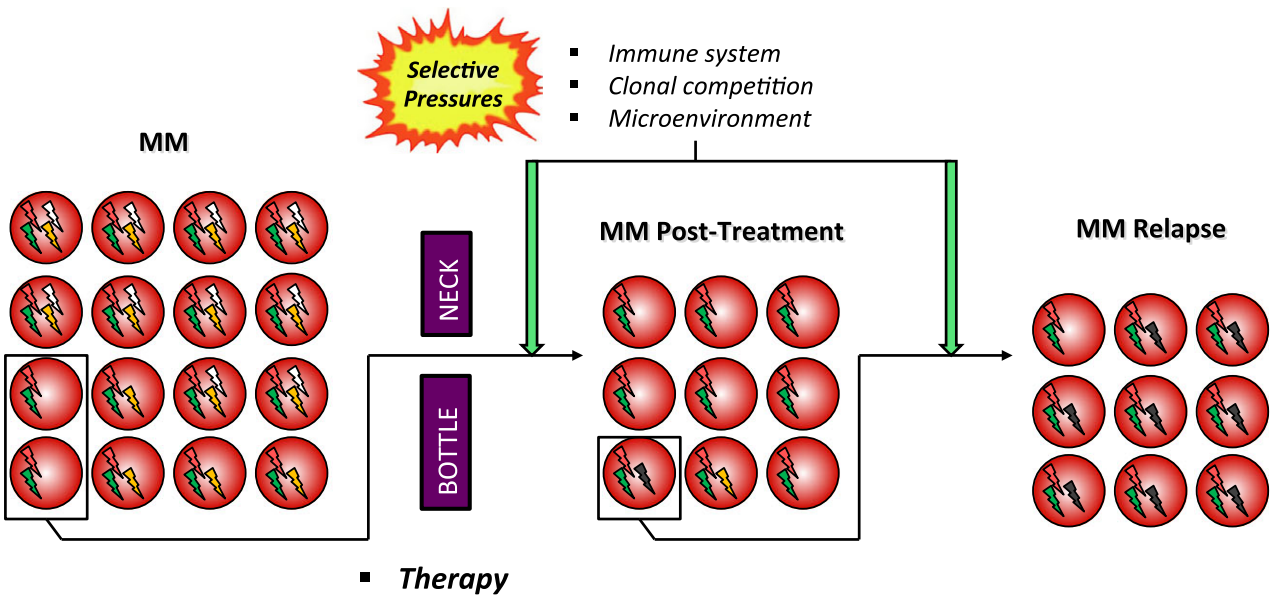


**Fig 5.** The impact of therapy on intraclonal heterogeneity in MM: The introduction of treatment presents a new selective pressure on a heterogeneous multiple myeloma (MM) tumour, in conjunction with those already existing due to the bone marrow microenvironment, immune system and competing clonal architecture. While therapy is effective in eliminating dominant plasma cell (PC) populations harbouring critical "driver" mutations, it may be ineffective against indolent PC populations that do not have "driver" mutations. These residual PC clones that survive treatment may mutate further, acquiring "driver" mutations, thereby conferring improved fitness and malignant potential, which in turn leads to MM disease relapse.

treatment of relapsed MM patients (who have undergone previous lines of treatment) has seen promising results with the clinical trials and approval of monoclonal antibodies daratumumab (targeting cell surface protein CD38) and elotuzumab (targeting cell surface protein CD319) in 2015. Daratumumab has shown great efficacy in clinical trials with the ability to induce a deep response as both a monotherapy (Lonial *et al*, 2016; Usmani *et al*, 2016) and in combination therapy with bortezomib-dexamethosone (Palumbo *et al*, 2016) or lenalidomide-dexamethosone (Dimopoulos *et al*, 2016). Similarly, elotuzumab has shown efficacy in its clinical trial as a combination therapy with lenalidomide-dexamethosone, but does not show any single-agent activity (Lonial *et al*, 2015; Rajkumar, 2016). These novel and combination therapies have been observed to provide a durable response and greater progression-free survival for MM patients, although there are no current studies performed that investigate how a patient tumour composition may change genetically with the administration of these new treatments.

Heterogeneity is thought to be characteristic of MM, and the administration of therapy acts as a potent source of artificial selection, which changes the dynamics of cancer clones (Fig 5). The therapeutic strategies employed to control tumour growth are genotoxic and result in massive death of aggressive tumour PC clones with "driver" mutations, but also provides a selective pressure for the proliferation of indolent tumour PC clones with "passenger" mutations that may resist treatment (Greaves & Maley, 2012; Brioli *et al*, 2014). Thus, therapy can initiate a selective bottleneck, facilitating the death or survival of different subclones based on fitness (Greaves & Maley, 2012; Brioli *et al*, 2014). Indolent clones surviving treatment may mutate further, acquiring "driver" mutations, thereby conferring improved fitness and malignant potential, which in turn, leads to disease relapse (Greaves & Maley, 2012; Brioli *et al*, 2014). Clonal tiding, the rise and fall in dominance of subclones as selective pressures change, has been associated with the occurrence of MM disease relapse (Melchor *et al*, 2014). As a result, distinct clones may dominate at different times during the disease course making MM disease control difficult (Greaves & Maley, 2012; Brioli *et al*, 2014).

In view of these new findings, the impact of therapy on clonal evolution and disease progression in MM should be considered at the outset of treatment. To date, a limited number of studies have investigated the changing clonal architecture in MM associated with treatment. Longitudinal WGS study of a single patient tumour through transformation stages – diagnosis, first relapse, second relapse and secondary PCL – has revealed substantial tumour heterogeneity, with clonal tiding in response to selective pressures from treatment, and resulting clonal evolution (Egan *et al*, 2012). These findings have been validated by WES analysis of a patient tumour at diagnosis and first relapse where 81 novel NS-SNVs were identified (33 shared at diagnosis and relapse, while 48 were new) following relapse after first line therapy (Weston-Bell *et al*, 2013). Genomic analysis of paired diagnosis-relapse samples ($n = 24$) using the Genome-Wide Human SNP array has identified patients exhibiting

branching, non linear evolution following therapy driven by the survival of a minor subclone that expanded at relapse (Magrangeas *et al*, 2013). Similarly, targeted genomic mutation panel sequencing of sequential pre- and post-therapy MM samples ($n = 25$) investigating the most commonly mutated genes in MM has revealed clonal evolution in the majority of patients, including clonal expansion, retraction and/or extinction (Kortum *et al*, 2015). To this end, the complete extinction of subclones (with mutations of *KRAS* and *TP53*) and emergence of new subclones (with mutations of *FAM46C, FAT1, SPEN* and *TP53*) was identified following therapy (Kortum *et al*, 2015). Conversely, however, WES on paired high-risk SMM-MM post-treatment samples has also identified that therapy is able to reduce the clonal complexity of disease (Walker *et al*, 2014). These observations suggest inherent disease complexity at relapse in response to changing selective pressures attributed to the different chemotherapeutic agents and illustrates the need for tumour clones to be monitored for regressing or reappearing subclones, which may contribute to disease aggressiveness following specific treatment regimes.

It has been suggested that combinatory treatment regimens should be utilised for a deeper response to reduce both bulk tumour and eliminate clonal and subclonal populations. It has also been suggested that continued therapy versus selective therapy at specific stages of progression for disease control may provide better treatment outcomes. Unfortunately, there is no evidence to support the notion that continuous therapy is more effective than repeated therapy following disease relapse (Kumar & Russell, 2014). Ultimately, improvements in the outcomes of future treatment will need to take into account the plasticity of MM PCs and altering dominance of genetically distinct subclones that occur as results of previous treatment (Zhou *et al*, 2009; Hajek *et al*, 2013).

## Summary and future directions

In recent years, rapid advances in genomic technologies, including the application of NGS and single cell analysis techniques, has led to a revolution in our understanding of MM biology and provides direct evidence that MM is a genetically complex disease. Studies also suggest that MM development can be accounted for by a number of tumour development models including the linear model, expansionist model and intraclonal heterogeneity model. In addition, these methods have shown that MM development is associated with significant recurrent probable "driver" mutations in *KRAS, NRAS, TP53, BRAF, FAM46C* and *DIS3* which are central to MM disease pathogenesis. Additionally, these new insights will impact current therapeutic strategies used to control MM disease. At this stage, research has mainly been performed on unpaired MGUS/SMM/MM samples, which limits our full understanding of the key "drivers" of MGUS to MM transition. Furthermore, few studies have examined the impact of treatment on intraclonal heterogeneity. With the progress of NGS technologies and the development of more cost-effective methods, thorough analysis of paired MGUS-MM samples and analysis of post-treatment samples should reveal the genetic and molecular mechanisms that play a central role in MM tumour development and disease progression. Ultimately, these insights will heavily influence the future therapeutic strategies used to control MM disease development and relapse.

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## Authorship

All authors were involved in discussion, writing, editing and reviewing the manuscript before submission.

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All authors declare no competing interests.

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