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## Clonal evolution in myeloma: the impact of maintenance lenalidomide and depth of response on the genetics and sub-clonal structure of relapsed disease in uniformly treated newly diagnosed patients

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### ABSTRACT

The emergence of treatment resistant sub-clones is a key feature of relapse in multiple myeloma. Therapeutic attempts to extend remission and prevent relapse include maximizing response and the use of maintenance therapy. We used whole exome sequencing to study the genetics of paired samples taken at presentation and at relapse from 56 newly diagnosed patients, following induction therapy, randomized to receive either lenalidomide maintenance or observation as part of the Myeloma XI trial. Patients included were considered high risk, relapsing within 30 months of maintenance randomization. Patients achieving a complete response had predominantly branching evolutionary patterns leading to relapse, characterized by a greater mutational burden, an altered mutational profile, bi-allelic inactivation of tumor suppressor genes, and acquired structural aberrations. Conversely, in patients achieving a partial response, the evolutionary features were predominantly stable with a similar mutational and structural profile seen at both time points. There were no significant differences between patients relapsing after lenalidomide maintenance *versus* observation. This study shows that the depth of response is a key determinant of the evolutionary patterns seen at relapse. This trial is registered at *clinicaltrials.gov* identifier: 01554852.

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### Introduction

Multiple myeloma (MM) results from the malignant transformation of a normal plasma cell and has a mutational load which lies in an intermediate position between genetically simple malignancies, such as chronic myelogenous leukemia, and the more complex solid cancers.<sup>1,2</sup> As such, MM provides an excellent model system through which to understand the mutational and evolutionary processes underlying disease relapse. MM is a genetically diverse condition, which is manifested by variations in clinical outcome even in uniformly treated populations.<sup>3,4</sup>

The treatment of newly diagnosed (ND) MM has improved over the last decade following the introduction of immunomodulatory drugs and proteasome inhibition, which, together with autologous stem cell transplantation, have increased the median overall survival from three to eight years.<sup>5-7</sup> However, patients frequently

relapse, and to improve outcomes further, strategies have been designed to eliminate the residual clonal cells that mediate relapse.<sup>8-10</sup> In this context, an important strategy has been the use of maintenance therapy with lenalidomide.<sup>11-13</sup> This agent is well tolerated and has a bifunctional mode of action directly killing the tumor cells and enhancing the immune response to the malignant plasma cells that is mediated by directing Aiolos and Ikaros to the proteasome for degradation.<sup>14-17</sup>

Understanding the features of residual cells that lead to relapse is an important challenge; however, this is technically difficult, especially in patients who have achieved stringent complete responses. One way of understanding the characteristics of cells in remission is to study the characteristics of cells at relapse. Previous studies of the genetic features of paired presentation and relapse samples have shown that, after intensive chemotherapy, bi-allelic loss of tumor suppressor genes, in particular *TP53*, and the deregulation of *MYC* by structural chromosomal changes are important features.<sup>18</sup> In addition to mutational change, subclonal structure also varies at relapse and three main patterns have been described; branching, linear and shifting

patterns of clonal dominance<sup>18-23</sup> (Table 1). Such analyses have not, however, studied uniformly treated patients for whom the depth of response or treatment information is available. To address the impact of maintenance and response depth on mutational patterns and clonal structure at relapse, we performed whole exome sequencing (WES) on paired presentation and relapse samples from 56 NDMM patients, 30 of whom had received lenalidomide maintenance and 26 who had not. All were treated at first presentation with a triple induction regimen containing an immunomodulatory agent, cyclophosphamide, and dexamethasone, making this the largest analysis of genetic evolution in a uniformly treated series of MM patients to date.

## Methods

Samples were selected from NDMM patients enrolled into the Myeloma XI trial (*clinicaltrials.gov identifier: 01554852*) for whom adequate DNA volumes were available.<sup>24</sup> The study was undertaken after written informed consent from patients and ethical approval was obtained from the Oxfordshire Research Ethics

**Table 1.** Previous studies assessing clonal evolution in myeloma.

Study	N. of patients	Disease status	Technique	Findings
Keats <i>et al.</i> Blood 2012 <sup>19</sup>	28	Presentation <i>vs.</i> Relapse	Array CGH	Three major evolutionary mechanisms identified: 1. Genetically stable 2. Linear evolution 3. Heterogeneous clonal mixtures and shifting predominant clones.
Magrangeas <i>et al.</i> Leukaemia 2013 <sup>20</sup>	24	Presentation <i>vs.</i> Relapse	SNP array	Branching evolution outlined as a cause for relapse in one-third of patients. The remaining patients displayed linear and stable pathways to relapse.
Bolli <i>et al.</i> Nat Commun 2014 <sup>21</sup>	67	Presentation <i>vs.</i> Relapse	NGS/SNP array and cytogenetics	Linear, branching, stable and differential clonal evolutionary responses described. Description of kataegis and somatic hypermutation.
Melchor <i>et al.</i> Leukaemia 2014 <sup>23</sup>	6	Presentation <i>vs.</i> Relapse	NGS and single cell analysis	Linear and branching evolution shown at a single cell level.
Kortum <i>et al.</i> Ann Hematol 2015 <sup>22</sup>	25	Presentation <i>vs.</i> Relapse	NGS gene panel	Use of a targeted sequencing panel to demonstrate gain and loss of significantly mutated genes confirming clonal evolution.
Weinhold <i>et al.</i> Blood 2016 <sup>18</sup>	33	Presentation <i>vs.</i> Relapse	NGS and GEP	Mutational load increases at relapse in association with enrichment of driver gene mutations and bi-allelic TSG events. Branching evolution as the main mechanism leading to relapse.

CGH: comparative genomic hybridization; SNP: single nucleotide polymorphism; NGS: next generation sequencing; GEP: gene expression profiling; TSG: tumor suppressor genes.



dependant probe amplification (MLPA) (SALSA MLPA P425-B1 multiple myeloma probemix, MRC Holland, Amsterdam, the Netherlands) and the bioinformatics assessment tool Sequenza (v.2.1.2).<sup>33,34</sup> Paired MLPA and Sequenza data was available for 90 of 112 (80%) tumor samples, with a consensus between MLPA and Sequenza being seen in 85 of 90 samples (94%). For the five patients for whom a mismatch was observed, Sequenza was used to call the copy number profile. Translocations were determined using MANTA (v.0.29.3).<sup>35</sup> For 46% (51 of 112) of patient samples, translocations involving the immunoglobulin heavy chain (IGH) were also assessed using multiplexed qRT-PCR.<sup>36</sup> A consensus between MANTA and qRT-PCR was observed in 84% (43 of 51). In the eight patients for whom a mismatch was seen, the integrative genomics viewer (IGV) was used to confirm or exclude the translocation. All suspected bi-allelic copy number abnormality (CNA) events were confirmed by manual interrogation of BAM files using IGV. Bi-allelic inactivation was also called in patients with evidence of a non-synonymous mutation with mono-allelic loss or a single mutation with a VAF of  $\geq 80\%$ . A summary of the methods, bioinformatics tools and filters used to complete this analysis is available in *Online Supplementary Figure S2*.

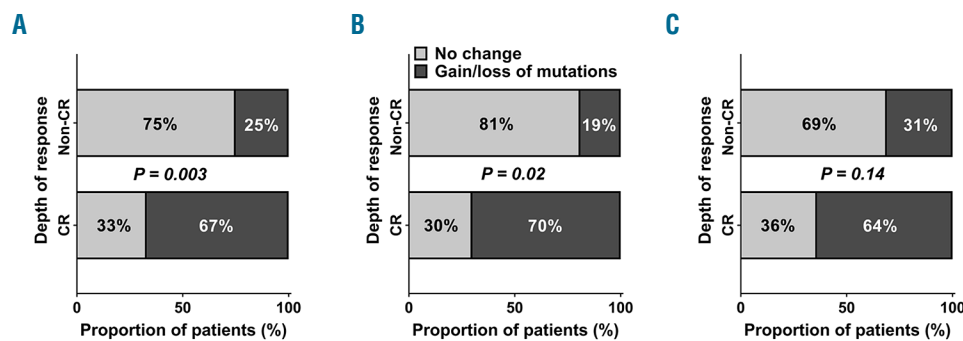
Statistical analysis was performed using GraphPad Prism v.7.01 and R v.3.2.1.  $P=0.05$  (two-sided) was considered statistically significant. Wilcoxon matched-pairs signed rank tests were conducted to determine significance between paired data sets, including the mutational load at presentation/relapse and PFS according to maintenance allocation, depth of response, and induction treatment. Fisher exact test was used to determine differences between

two nominal variables, including the change in mutational profile from presentation to relapse and the evolutionary mechanism leading to relapse.

## Results

### Relapse is characterized by a change in mutational spectrum

At presentation, genes mutated in >10% of patients were *NRAS* (23%, n=13), *KRAS* (23%, n=13), and *DIS3* (13%, n=7) (Figure 1A and B). At relapse, *TP53* was also seen in >10% of patients (11%, n=6). We examined genes that have previously been shown to be recurrently mutated in myeloma, including tumor suppressor genes, epigenetic modifiers, and genes within the RAS, NF $\kappa$ B and apoptosis pathways, the results of which are summarized (Figure C).<sup>18,28,37-44</sup> Non-synonymous mutations were seen in one or more of these genes in 79% (44 of 56) of patients at presentation and 80% (45 of 56) at relapse. Importantly, gain and/or loss of one or more of these lesions at relapse was seen in 37% of patients (21 of 56), with 21% (12 of 56) of patients gaining a new lesion, 11% (6 of 56) losing a lesion, and 5% (3 of 56) both gaining and losing lesions. The most common new mutations at relapse were *KRAS* and *PRDM1*, both seen in 5% of patients (3 of 56), and *NRAS* and *TP53*, each seen in 4% (2 of 56). However, mutations in *KRAS* and *NRAS* were just as likely to be lost



Maintenance	Response	Patients gaining a mutation (%)	Patients losing a mutation (%)	Patients gaining and losing a mutation (%)	Patients with no mutational change (%)	Number of patients with a change at relapse (%)	P value
Lenalidomide (n=30)	CR (n=14)	4 (29)	4 (29)	1 (7)	5 (36)	9/14 (64)	0.14
	Non-CR (n=16)	2 (13)	1 (6)	2 (13)	11 (69)	5/16 (31)	
Observation (n=26)	CR (n=10)	4 (40)	1 (10)	2 (20)	3 (30)	7/10 (70)	0.02
	Non-CR (n=16)	2 (13)	1 (6)	0	13 (81)	3/16 (19)	
Whole series (n=56)	CR (24)	8 (33)	5 (21)	3 (13)	8 (33)	16/24 (67)	0.003
	Non-CR (32)	4 (13)	2 (6)	2 (6)	24 (75)	8/32 (25)	

**Figure 2. The proportion of patients with a change in the profile of mutations known to be recurrent in myeloma or important in immunomodulatory mechanism of action at relapse.** (A) The proportion of all patients (n=56) with a change in the profile of recurrent mutations at relapse. The majority of complete remission (CR) series patients had a change in the mutational profile at relapse, 67% (16 of 24) versus 25% (8 of 32) of non-CR patients (Wilcoxon matched pairs,  $P=0.003$ ). (B) The proportion of observation patients (n=26) with a change in the profile of recurrent mutations at relapse. Only 19% (3 of 16) of non-CR patients under observation had a change in the profile of mutations at relapse, compared to 70% (7 of 10) of CR patients under observation ( $P=0.02$ ). (C) The proportion of lenalidomide maintenance patients (n=30) with a change in the profile of recurrent mutations at relapse. The same pattern of mutational profile change was seen in the lenalidomide maintenance patients, with 64% (9 of 14) of the CR patients having a mutational profile change at relapse compared to 31% of non-CR patients ( $P=0.14$ ).

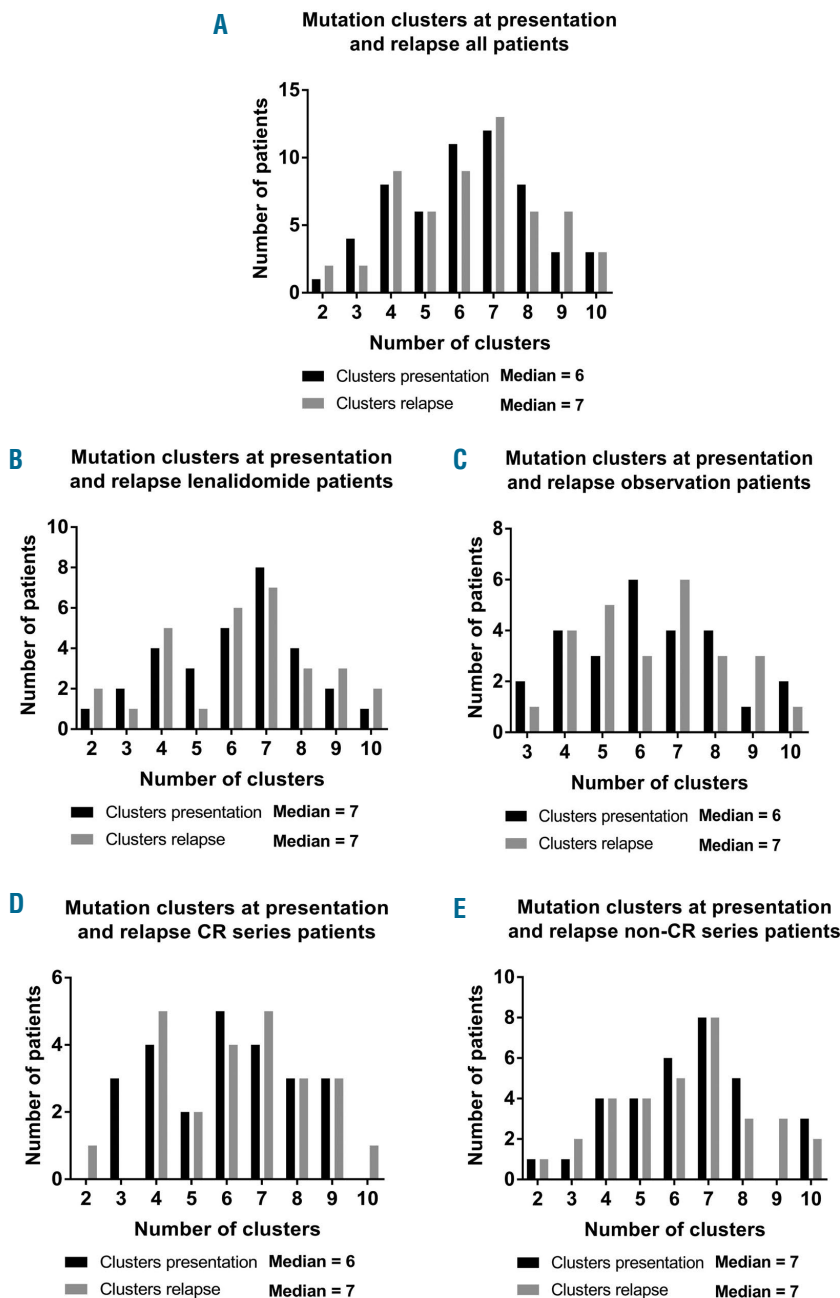


at relapse, with 5% (3 of 56) of patients losing *KRAS* mutations and 4% (2 of 56) losing *NRAS* (Figure 1B). The most commonly mutated pathway was the RAS-MAPK pathway, with mutations of one or more of *NRAS*, *KRAS*, *BRAF*, *NF1*, and *EGFR* being seen in 57% (33 of 56) of patients at some point during the disease course. Loss of one or more of these mutations was noted in 9% (5 of 56) of patients at relapse while new mutations were seen in 13% (7 of 56). The majority of these new mutations were clonal (CCF>80%) and all had a CCF >20% (range, 0.29-1) (*Online Supplementary Figure S3*). The results of these mutational studies are consistent with there being important changes in subclonal structure at relapse, rather than the simple accumulation of new mutations. The profile of mutations at presentation and relapse in patients who

received lenalidomide or thalidomide induction was well matched (*Online Supplementary Table S2*).

**Acquired structural change is a frequent feature of relapse**

We interrogated regions of recurrent CNA that are known to be associated with prognosis: 1p32.3 (*FAF1/CDKN2C*), 1p12 (*FAM46C*), 13 (RB1), 14q (*TRAF3*), and 17p (*TP53*). Copy number loss of  $\geq 1$  of these regions was seen in 63% (35 of 56) of patients at presentation and 59% (33 of 56) at relapse (*Online Supplementary Figure S4*). There was an evident change in the profile at relapse, with regions of loss seen at presentation returning to a diploid status in 9% (5 of 56) of patients while new losses were seen in 13% (7 of 56). Once again, these features are con-



**Figure 3. Number of mutational clusters at presentation and relapse.** (A) For all 56 patients, the number of mutational clusters was similar at presentation and relapse. The same pattern was seen irrespective of maintenance strategy (B and C) or depth of response (D and E). This suggests that a change in clonal number is not a major factor in disease progression.

sistent with an alteration in subclonal structure and a change in the nature of the dominant clone observed at relapse.

Gain(1q) was the most common new event at relapse, occurring in 13% (7 of 56) patients. Secondary translocations to chromosome 8q, the site of *MYC*, also occurred more frequently at relapse, increasing from 21% (12 of 56) to 27% (15 of 56) (*Online Supplementary Table S3*).<sup>45</sup> Consistent with translocations to *MYC* being late events, five patients had evidence of two separate *MYC* translocations at relapse. All patients with gain(1q) or t*MYC* at presentation had evidence of the lesion at relapse, illustrating the driver status.

Bi-allelic inactivation of tumor suppressor genes located at sites of recurrent CNA are likely to be relevant mediators of relapse. We show that bi-allelic inactivation events of *RB1*, *TRAF3*, and *TP53* are important with 18% (10 of 56) of patients having evidence of bi-allelic inactivation of  $\geq 1$  gene at relapse, in comparison to 14% (8 of 56) at presentation. One patient harboring both a *TP53* mutation and del(17p) at presentation lost evidence of the del(17p) at relapse, but maintained bi-allelic inactivation *via* expansion of a different subclone characterized by a *TP53* mutation which had a higher CCF at relapse (0.55 vs. 0.83).

### Relapse following a complete response is characterized by a greater mutational load and a change in genetic profile

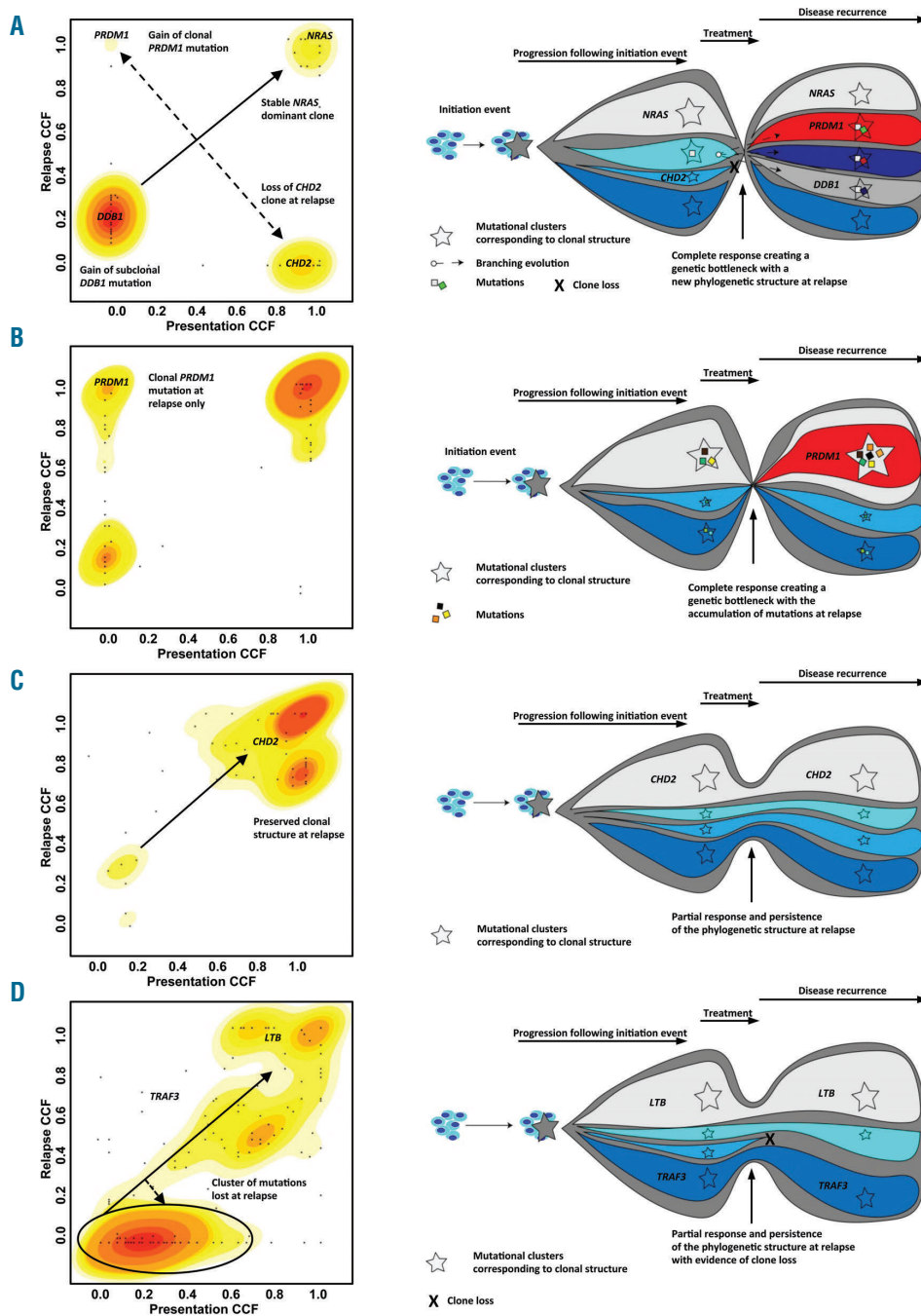
Patients achieving a CR had a significantly higher non-synonymous mutational load at relapse, with a median of 59 mutations compared to 40 at presentation ( $P < 0.001$ ). Non-CR patients had a similar mutational load at relapse, with a median of 39 mutations at both time points ( $P = 0.63$ ). Similar patterns were also seen in patients receiving or not receiving lenalidomide maintenance (*Online Supplementary Table S4*).

By comparing the profile of known recurrently mutated genes, we show that 67% (16 of 24) of CR cases had a change in mutational profile at relapse compared to only 25% (8 of 32) of non-CR cases ( $P = 0.003$ ) (Figure 2A); these findings were a feature of both the observation and lenalidomide maintenance series (Figure 2B and C). Consistent with the mutation profile changes described, gain and loss of the structural lesions del(1p), del(13), del(14), del(17p), gain (1q), and t*MYC* at relapse was more common in the CR series, with 42% (10 of 24) of patients having a change in the profile of these aberrations compared to 28% (9 of 32) of non-CR patients (*Online Supplementary Figure 5S*). The changes in mutational load

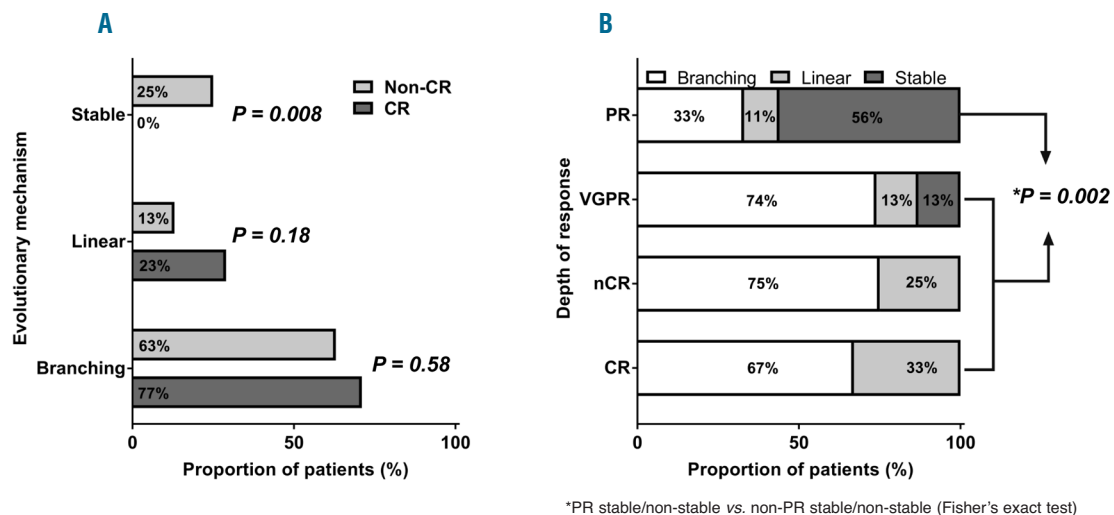
**Table 2. Series characteristics.**

	Whole series (% of all)	Maintenance randomization	
		Lenalidomide (% of group)	Observation (% of group)
N. of patients	56	30	26
Median age	68	67	69
Gender			
Male	28 (50)	13 (43)	15 (58)
Female	28 (50)	17 (57)	11 (42)
Pathway			
TE	22 (39)	11 (37)	11 (42)
TNE	34 (61)	19 (63)	15 (58)
Induction			
Thalidomide	29 (52)	15 (52)	14 (48)
Lenalidomide	27 (48)	15 (56)	12 (44)
Best response			
CR series (CR/nCR)	24 (43)	14 (47)	10 (38)
Non-CR series (VGPR/PR)	32 (57)	16 (53)	16 (62)
Median maintenance duration (months, range)	10 (1-27)	10 (1-27)	9 (1-22)
Median number of maintenance cycles (range)	n/a	7 (1-28)	n/a
Median PFS (months, range)	19 (8-51)	19 (8-51)	19 (8-34)
Presentation ISS			
I	13 (23)	5 (17)	8 (31)
II	21 (38)	12 (40)	9 (35)
III	21 (38)	12 (40)	9 (35)
Missing	1 (2)	1 (3)	
Cytogenetic risk*			
High risk	20 (36)	12 (40)	8 (31)
Ultra-high risk	7 (13)	5 (13)	2 (8)

\*High risk defined as one of t(4;14), t(14;16), t(14;20), gain(1q), and del(17p). Ultra-high risk defined as two lesions. TE: transplant eligible; TNE: transplant ineligible; CR: complete response; nCR: near CR; VGPR: very good partial response; PR: partial response; PFS: progression-free survival; ISS: International Staging System; n/a: not applicable.



**Figure 4. The evolutionary patterns seen leading to relapse.** (A) Branching: branching evolution was the predominant mechanism seen and was characterized both by the gain and loss of mutational clusters at relapse. The cancer clonal fractions (CCF) for all coding mutations using kernel density estimation for a typical patient (left) is shown and reveals the presence of a new dominant *PRDM1* (CCF 1.0) containing clone at relapse only (each dot represents a mutation). In addition, a clone containing *CHD2* (CCF 0.91 presentation only) is lost at relapse while a clone containing *NRAS* remained dominant at presentation (CCF 1.0) and relapse (CCF 0.99). (Right) Illustration of the branching evolutionary process using the same patient. Prior to treatment, there are a number of competing sub-clones, but as a result of effective therapy, clonal extinction occurs leading to a genetic bottleneck. This leads to the emergence of a new clonal structure at relapse; in this case the loss of a dominant *CHD2* clone, the gain of a *PRDM1* clone, and a stable *NRAS* clone. In addition, the emergence of a new *DDB1* mutation was seen within a minor clone with a CCF of 0.21. (B) Linear: linear evolution was seen in 20% of patients, characterized by the gain of mutations at relapse but no evidence of clonal loss. The KDE plot is displayed and shows the emergence of a new clonal *PRDM1* mutation at relapse with a CCF of 1.0. (Right) Over time, successive generations of daughter cells acquire aberrations making them genetically distinct; in this example, we see the emergence of a new *PRDM1* mutation. (C) Stable progression: KDE plot (left) showing a typical patient with stable progression, revealing a preserved clonal structure at both time points, with CCF values for all mutations remaining consistent at both time points. The *CHD2* mutation was present within a dominant clone at presentation and relapse with a CCF of 0.83 and 0.87, respectively. Stable evolution was a characteristic of patients achieving a non-complete remission (non-CR), and in particular a partial remission (PR). These patients appeared to have a treatment resistant disease status and therefore the emergence of the same clonal structure was seen at relapse as had been seen at disease onset; in this case, with a *CHD2* dominant clone at both time points (right). (D) Stable with loss was seen in one patient and kernel density estimation (right) revealed the presence of a predominantly preserved clonal structure at relapse with clusters containing *TRAF3* and *LTB* present with similar CCF values at both points. There was evidence of the loss of a cluster of mutations at relapse, suggestive of clone loss (circled). The evolutionary process is shown. Treatment sensitive clone(s) are eliminated but the resistant clone(s) remain and lead to the relapse disease state.



**Figure 5. The evolutionary patterns seen leading to relapse according to the depth of treatment response.** (A) The evolutionary mechanism leading to relapse for the complete remission (CR) and non-CR series. Stable evolution was only seen in the non-CR patients (Fisher's exact test,  $P=0.008$ ). Branching evolution was the predominant mechanism leading to relapse in both CR and non-CR patients, with linear evolution also occurring, but in a smaller proportion of patients. (B) The evolutionary mechanism leading to response according to the depth of response. Over half (56%) of the patients who achieved a partial response (PR) as their best response prior to relapse progressed via a stable mechanism ( $P=0.002$ ). A smaller proportion of very good partial response (VGPR) patients displayed stable progression (13%). Branching evolution was dominant, and was seen in 75% of nCR patients and 67% of CR patients.

could not be linked to a change in the number of clones predicted by SciClone clustering, where a median number of seven clones was seen at relapse in both the CR and non-CR series (Figure 3). The same findings were noted for all 56 patients and when analyzed according to maintenance strategy. No patient had evidence of the loss or gain of  $>2$  mutational clusters at relapse, further suggesting that a change in the number of clones is not a major cause for change in the mutational load (*Online Supplementary Table S5*).

#### Lenalidomide maintenance has no impact on the mutational profile at relapse

There was no specific mutational, copy number or structural feature which characterized patients who received lenalidomide maintenance compared to observation patients. Of patients who received lenalidomide maintenance, 83% (25 of 30) had a mutation in one or more of the recurrently mutated myeloma genes at some point during the disease course compared to 85% (22 of 26) of observation patients. Gain and/or loss of one or more mutation at relapse, including those in genes implicated in the mechanism of action of an immunomodulatory agent, were seen in 43% (13 of 30) of patients receiving lenalidomide maintenance and 35% (9 of 26) of the observation patients (Figure 1B).

We did not identify a mutational signal consistent with the selection of clones carrying mutations associated with acquired resistance to lenalidomide. Five patients had mutations in *DDB1* ( $n=2$ ), *SLC16A1* ( $n=2$ ), and *CRBN* ( $n=1$ ), but these were not confined to the cases of lenalidomide maintenance, nor were they seen exclusively at relapse (Figure 1B). Mutations in other genes linked to the mechanism of action of lenalidomide, including regulator of cullins 1, cullin-4A, Ikaros, Aiolos, and Basigin, were not found at presentation or relapse in any patient.<sup>14,46-48</sup> Mutations in *MYC* and *IRF4*, transcription factors known

to be down-regulated in response to immunomodulation, were seen in 2% ( $n=1$ ) and 4% ( $n=2$ ) of patients, respectively. However, consistent with an acquired mutation having a possible role in drug resistance, we identified one patient who had been exposed to eight months of lenalidomide maintenance and achieved flow cytometric minimal residual disease (MRD) negativity (minimum  $5 \times 10^5$  cells interrogated) and who then went on to develop a novel *CRBN* mutation (p.Cys326Gly) at relapse; this is consistent with the emergence of resistance due to mutation at the immunomodulatory molecule binding site. The mutation was also present in a dominant clone, with a CCF of 0.71, further suggesting its presence may have impacted on the fitness of the tumor in relation to pressure from the immunomodulatory treatment (*Online Supplementary Figure S6*).

#### Branching evolution is the predominant process leading to relapse, particularly in patients achieving a deep treatment response

We observed three main evolutionary patterns at relapse: branching, linear, and stable. Branching evolution was characterized by both gain and loss of mutational clusters and was the predominant pattern, seen in 66% (37 of 56) of all cases (Figure 4A). Linear evolution characterized only by the gain of mutations at relapse was seen in 20% (11 of 56) of cases (Figure 4B). The remaining 14% (8 of 56) had either the same mutational profile at both time points, and were classified as stable progression ( $n=7$ ), or displayed a loss of a mutational cluster at relapse, classified as stable progression with clone loss ( $n=1$ ) (Figure 4C and D). There was no significant impact of induction or maintenance lenalidomide on the evolutionary pattern seen at relapse (*Online Supplementary Figure S7*).

We show that the depth of treatment response is the most important determinant of the evolutionary pattern seen at relapse. Although branching evolution was domi-



nant in both the CR and non-CR series, stable progression was only seen in the non-CR series, (25%, 8 of 32, vs. 0% of 25;  $P=0.008$ ) (Figure 5A). Breaking down the non-CR series further showed that stable progression was predominantly associated with a PR (56%, 5 of 9 of PR; 13%, 3 of 23 of VGPR patients). No CR or nCR patients (0 of 24) had evidence of stable progression, with all patients showing branching or linear evolution ( $P=0.002$ ) (Figure 5B). Consistent with a deep response being synonymous with a change in clonal architecture, 86% (6 of 7) of patients who achieved an MRD-negative state relapsed *via* a branching mechanism and 14% (1 of 7) *via* linear evolution. The type of evolution leading to relapse had no impact on the time to relapse or overall survival (*Online Supplementary Table S6*).

## Discussion

This study shows that an increase in mutational load, altered mutational profile, accumulation of deleterious structural lesions [particularly *tMYC* and gain(1q)], and a change in copy number profile are key molecular features of relapse. The depth of response to treatment has a significant impact on both the genetic landscape and the evolutionary patterns seen at relapse, with an increased mutational load being predominantly associated with the achievement of a CR. We also note that relapse from CR is associated with an altered mutational profile, with 63% (15 of 24) of the CR patients having evidence of loss or gain of known recurrently mutated genes in comparison to only 25% (8 of 32) of non-CR patients ( $P=0.006$ ). This pattern was also seen for CNA and structural variants, with del(17p), *tMYC*, gain(1q), and loss of tumor suppressor gene regions, including *CDKN2C*, *FAF1*, *FAM46C*, *RB1*, and *TRAF3*, being seen more frequently in the patients relapsing after CR.

It has been shown that the progression through the multistep transformation of MM is associated with an increased mutational load. This is well illustrated by studies that have compared monoclonal gammopathy of undetermined significance, MM, and plasma cell leukemia. Our results are consistent with this observation, and we clearly show that an increased mutational load is an important factor in early disease progression following a good response to treatment.<sup>49,50</sup> Similar findings are observed in other cancer types where a greater mutational load is associated with a more aggressive disease status, for example, lung cancer in smokers compared to non-smokers, and in malignant melanoma.<sup>51,52</sup>

A variety of subclonal patterns, including branching, linear, and stable patterns, are seen at relapse (Figure 4A-D). The pattern of clonal change is most consistent with the hypothesis that branching evolutionary pathways are the predominant mechanism underlying relapse, especially if spatial variation is taken into account. Treatment can be seen as causing an evolutionary bottleneck, particularly in patients who achieve a deep treatment response, providing a selective pressure for the emergence of pre-existent resistant clones. Importantly, these branching patterns and increased genetic damage in the clonal cells are hallmarks of effective treatment and achievement of deep responses. In contrast, the stable patterns seen in non-CR patients are most consistent with microenvironmental change, possibly as a consequence of the presence of treatment resistant

dominant clones at disease onset. This stable pattern is reminiscent of the results seen in the progression of smoldering MM to MM, where the emergence of new mutations is infrequent, yet there is a profound change in clinical behavior.

Mechanistically, relapse in the setting of effective therapy that results in the achievement of a CR is occurring either due to the emergence of low-level sub-clones that are undetectable at presentation, but which are selected for by treatment, or as a result of the acquisition of new mutations, albeit less likely given the short duration of remission. It may also be that the differences seen are the result of sampling bias, as previously shown by our group, although this is less likely given that all biopsies were obtained from the pelvis.<sup>53</sup> The pattern of results seen with eradication of dominant clones and emergence of low-level clones with different pattern of mutation, and its association with CR, supports the concept that treatment can result in subclonal eradication. These findings highlight the therapeutic importance of achieving a CR to eradicate dominant clones present at the initiation of therapy. Supporting this as a therapeutic aim, we have recently shown that high-risk patients achieving a molecular CR have very significantly improved 5-year survival rates.<sup>54</sup>

The results show that, even in MRD-negative CR, low-level resistant subclones remain, and therapeutic strategies need to be designed to address them. We believe this work supports the current best practice strategy of using different treatment regimens at successive relapses and shows why this approach is successful. We show that early relapse is either due to innate treatment resistance, requiring no change in the clonal structure, or due to a change of clonal architecture in response to effective therapy. Both mechanisms are consistent with a disease state at relapse that is resistant to the initial therapies used. This knowledge supports the use of combination regimens at relapse, incorporating agents with differing mechanisms of action to those used at presentation. This will be analyzed in a planned comparator series of patients who achieve long-term remission in the trial. Our work here does, however, provide new insights into the mechanisms of early relapsing disease, revealing a different mechanism in those who achieve a deep therapeutic response and those who do not.

Interestingly, a recent study looking at non-small cell lung cancer has shown that mutational load may direct treatment choices, whereby the use of immunotherapy was associated with a longer and durable remission in patients with a greater mutational load at presentation.<sup>55</sup> Therefore, as our understanding of myeloma biology increases, information such as mutational load may guide specific treatment modalities in the future. The use of lenalidomide maintenance is one of the first clinical strategies to address the residual cells that remain following initial treatment. Using this approach, we did not see a signal of any adverse impact on the clonal cells as a result of this therapy. In particular, we did not see a signal for selection of mutations that could confer resistance to lenalidomide either at presentation or at relapse. Previous studies have suggested that such mutations may exist and could be relatively frequent in cell lines and heavily pre-treated patients, but in this study of newly diagnosed patients they were rare.<sup>56</sup> However, we do describe a patient who received eight months of maintenance treatment, achieving an MRD-negative CR who did relapse with a new

*CRBN* mutation. It is likely that the mutation occurred by chance, but that, in true Darwinian fashion, in the presence of lenalidomide, it conferred a survival advantage to the malignant cells harboring it. Recent analyses indicating that prolonged exposure to lenalidomide is not linked to an increased incidence of second primary hematologic malignancies when used in combinations excluding oral melphalan may be supported by a lack of evidence of mutagenesis leading to treatment resistance in this work, although it is acknowledged that larger analyses using patients with prolonged remissions are required.<sup>24,57</sup> It is important to note that the patients studied here relapsed early and that they were selected because of this characteristic, as it had previously been suggested that exposure to lenalidomide could enhance progression of such cases post maintenance. However, we did not find any evidence to support such a hypothesis.

It does remain important to evaluate the impact of maintenance on low-risk cases who are long-term survivors, a question not addressed in this study. In addition, we acknowledge that clonal structure may be further assessed using single cell analysis. However, for the moment, the challenges of obtaining individual malignant plasma cells from large series of patients at multiple disease time points has proved to be a significant barrier. In this series of patients, however, we do see evidence of parallel evolution, as previously described using single cell technology, further validating the clonal changes described here.<sup>25</sup> An example is seen in a patient with bi-

allelic inactivation of *TP53* at presentation characterized by *del(17p)* and *TP53* mutation, but only the presence of a *TP53* mutation at relapse. In this case the clone with *del(17p)* was no longer detectable at relapse, suggesting it was treatment sensitive and/or out-competed by a more aggressive treatment insensitive clone harboring a *TP53* mutation that had expanded following treatment, confirmed by a higher CCF at relapse (0.55 vs. 0.83).

In this group of high-risk early-relapsing patients, we show that relapse is a result of the dynamic interplay of evolutionary processes based on the capacity of clonal cells to adapt to their bone marrow microenvironment as a result of new mutations, copy number change, and the selective pressure of the treatment used. Specifically, the depth of response is a critical feature that impacts the evolutionary patterns seen at relapse, rather than the use of lenalidomide maintenance.

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