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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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Running Title: Maintenance, response status, and subclonal structure at MM relapse.

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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 the maximisation of response and use of maintenance therapy. We used whole exome sequencing to study the genetics of paired presentation and relapse samples from 56 newly diagnosed patients, following induction therapy, randomised 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 randomisation. Patients achieving a complete response had predominantly branching evolutionary patterns leading to relapse, characterised by a greater mutational burden, an altered mutational profile, bi-allelic inactivation of tumour 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 maintenance lenalidomide versus observation. This study shows that the depth of response is a key determinant of the evolutionary patterns seen at relapse.

Clinical trial: NCT01554852

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 (1, 2). As such, MM provides an excellent model system with 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 (3, 4).

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 3 to 8 years (5-7). However, relapse remains frequent, and to improve outcomes further strategies have been designed to eliminate the residual clonal cells that mediate relapse (8-10). In this context an important strategy has been the use of maintenance therapy with lenalidomide (11-13). This agent is well tolerated and has a bifunctional mode of action directly killing the tumour cells and enhancing the immune response to the malignant plasma cells that is mediated by directing Aiolos and Ikaros to the proteasome for degradation (14-17).

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 (18). In addition to mutational change, subclonal structure also varies at relapse with three main patterns having been described; branching, linear and shifting patterns of clonal dominance (18-23), **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 maintenance lenalidomide and 26 who did 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 (NCT01554852) for whom adequate DNA volumes were available (24). The study was undertaken with written informed consent from patients and ethical approval was obtained from the Oxfordshire Research Ethics Committee (MREC 17/09/09, ISRCTN49407852). A nested case control analysis was performed on 56 patients who received either lenalidomide maintenance (n=30) or underwent observation (n=26) and subsequently relapsed (25, 26), **Supplementary Figure 1**. All patients included had phenotypical high risk disease, defined as relapse within 30 months of maintenance randomisation, irrespective of classical genetic risk status and best response. The median time from trial entry to relapse was 19 months, notably shorter than the Myeloma XI trial reported PFS of 35.9 and 32.9 months for patients treated with lenalidomide and thalidomide induction, respectively (27). Response was determined using International Myeloma Working Group (IMWG) criteria and a near CR (nCR), defined as no detectable paraprotein, a normal light chain ratio but where immunofixation and or bone marrow sampling was not performed or if immunofixation was positive. Prior to relapse, 21% (12/56) achieved a complete response (CR), 21% (12/56) a nCR, 42% (23/56) a very good partial response (VGPR), and 16% (9/56), a partial response (PR). To determine the impact of response on the genetic landscape at relapse we grouped patients according to the best response achieved prior to relapse, complete responders (CR/nCR) and non-complete responders (VGPR/PR). Clinical characteristics were well matched between the maintenance groups, **Table 2**. The characteristics of patients according to induction regimen were also well matched, **Supplementary Table 1**.

DNA was isolated from plasma cells following selection using CD138+ MACSorting (Miltenyi Biotech, Bisley, United Kingdom) from bone marrow aspirate samples. Control DNA was obtained from peripheral blood samples. Libraries for WES were prepared using the SureSelectQXT sample prep kit and the SureSelect Clinical Research Exome kit (Agilent), with additional baits covering the immunoglobulin and *MYC* loci, as previously described (28). Paired-end sequencing was performed to a median sequencing depth of

122x for tumour samples and 58x for controls using a HiSeq2500 (Illumina). Single nucleotide variants, including those of tumour suppressor genes and oncogenes were determined using Strelka (v1.0.14) and MuTect (29, 30). The distribution of mutant alleles determined by the variant allele frequency (VAF) was mapped using the R package SciClone (31). Cancer clonal fractions (CCF) were calculated for all mutations and plotted using Kernal density estimation to infer clonal structure at presentation and relapse (32).

Copy number was assessed using both multiplexed ligation-dependant probe amplification (MLPA) (SALSA MLPA P425-B1 multiple myeloma probemix, MRC Holland, Amsterdam, The Netherlands) and the bioinformatics assessment tool Sequenza (version 2.1.2) (33, 34). Paired MLPA and Sequenza data was available for 90/112 (80%) tumour samples, with a consensus between MLPA and Sequenza being seen in 85/90 samples (94%). For the five patients where a mismatch was observed Sequenza was used to call the copy number profile. Translocations were determined using MANTA (version 0.29.3) (35). For 46% (51/112) of patient samples, translocations involving the immunoglobulin heavy chain (IGH) were also assessed using multiplexed qRT-PCR (36). A consensus between MANTA and qRT-PCR was observed in 84% (43/51). In the 8 patients where 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 ≥80%. A summary of the methods, bioinformatics tools and filters used to complete this analysis are covered in **Supplementary Figure 2**.

Statistical analysis was performed using GraphPad Prism version 7.01 and R version 3.2.1. A P-value of 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's exact test was

used to determine differences between two nominal variables, including the change in mutational profile at presentation/relapse and evolutionary mechanism leading to relapse.

Results

Relapse is characterised 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**. 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 tumour suppressor genes, epigenetic modifiers, and genes within the RAS, NFκB and apoptosis pathways, the results of which are summarized, **Figure 1B** (18, 28, 37-44). Non-synonymous mutations were seen in one or more of these genes in 79% (44/56) of patients at presentation and 80% (45/56) at relapse. Importantly, gain and/or loss of one or more of these lesions at relapse was seen in 37% of patients (21/56), with 21% (12/56) of patients gaining a new lesion, 11% (6/56) losing a lesion, and 5% (3/56) both gaining and losing lesions. The most common new mutations at relapse were *KRAS* and *PRDM1* both seen in 5% of patients (3/56) and *NRAS* and *TP53* each seen in 4% (2/56). However, mutations in *KRAS* and *NRAS* were just as likely to be lost at relapse with 5% (3/56) of patients losing *KRAS* mutations and 4% (2/56) losing *NRAS,* **Figure 1C**. 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% (33/56) of patients at some point during the disease course. Loss of one or more of these mutations was noted in 9% (5/56) of patients at relapse whilst new mutations were seen in 13% (7/56). The majority of these new mutations were clonal (CCF>80%) and all had a CCF of >20% (range 0.29 – 1), **Supplementary Figure 3**. 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, **Supplementary table 2**.

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 ≥1 of these regions was seen in 63% (35/56) of patients at presentation and 59% (33/56) at relapse, **Supplementary Figure 4**. A change in the profile at relapse was evident, with regions of loss seen at presentation returning to a diploid status seen in 9% (5/56) of patients and new losses being seen in 13% (7/56). These features are again consistent with an alteration in subclonal structure and a change in the nature of the dominant clone being seen at relapse.

Gain(1q) was the most common new event at relapse, occurring in 13% (7/56) patients. Secondary translocations to chromosome 8q, the site of *MYC,* also occurred more frequently at relapse, increasing from 21% (12/56) to 27% (15/56), **Supplementary Table 3** (45). 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 tumour 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% (10/56) of patients having evidence of bi-allelic inactivation of ≥1 gene at relapse, in comparison to 14% (8/56) at presentation. One patient lost evidence of bi-allelic inactivation of *TP53* at relapse due to expansion of a different subclone also characterized by a *TP53* mutation which had a higher CCF at relapse (0.55 vs 0.83).

Relapse following a complete response is characterised 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 lenalidomide maintenance or not, **Supplementary Table 4**.

By comparing the profile of known recurrently mutated genes we show that 67% (16/24) of CR cases had a change in mutational profile at relapse compared to only 25% (8/32) 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% (10/24) of patients having a change in the profile of these aberrations compared to 28% (9/32) of non-CR patients, **Supplementary Figure 5.** The changes in mutational load could not be linked to a change in the number of clones predicted by SciClone clustering, where a median number of 7 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 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 mutational load change, **Supplementary Table 5**.

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 those being observed. Of patients who received lenalidomide maintenance, 83% (25/30) had a mutation in one or more of the recurrently mutated myeloma genes at some point during the disease course compared to 85% (22/26) of patients being observed. Gain and/or loss of one or more mutation at relapse, including those in genes implicated in immunomodulatory agent mechanism of action were seen in 43% (13/30) of lenalidomide maintenance patients and 35% (9/26) of the observation patients (figure 1B).

We did not identify a mutational signal consistent with the selection of clones carrying mutations associated with the 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 lenalidomide maintenance cases nor were they seen exclusively at relapse (figure 1B). Mutations in other genes linked to lenalidomide mechanism of action including

regulator of cullins 1, cullin-4A, *Ikaros*, *Aiolos,* and Basigin were not found at presentation or relapse in any patient (14, 46-48). Mutations in *MYC* and *IRF4*, transcription factors known to be downregulated 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 a patient, exposed to 8 months of lenalidomide maintenance who achieved flow cytometric MRD negativity (minimum 5 x 10^5 cells interrogated) that went on to develop a novel *CRBN* mutation (p.Cys326Gly) at relapse 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 tumour in relation to immunomodulatory treatment pressure, **Supplementary Figure 6**.

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 characterised by both gain and loss of mutational clusters and was the predominant pattern, seen in 66% (37/56) of all cases, **Figure 4A**. Linear evolution, characterised only by the gain of mutations at relapse was seen in 20% (11/56) of cases, **Figure 4B**. The remaining 14% (8/56) 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), **Figures 4C** and **4D.** There was no significant impact of induction or maintenance lenalidomide on the evolutionary pattern seen at relapse, **Supplementary Figure 7**.

We show that the depth of treatment response is the most important determinant of the evolutionary pattern seen at relapse. Although branching evolution was dominant in both the CR and non-CR series, stable progression was only seen in the non-CR series, (25% n=8/32 vs 0%, 0/25, p=0.008), **Figure 5A**. Breaking down the non-CR series further showed that stable progression was predominantly associated with a PR (56% (5/9) of PR and 13% (3/23) of VGPR patients). No CR or nCR patients (0/24) 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% (6/7) of patients who achieved an MRD-ve state relapsed via a branching mechanism and 14% (1/7) via linear evolution. The type of evolution leading to relapse had no impact on the time to relapse or overall survival, **Supplementary Table 6**.

Discussion

This study shows that an increase in mutational load, altered mutational profile, accumulation of deleterious structural lesions (particularly t*MYC* 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% (15/24) of the CR patients having evidence of loss or gain of known recurrently mutated genes in comparison to only 25% (8/32) of non-CR patients (p=0.006). This pattern was also seen for CNA and structural variants with del(17p), t*MYC,* gain(1q), and loss of tumour suppressor gene regions including *CDKN2C, FAF1, FAM46C, RB1,* and *TRAF3* being seen more frequently in the patients relapsing from 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 MGUS, MM and plasma cell leukaemia. 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 (49, 50). Similar findings are observed in other cancer types where a greater mutational load is associated with a more aggressive disease state, for example lung cancer in smokers compared to non-smokers and in malignant melanoma (51, 52).

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 is the hallmark of effective treatment and the 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 innately treatment resistance dominant clones at disease onset, rather than the selection of “resistance mutations” as being the predominant mechanism for relapse. This stable pattern is reminiscent of the results seen in the progression of SMM to MM, where the emergence of new mutations is infrequent, yet there is a profound change in clinical behaviour.

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 undetectable at presentation, but which are selected for by the 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 sample bias resulted in the differences seen, as previously shown by our group, although this is less likely given all biopsies were obtained from the pelvis (53). 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 (54).

The results show that even in CR and MRD-ve states, there are low level resistant subclones and that strategies need to be designed to address these therapeutically. We believe this work supports the current best practice strategy of using different treatment regimens at successive relapses and 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. A

comparator series looking at patients who achieved long-term remission is planned and as the trial matures this analysis will be undertaken. This work does however provide new insights into the mechanisms of relapse in early disease, revealing a different mechanism in those who achievement of a deep therapeutic response and those who do not.

It is of interest that a recent study looking at non-small cell lung cancer has shown that mutational load my 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 (55). It may therefore be that 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 the first clinical strategy 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 the 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 (56). However, we do describe a patient who received 8 months of maintenance treatment, achieving an MRD-ve CR who did relapse with a new CRBN mutation. It is likely the mutation occurred by chance, but in true Darwinian fashion, in the presence of lenalidomide, it conferred a survival advantage to the malignant cells harbouring it. Recent analyses indicating that prolonged exposure to lenalidomide is not linked to an increased incidence of second primary haematological 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 (24, 57). It is important to note that the patients studied here relapsed early and were selected for this behavior as it has previously been suggested that exposure to lenalidomide could enhance progression of such cases post-maintenance. 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, but at present the challenges of obtaining individual malignant plasma cells from large series of patients at multiple disease time points has been 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 (23). An example is seen in a patient with bi-allelic inactivation of *TP53* at presentation but only the presence of a *TP53* mutation at relapse. In this case the clone with bi-allelic inactivation was no longer detectable at relapse, suggesting it was treatment sensitive and/or out-completed by a more aggressive treatment insensitive clone harbouring 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 around the adaptation of clonal cells capacity 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 but this is not impacted by the use of maintenance lenalidomide.

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Author contributions:
Conception and design JJ, GJM and MG; Development of methodology JJ, NW, CA, BW, LM and CW; Acquisition of data JJ, NW, CA, LM, PB and SE; Analysis and interpretation of data JJ, NW, GJM, MG, CA, CP and CW; Writing, review and/or revision of the manuscript All authors.

Conflict of interest and disclosure:
No potential conflicts of interest were disclosed.

Supplementary information is available at Leukemia's website.

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Tables

Table 1. Previous studies assessing clonal evolution in myeloma.

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

Abbreviations: CGH – comparative genomic hybridization, SNP – single nucleotide polymorphism, NGS – next generation sequencing, GEP – gene expression profiling.

Table 2. Series characteristics.

	Whole series (% of all)	Maintenance randomisation	
		Lenalidomide (% of group)	Observation (% of group)
Patient numbers	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.

Figure legends

Figure 1. The mutational profile at presentation and relapse.

A) Recurrent mutations in myeloma and mutations within the genes associated with immunomodulatory agent action. The number of patients with these mutations at presentation, relapse and at both time points is shown (dotted line denotes 10% level).

B) Mutational profile for each patient at presentation and relapse. Maintenance strategy and best response prior to relapse is shown. The gain and loss of mutated genes typical of MM was a dominant feature. **C)** Summary of mutations gained and lost at relapse**.** New mutations at relapse were seen in *PRDM1*, *TP53*, *NF1*, *TET2*, *EGFR*, *MYC*, *DDB1*, *CRBN,* and *FAF 1* (red bars). Loss of mutations in *FANCA*, *DIS3*, *FAM46C*, *BRAF* and *CDH2* were noted at relapse (blue bars). Mutations in *NRAS, KRAS,* and *SLC16A1* were gained and lost at relapse.

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 CR series patients had a change in the mutational profile at relapse, 67% (16/24) vs 25% (8/32) of non-CR patients (p=0.003, Wilcoxon matched pairs). **B)** The proportion of observation patients (n=26) with a change in the profile of recurrent mutations at relapse. Only 19% (3/16) of non-CR observation patients had a change in the profile of mutations at relapse, compared to 70% (7/10) of CR observation patients (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% (9/14) of the CR patients having a mutational profile change at relapse compared to 31% of non-CR patients (p=0.14).

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)** and depth of response **(D and E)**. This suggests a change in clonal number is not a major factor in disease progression.

Figure 4. The evolutionary patterns seen leading to relapse.

A) Branching: Branching evolution was the predominant mechanism seen and was characterised both by the gain and loss of mutational clusters at relapse. The CCF for all coding mutations using kernel density estimation for a typical patient (left image) 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 *CHD2* (CCF 0.91 presentation only) containing clone is lost at relapse whilst an *NRAS* containing clone remained dominant at presentation (CCF 1.0) and relapse (CCF 0.99). The right sided image is an 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, characterised 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. The right sided illustration shows that over time, successive generation of daughter cells acquire aberrations making them genetically distinct, in this example the emergence of a new *PRDM1* mutation.

C) Stable progression: The left KDE plot shows 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-CR, particularly a PR. These patients appeared to have a treatment resistant disease state and therefore the emergence of the same clonal structure was seen at relapse as seen at disease onset. This is illustrated by the right side schematic, in this case with a *CHD2* dominant clone at both time points.

D) Stable with loss was seen in one patient and kernel density estimation 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 schematic illustrates the evolutionary process. 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 the depth of treatment response.

A) The evolutionary mechanism leading to relapse for the CR and non-CR series. Stable evolution was only seen in the non-CR patients (Fishers 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 the depth of response. Over half (56%) of the patients who achieved a PR as their best response prior to relapse progressed via a stable mechanism (p=0.002). A smaller proportion of VGPR patients displayed stable progression (13%). Branching evolution was dominant, seen in 75% of nCR patients and 67% of CR patients.

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Supplementary Table 1. Characteristics according to induction treatment

	Induction	
	Lenalidomide (% of group)	**Thalidomide (% of group)**
Patient numbers	27 (52)	29 (48)
Best response		
CR series (CR/nCR)	12 (44)	12 (41)
Non-CR series (VGPR/PR)	15 (56)	17 (59)
Median PFS (months, range)	19 (8-51)	19 (8-34)
Presentation ISS		
I	6 (22)	7 (24)
II	11 (41)	10 (34)
III	10 (37)	11 (38)
Missing		1 (3)
Evolutionary mechanism		
Branching	20 (74)	17 (59)
Linear	3 (11)	8 (28)
Stable	4 (15)	4 (14)
Non-synonymous mutational load		
Presentation	37	40
Relapse	41	58
All coding mutations		
Presentation	70	81
Relapse	86	102

Supplementary Table 2. The profile of mutations known to be important in myeloma at presentation and relapse according to induction therapy. Mutations in *KRAS*, *NRAS*, *DIS3*, *FAM46C*, *TET2*, *TRAF3* and *TP53* were seen in >5% of patients at either time point in both series. Mutations were gained and lost at relapse in both the lenalidomide and thalidomide series.

Lenalidomide induction		
Gene	Presentation	Relapse
KRAS	**26 (7)**	**30 (8)**
NRAS	**30 (8)**	**22 (6)**
DIS3	**22 (6)**	**19 (5)**
RB1	15 (4)	15 (4)
ATM	7 (2)	7 (2)
FAM46C	**7 (2)**	**7 (2)**
TET2	**7 (2)**	**7 (2)**
TRAF3	**7 (2)**	**7 (2)**
EGFR	4 (1)	7 (2)
TP53	**4 (1)**	**7 (2)**
DDB1	4 (1)	7 (2)
BRAF	7 (2)	4 (1)
NF1	4 (1)	4 (1)
LTB	4 (1)	4 (1)
ATR	4 (1)	4 (1)
HIST1H1E	4 (1)	4 (1)
SETD2	4 (1)	4 (1)
SLC16A1	4 (1)	4 (1)
EGR1	4 (1)	4 (1)
FGFR3	4 (1)	4 (1)
IRF4	4 (1)	4 (1)
PRDM1	0	4 (1)
CHD2	4 (1)	0
FANCA	4 (1)	0
CRBN	0	0
FAF1	0	0
MYC	0	0

Thalidomide induction		
Gene	Presentation	Relapse
NRAS	**21 (6)**	**24 (7)**
KRAS	**17 (5)**	**17 (5)**
TP53	**10 (3)**	**14 (4)**
TRAF3	**10 (3)**	**10 (3)**
DIS3	**7 (2)**	**7 (2)**
NF1	3 (1)	7 (2)
TET2	**3 (1)**	**7 (2)**
FAM46C	**7 (2)**	**3 (1)**
PRDM1	0	7 (2)
ATM	3 (1)	3 (1)
ATR	3 (1)	3 (1)
HIST1H1E	3 (1)	3 (1)
CHD2	3 (1)	3 (1)
RB1	3 (1)	3 (1)
EGR1	3 (1)	3 (1)
FGFR3	3 (1)	3 (1)
IRF4	3 (1)	3 (1)
CRBN	0	3 (1)
MYC	0	3 (1)
FAF1	0	3 (1)
FANCA	3 (1)	0
SETD2	0	0
DDB1	0	0
SLC16A1	0	0
BRAF	0	0
EGFR	0	0
LTB	0	0

Mutations in bold indicates they were seen in >5% of patients at either presentation or relapse in both series.

Supplementary Table 3. *MYC* translocations at presentation and relapse

Patient	*MYC* time point	*IGH* translocation	1st Chr.	1st gene	2nd Chr.	2nd gene	Induction	Maintenance	Best response
Presentation and relapse									
7*	Presentation and relapse	14;16 P+R	16	WWOX	8	LOC727677-MYC	CTD	Lenalidomide	VGPR
7*	Presentation and relapse	14;16 P+R	14	BRF1	8	PVT1-LOC728724			
11	Presentation and relapse	Nil	14	C14orf80-TMEM121	8	POU5F1B-LOC727677	RCD	Lenalidomide	VGPR
12*	Presentation and relapse	Nil	15	BCL2A1-ZFAND6	8	PVT1	CTDa	Lenalidomide	PR
12*	Presentation and relapse	Nil	21	PRDM15	8	PVT1-LOC728724			
29	Presentation and relapse	Nil	5	ZNF131	8	LOC727677	RCDa	Lenalidomide	VGPR
34*	Presentation and relapse	10;14 P only	9	SYK	8	LOC727677-MYC	CTD	Observation	VGPR
34*	Presentation and relapse	10;14 P only	2	EIF2AK-RPIA	8	MYC-PVT1			
36	Presentation and relapse	14;19 P only	22	TTC28	8	PCAT1-POU5F1B	RCD	Observation	PR
38	Presentation and relapse	Nil	22	TTC28	8	PCAT1-POU5F1B	RCDa	Observation	nCR
42*	Presentation and relapse	Nil	1	FAM46C	8	PVT1	CTDa	Observation	PR
42*	Presentation and relapse	Nil	1	FAM46C	8	PVT1			
46	Presentation and relapse	Nil	4	TMEM155	8	PVT1-LOC728725	CTD	Observation	PR
55	Presentation and relapse	Nil	6	DUSP22	8	PVT1-LOC728725	CTD	Observation	VGPR
56	Presentation and relapse	11;14 P+R	14	ELK2AP-KIAA0125	8	LOC727677-MYC	CTD	Observation	VGPR
Relapse only									
18*	Relapse only	14;16 P+R	2	LAPTM4A-SDC1	8	PVT1-LOC728724	CTDa	Lenalidomide	CR
18*	Relapse only	14;16 P+R	22	IGLL5-RTDR1	8	PVT1-LOC728724			
31	Relapse only	Nil	22	IGLL5-RTDR1	8	PVT1-LOC728724	RCDa	Observation	CR
47	Relapse only	11;14 P+R	7	COBL-POM121K12	8	PVT1	CTDa	Observation	VGPR
Loss and gain									
53*	Presentation only	Nil	22	IGLL5-RTDR1	8	PVT1-LOC728725	CTDa	Observation	nCR
53*	Relapse only	Nil	3	SPTA16-NLGN1	8	PCAT1-POU5F1B			

Supplementary Table 4. Mutational load according to depth of response and maintenance allocation

Treatment/time point		Median number of mutations	Interquartile range	p value
CR series - all mutations and non-synonymous mutations only				
Lenalidomide all n=14	Presentation Relapse	82 117	59 - 271 80 - 288	0.046
Lenalidomide NS n=14	Presentation Relapse	42 58	25 - 110 41 - 124	0.01
Observation all n=10	Presentation Relapse	65 93	53 - 95 64 - 152	0.07
Observation NS n=10	Presentation Relapse	38 59	30 - 52 32 - 78	0.09
Whole series all n=24	Presentation Relapse	76 102	58 - 115 71 - 177	0.008
Whole series NS n=24	Presentation Relapse	40 59	29 - 52 40 - 81	<0.001
Non-CR series				
Lenalidomide all n=16	Presentation Relapse	64 67	54 - 86 44 - 87	0.68
Lenalidomide NS n=16	Presentation Relapse	37 37	26 - 46 22 - 44	0.75
Observation all n=16	Presentation Relapse	85 97	69 - 107 69 - 140	0.59
Observation NS n=16	Presentation Relapse	45 51	34 - 54 33 - 77	0.56
Whole series n=32	Presentation Relapse	71 82	60 - 105 60 - 117	0.53
Whole series n=32	Presentation Relapse	39 39	32 - 54 33 - 62	0.56

NS = Non-synonymous
All = all coding mutations

Supplementary Table 5. Mutational clusters at presentation and relapse

Patient	Maintenance	Cluster change at relapse	Clusters presentation	Clusters relapse
1	Lenalidomide	Gain	7	8
2	Lenalidomide	Neutral	4	4
3	Lenalidomide	Gain	8	10
4	Lenalidomide	Gain	8	9
5	Lenalidomide	Neutral	4	4
6	Lenalidomide	Neutral	7	7
7	Lenalidomide	Neutral	6	6
8	Lenalidomide	Neutral	8	8
9	Lenalidomide	Neutral	7	7
10	Lenalidomide	Neutral	10	10
11	Lenalidomide	Loss	7	6
12	Lenalidomide	Neutral	7	7
13	Lenalidomide	Loss	8	7
14	Lenalidomide	Loss	5	4
15	Lenalidomide	Neutral	5	5
16	Lenalidomide	Neutral	2	2
17	Lenalidomide	Neutral	3	3
18	Lenalidomide	Neutral	9	9
19	Lenalidomide	Neutral	4	4
20	Lenalidomide	Gain	6	6
21	Lenalidomide	Neutral	9	9
22	Lenalidomide	Loss	3	2
23	Lenalidomide	Gain	6	7
24	Lenalidomide	Gain	5	6
25	Lenalidomide	Neutral	7	7
26	Lenalidomide	Neutral	6	6
27	Lenalidomide	Neutral	6	6
28	Lenalidomide	Gain	7	8
29	Lenalidomide	Neutral	4	4
30	Lenalidomide	Neutral	7	7
31	Observation	Neutral	4	4
32	Observation	Loss	10	9
33	Observation	Neutral	5	5
34	Observation	Gain	8	9
35	Observation	Gain	7	8
36	Observation	Loss	8	7
37	Observation	Neutral	8	8
38	Observation	Gain	6	7
39	Observation	Neutral	6	6
40	Observation	Loss	4	5
41	Observation	Loss	6	5
42	Observation	Neutral	6	6
43	Observation	Neutral	7	7
44	Observation	Gain	4	5
45	Observation	Gain	3	4
46	Observation	Neutral	5	5
47	Observation	Neutral	10	10
48	Observation	Neutral	4	4
49	Observation	Gain	6	7
50	Observation	Loss	5	3
51	Observation	Loss	8	7
52	Observation	Gain	3	4
53	Observation	Neutral	9	9
54	Observation	Neutral	6	6
55	Observation	Neutral	7	7
56	Observation	Gain	7	8

Supplementary Table 6 – Impact of evolution on outcome according to maintenance allocation

Treatment group	Time point	PFS	p value	OS	p value
Lenalidomide	Branching	19	0.69	36	0.58
	Non-branching	18		36	
Observation	Branching	22	0.18	46	0.73
	Non-branching	16		44	
All patients	Branching	19	0.24	37	0.13
	Non-branching	16		41	

Supplementary Figure 1. Pathway for all patients included in the nested case control analysis.
Abbreviations; TE, transplant eligible; TNE, transplant non-eligible; CTD, cyclophosphamide, thalidomide and dexamethasone; RCD, lenalidomide, cyclophosphamide and dexamethasone; a, attenuated.

Supplementary Figure 2. Analysis pipeline. Following preparation and indexing all samples were run on the Illumina HiSeq 2500. Conversion of BCL files to compressed Fastq files required de-multiplexing (for pooled samples) which was conducted using the package CASAVA v1.8.4 (Illumina). The package FASTQC (version 0.11, Babraham Bioinformatics) was used for basic quality checking of all Fastq files. All files were aligned to the reference human genome (GRCh37) using Burrow-Wheeler Aligner (BWA-mem version 0.7.12 (Broad institute)). This consists of a package of three algorithms that enable mapping of reads from between 70 bp – 1Mbp (1, 2). Additional indexing steps were conducted using Sambamba (version 0.5.6, GitHub), to index and mark duplicates and the Genome Analysis Toolkit (version 3.5, GATK) for base recalibration and indel realignment (3). Determination of coverage, number of duplicates and on-target percentage was performed using Picard (version 1.119, Broad institute).

Translocations involving the IgH and MYC locus were determined in all patients using the bioinformatics package Manta (version 0.29.3) (4). For 51/112 (46%) patient samples, for which enough DNA was available, translocations involving the IgH were also assessed using multiplexed real-time quantitative reverse transcriptase-PCR (qRT-PCR) and a fluorescence in-situ hybridisation-validated translocation analysis using a cyclin-D classification-based hierarchical algorithm was applied to determine the IgH translocation status (5). This formed an internal quality control and a consensus between MANTA and qRT-PCR was observed in 43/51 samples (84%). For the 8 patients where a mismatch was observed the Integrative Genomics Viewer (IGV) (version 2.3.90) was used to confirm whether a translocation was present or not (6). IGV was used to confirm all suspected MYC translocations.

CNAs for all samples were determined using the bioinformatics assessment tool Sequenza (version 2.1.2, CRAN) and where there was enough DNA multiplexed ligation-dependent probe amplification (MLPA) (SALSA MLPA P425-B1 multiple myeloma probemix, MRC Holland) was also undertaken (7, 8). Paired MLPA and Sequenza data was available for 90/112 (80%) tumour samples. A consensus between MLPA and Sequenza was observed in 85/90 samples (94%). For the five patients where a mismatch was observed Sequenza was used to determine the final profile.

Single nucleotide variants and indels were identified using MuTect (version 1.1.17, broad) and Strelka (version 1.0.14, GitHub) using the default settings (9, 10). Filtering of MuTect output was undertaken with fpfilter (github.com) with a set variant allele frequency (VAF = reference allele/variant allele corrected for copy number) threshold of 5%. Indels were called using Strelka only (VAF filter 5%). A VAF filter was required to ensure mutations were not called inappropriately, as a consequence of low level cross contamination or sequencing errors. Single nucleotide variants located with the immunoglobulin loci were excluded due to expectant non-significant variation. The R package Rsamtools (version 1.24.0, Bioconductor) for aligned sequences was used to determine read counts for each mutation. The set inclusion criteria for mutation calling included; the presence of unique reads, a mapping quality of a minimum of 20 reads and the same for base quality at variant sites and a minimum coverage of 50x for presentation, relapse and control samples for the patient. Non-silent mutations were defined as missense, frameshift, non-sense, and splice site.

For all mutations the CCF was calculated according to Stephens *et al* (2012) (11). To determine the CCF the copy number at the mutation site, tumour purity and VAF was required and the following equation applied;

$$n_{mut} = f_s * \frac{1}{p}\left[pn_{locus}^{t} + 2(1-p)\right]$$

Where; n_{mut} = mutation copy number, f_s = VAF, p = tumour purity, n_{locus}^{t} = mutation site copy number

Purity and mutation site copy number where determined using Sequenza, with purity manually checked against the mutant allele frequency on Chromosome 14. The expected VAF was compared to values assuming the mutation was on 1, 2, …., C chromosomes and assigned ^{n}chr the value of C with binomial distribution used to determine the maximum likelihood. The CCF was then calculated by dividing ^{n}mut by ^{n}chr.

SciClone clustering, using the VAF for all coding mutations (R package version 1.1.0, GitHub) was performed to infer clusters of mutations in all patients at presentation and relapse (12). In addition, kernel density estimation plots for determining clusters of mutations according to CCF was used (R package).

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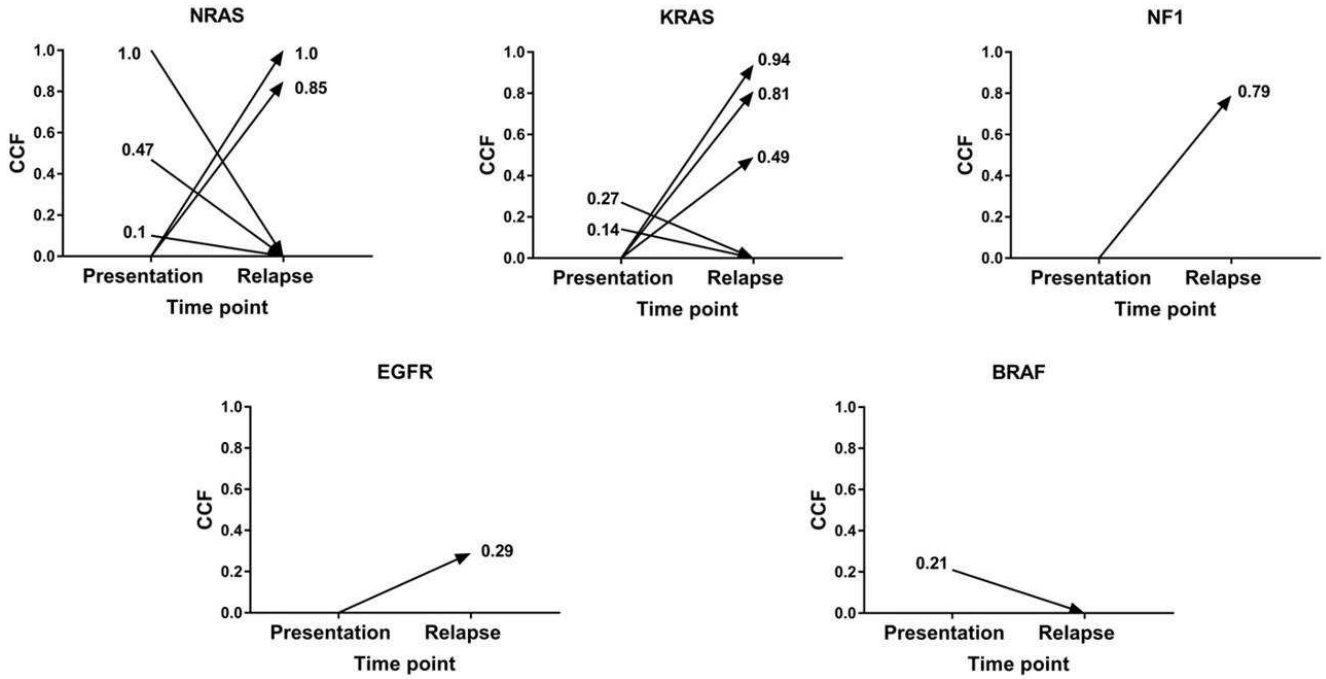
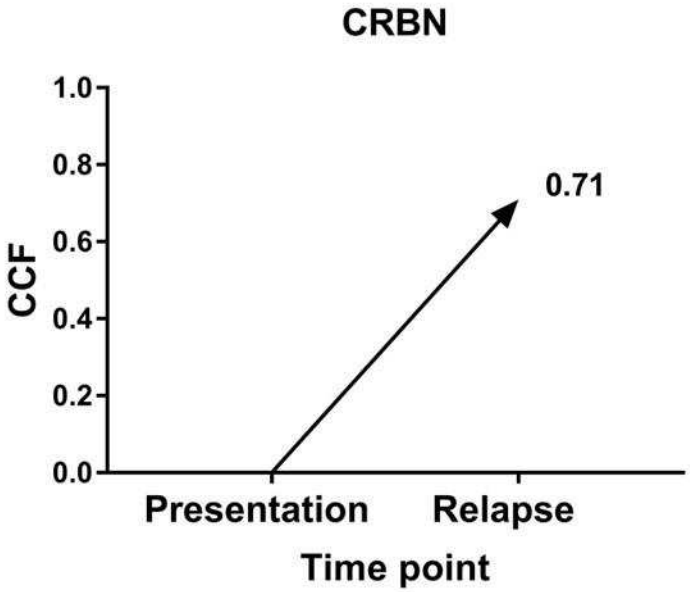


Supplementary Figure 3. Cancer clonal fractions of RAS pathway mutations gained and lost at relapse. New mutations of the MAPK pathway genes, *NRAS*, *KRAS*, *NF1* and *EGFR* were seen in 13% (7/56) patients at relapse. Importantly most of these new mutations were clonal at relapse, with CCF values of greater than 80%, suggesting a marked change in clonal dominance. Mutations in *NRAS*, *KRAS* and *BRAF* were also lost in 9% (5/56) patients at relapse.

Supplementary Figure 4. Structural aberration profile at presentation and relapse. The number of patients with one or more prognostic tumour suppressor gene deletion remained stable at both time points, with 63% (35/56) of patients having a deletion or one or more region at presentation compared to 59% (33/56) at relapse. There was a slightly higher proportion of patients with three or more deleted regions at relapse, 16% versus13% at presentation.

Supplementary Figure 5. Proportion of patients with structural change at presentation and relapse. A) CR series. A change in the profile of structural aberrations was seen in 42% (10/24) of the CR patients at relapse when compared to presentation. The gain of lesions predominated, particularly tMYC, del(17p), del(1p) and gain(1q), all of which were seen in a greater proportion of patients at relapse. **B) Non-CR series.** Only 28% of non-CR patients had a change in the structural aberration profile at relapse, with only gain(1q) and del(1p) seen in a greater proportion of patients when compared to presentation.



Supplementary Figure 6. *CRBN* mutation cancer clonal fraction at presentation and relapse. At presentation no *CRBN* was evident but at relapse a new mutation was found with a CCF of 0.71 suggesting it was present within a dominant clone at relapse.

Supplementary Figure 7. Evolutionary mechanism leading to relapse according to induction and maintenance randomisation. A) Evolution according to maintenance. Branching evolution was the predominant mechanism leading to relapse, seen in 54% of observation patients and 77% of lenalidomide maintenance patients (p=0.09, Fisher's Exact). There was also no statistical difference between the proportions of patients relapsing via linear and stable mechanisms. **B) Evolution according to induction.** Branching evolution was the predominant mechanism leading to relapse, seen in 59% of thalidomide treated patients and 74% of lenalidomide treated patients (p=0.27). Although there was a slightly higher proportion of thalidomide patients displaying linear evolution, 28% vs 11%, this was not significant (p=0.18). Stable progression was seen in 14% and 15% of thalidomide and lenalidomide patients respective (p=1.0).