c-MYC expression and maturity phenotypes are associated with outcome benefit from addition of ixazomib to lenalidomide-dexamethasone in myeloma

Alessandra Di Bacco1 | Nizar J. Bahlis2 | Nikhil C. Munshi3 | Hervé Avet-Loiseau4 | Tamás Masszi5,6 | Luísa Viterbo7 | Ludek Pour8 | Peter Ganly9 | Michele Cavo10 | Christian Langer11 | Shaji K. Kumar12 | S. Vincent Rajkumar12 | Jonathan J. Keats13 | Deborah Berg1 | Jianchang Lin1 | Bin Li1 | Sunita Badola1 | Lei Shen1 | Jacob Zhang1 | Dixie-Lee Esseltine1 | Katarina Luptakova1 | Helgi van de Velde1 | Paul G. Richardson3 | Philippe Moreau14

1Millennium Pharmaceuticals, Inc. (a wholly owned subsidiary of Takeda Pharmaceutical Company Limited), Cambridge, MA, USA,
2Southern Alberta Cancer Research Institute, University of Calgary, Calgary, AB, Canada
3Hematologic Oncology, Dana-Farber Cancer Institute, Boston, MA, USA
4Hematology, IUC-Oncopole, Toulouse, France
5Department of Haematology and Stem Cell Transplantation, St. István and St. László Hospital of Budapest, Budapest, Hungary
63rd Department of Internal Medicine, Semmelweis University, Budapest, Hungary
7Instituto Português de Oncologia do Porto Francisco Gentil, Entidade Pública Empresarial (IPOPFGEPE), Porto, Portugal
8Hematology and Oncology, University Hospital Brno, Brno, Czech Republic
9Department of Haematology, Christchurch Hospital, Christchurch, New Zealand
10Institute of Hematology and Medical Oncology “Seràgnoli”, Bologna University School of Medicine, S. Orsola’s University Hospital, Bologna, Italy
11University Hospital of Ulm, Ulm, Germany
12Division of Hematology, Mayo Clinic, Rochester, MN, USA
13Translational Genomics Research Institute (TGEN), Phoenix, AZ, USA
14Department of Hematology, University Hospital Hôtel Dieu, University of Nantes, Nantes, France

Correspondence
Alessandra Di Bacco, Millennium Pharmaceuticals, Inc., a wholly owned subsidiary of Takeda Pharmaceutical Company Limited, 40 Landsdowne Street, Cambridge, MA 02139, USA.
Email: Alessandra.DiBacco@Takeda.com

Funding Information
Millennium Pharmaceuticals, Inc., Cambridge, MA, USA, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited

Abstract
Objectives: In the TOURMALINE-MM1 phase 3 trial in relapsed/refractory multiple myeloma, ixazomib-lenalidomide-dexamethasone (IRd) showed different magnitudes of progression-free survival (PFS) benefit vs placebo-Rd according to number and type of prior therapies, with greater benefit seen in patients with >1 prior line of therapy or 1 prior line of therapy without stem cell transplantation (SCT).
Methods: RNA sequencing data were used to investigate the basis of these differences.
1 | INTRODUCTION

The advent and widespread use of next-generation sequencing has revealed the full complexity of tumor heterogeneity and its implication for cancer prognosis and therapeutic response. Multiple myeloma (MM), a clonal plasma cell disorder, is genetically complex and heterogeneous, with clonal diversity evolving over the disease course. Several studies have investigated the genomic heterogeneity of relapsed/refractory MM (RRMM), but few have explored the relationship between clonal heterogeneity and types of prior therapies received, and the impact of the resulting differences in tumor biology on the outcomes seen with novel therapies.

The phase 3 TOURMALINE-MM1 study compared the oral proteasome inhibitor (PI) ixazomib plus lenalidomide-dexamethasone (IRd) vs placebo-Rd in 722 patients with RRMM. Results from this study demonstrated a significant progression-free survival (PFS) benefit in the intent-to-treat (ITT) analysis. A subgroup analysis showed that while IRd was associated with clinical benefit compared with placebo-Rd, the greatest benefit was observed in patients who had received 2 or 3 prior lines of therapy (LoT; 2/3LoT patients) compared to patients who had received 1 prior LoT (1LoT patients). We therefore conducted analyses of RNA sequencing (RNAseq) data from tumors collected during TOURMALINE-MM1 and investigated the molecular differences between tumors from patients according to number and/or type of prior therapies.

In particular, we evaluated outcomes according to expression of c-MYC. Increased expression of c-MYC is involved in MM pathogenesis/progression, making c-MYC one of the key genes deregulated in MM. Translocations of c-MYC are present in approximately 15% of patients with newly diagnosed MM (NDMM) and RRMM. Gene expression studies indicate that the transcriptional signature of c-MYC is present in 67% of primary MM but not in the pre-malignant condition monoclonal gammopathy of undetermined significance. Recent genetic evidence also suggests a novel role for c-MYC during the first steps of hematopoietic stem cell differentiation, along
with the B-cell receptor (BCR) complex, of which CD19, CD81, and CD79A make up part of the co-receptor. Thus, differences in c-MYC expression and level of cell maturity may be associated with differential activity of agents and regimens.

2 | METHODS

2.1 | Study design and patients

TOURMALINE-MM1 (NCT01564537) study design and clinical results have been reported previously. Briefly, 722 patients with RRMM after 1-3 LoT were randomized to receive IrD or placebo-Rd until disease progression or unacceptable toxicity. Bone marrow aspirates were collected from patients at screening, and purified CD138-positive cells were used for RNAseq. Data reported here are from the first prespecified statistical analysis of PFS (median follow-up ~15 months; range 13.6-15.4), as reported by Moreau et al.

2.2 | Myeloma cell purification and RNA isolation

Bone marrow aspirate samples were collected at screening from patients enrolled in the trial for tumor gene expression analysis. CD138-positive cells were isolated within 24-48 hours using the Miltenyi CD138+ isolation kit according to the manufacturer's instructions. Frozen CD138-positive cell pellets were then shipped to a different laboratory for DNA and RNA co-extraction using the Qiagen AllPrep DNA/RNA kit. Extracted DNA and RNA were measured for quantity, quality, and purity. Each sample was aliquoted in two vials, frozen at -80°C and shipped to the Broad Institute (Cambridge, MA) for sequencing. All RNA samples were quantified again using a Nanodrop spectrophotometer (Thermo Scientific), and their integrity was assessed using the Agilent Bioanalyzer and normalized before starting the sequencing. The minimum Nanodrop and BioAnalyzer input requirement was ≥250 ng RNA and an RNA integrity number (RIN) ≥6.

2.3 | Gene expression profiling and QC steps

Whole transcriptome sequencing (RNAseq) of bone marrow-sorted CD138-positive cells was performed as described in the Methods S1. The purity of the MM samples was assessed, and samples with at least one specific variable region isoform (either heavy or light chain) having more than 90% of the reads mapped were automatically deemed as having high purity and were used in the present analyses (“RNAseq population”).

2.4 | Gene expression analyses in the RNAseq population

Details of the gene expression analyses in the RNAseq population are provided in the Methods S1. c-MYC, CD19, CD81, and CD138 expression were evaluated by treatment arm and according to the number (1 vs 2/3) and type (stem cell transplant [SCT] vs no SCT) of prior therapies received. A multivariate analysis, using logistic regression with stepwise selection, was performed to understand the influence of different disease characteristics on c-MYC expression level among 1LoT patients. For subsequent analyses, patients were dichotomized into two subgroups, defined as c-MYC-high and c-MYC-low, using median c-MYC expression as the cutoff, or into four subgroups based on c-MYC expression quartiles. Patients' data were analyzed based on median expression levels of CD19 and/or CD81 (CD19-high vs CD19-low; CD81-high vs CD81-low). PFS was analyzed in subgroups based on prior LoTs (1LoT, 2/3LoT) and by whether patients had SCT in a prior line of therapy (1LoT-SCT, 1LoT-noSCT, 2/3LoT-SCT, 2/3LoT-noSCT). All analyses were exploratory; no correction for multiplicity of testing was done.

3 | RESULTS

3.1 | Baseline characteristics of patients with RNAseq data

Of 419 samples collected, 399 were deemed of high purity for the genomic analyses, and so RNAseq data were available for 399 of 722 (55.2%) patients enrolled in TOURMALINE-MM1, including 189 and 210 randomized to IrD and placebo-Rd, respectively (RNAseq population). Baseline characteristics were similar between patients with or without RNAseq data, including disease stage, performance status, frequency of high-risk vs standard-risk cytogenetic abnormalities, prior LoTs, and prior exposure to immunomodulatory drugs or PIs (Table S1).

3.2 | c-MYC expression levels differ between patients with different numbers of prior LoT

Since cereblon is essential for lenalidomide activity in MM, we initially assessed if cereblon's expression level varied across patients with different types and numbers of prior LoT and did not observe any significant differences (data not shown). We then analyzed c-MYC expression across these subgroups of patients, as prior observations had suggested that c-MYC activity was likely to increase benefit in patients treated with bortezomib-based therapy. c-MYC was significantly upregulated in tumors from 2/3LoT vs 1LoT patients (P = .0275) (Figure 1A). Analysis of 1LoT patients according to prior SCT showed that tumors from 1LoT-SCT patients had significantly lower c-MYC expression levels vs those from 1LoT-noSCT patients (P = .013) and vs those from 2/3LoT patients (P = .0019). No differences were observed between 1LoT-noSCT and 2/3LoT patients (P = .71) (Figure 1B). To investigate if other parameters were correlated with lower c-MYC expression in tumors, we performed a multivariate analysis and showed that the best predictor for c-MYC expression level (above vs below the median) in 1LoT patients was prior SCT (no
vs yes; odds ratio 2.296, $P = .0018$). Using c-MYC expression level as a continuous dependent variable, linear regression analyses with stepwise selection showed a strong correlation between c-MYC expression level and prior SCT (no vs yes; coefficient .696; $P = .0015$), disease stage (I vs II or III; coefficient .510; $P = .0282$), and baseline serum M-protein (coefficient .0270; $P = .0001$).

### 3.3 | Prognostic impact of c-MYC expression based on treatment received

Since c-MYC is a known negative prognostic factor in MM, we assessed the impact of its expression on PFS in the RNAseq population, dichotomized per median c-MYC expression level and regardless of therapy (IRD and placebo-Rd patients pooled). Median PFS was similar—18.5 vs 17.6 months—in the c-MYC-high vs c-MYC-low groups (HR 0.99; 95% CI, 0.73, 1.4; $P > .05$), suggesting that in this population c-MYC expression did not per se have a negative impact on outcome. Similar results were obtained in an analysis of patients in the top vs bottom quartiles of c-MYC expression (median PFS 18.4 vs 15.7 months; HR 0.92; 95% CI, 0.6, 1.4; $P > .05$) (Figure 2A). PFS was then assessed based on c-MYC expression level and study treatment. A statistically significant improvement in PFS was seen in the c-MYC-high group with IRD vs placebo-Rd (median PFS not reached vs 12.9 months; HR 0.41; 95% CI, 0.24, 0.7; $P < .001$) compared to 1LoT patients (median 20.6 vs 15.7 months; HR 0.68; 95% CI, 0.45, 1.0; $P > .05$). When 1LoT patients were divided by prior SCT, a difference in PFS benefit was observed only for 1LoT-noSCT patients. In this group, the median PFS with IRD vs placebo-Rd was 18.5 vs 11.1 months (HR 0.43; 95% CI, 0.24, 0.79; $P = .01$; Figure 2B). When each prior therapy subgroup was stratified as c-MYC-high or c-MYC-low (based on median c-MYC expression level), the magnitude of PFS benefit with IRD vs placebo-Rd appeared greater among the c-MYC-high vs c-MYC-low patient subgroups (based on the respective HRs). While PFS with IRD and placebo-Rd was similar among the c-MYC-high 1LoT-SCT patients, the PFS HR favored placebo-Rd in the c-MYC-low 1LoT-SCT subgroup (Figure 2B). Notably, the longest median PFS with placebo-Rd was observed in 1LoT-SCT patients.

### 3.4 | PFS by c-MYC expression and prior treatment exposure

Similar to what was reported previously for the ITT population, analyses of PFS in the RNAseq population according to prior therapy showed that the PFS benefit with IRD vs placebo-Rd was greater in 2/3LoT patients (median not reached vs 12.9 months; HR 0.41; 95% CI, 0.24, 0.7; $P < .001$) compared to 1LoT patients (median 20.6 vs 15.7 months; HR 0.68; 95% CI, 0.45, 1.0; $P > .05$). When 1LoT patients were divided by prior SCT, a difference in PFS benefit was observed only for 1LoT-noSCT patients. In this group, the median PFS with IRD vs placebo-Rd was 18.5 vs 11.1 months (HR 0.43; 95% CI, 0.24, 0.79; $P = .01$; Figure 2B). When each prior therapy subgroup was stratified as c-MYC-high or c-MYC-low (based on median c-MYC expression level), the magnitude of PFS benefit with IRD vs placebo-Rd appeared greater among the c-MYC-high vs c-MYC-low patient subgroups (based on the respective HRs). While PFS with IRD and placebo-Rd was similar among the c-MYC-high 1LoT-SCT patients, the PFS HR favored placebo-Rd in the c-MYC-low 1LoT-SCT subgroup (Figure 2B). Notably, the longest median PFS with placebo-Rd was observed in 1LoT-SCT patients.

### 3.5 | Characteristics of 1LoT-SCT patients

To understand if any baseline characteristics of 1LoT-SCT patients might have influenced the differential PFS benefit with
FIGURE 2  c-MYC as prognostic factor in RNAseq population. A, c-MYC level did not appear to be a negative prognostic factor in the population analyzed. A significant difference in outcome was observed when treatment arm was included in the analysis. A significant difference in PFS with IRd vs placebo-Rd was observed only in c-MYC-high patients. B, PFS with IRd vs Rd according to prior therapy and by high or low c-MYC expression level. CI, confidence interval; HR, hazard ratio; IRd, ixazomib-lenalidomide-dexamethasone; ITT, intent-to-treat; LoT, line of therapy; PFS, progression-free survival; Rd, lenalidomide-dexamethasone; RNAseq, RNA sequencing; SCT, stem cell transplantation [Colour figure can be viewed at wileyonlinelibrary.com]
IRd vs placebo-Rd, we compared the baseline characteristics of 1LoT-SCT patients to those of 1LoT-noSCT and 2/3LoT patients combined (Table S2). We found that 1LoT-SCT patients had better prognostic features compared with the rest of the RNAseq.
population: more of them had an Eastern Cooperative Oncology Group performance status of 0 (65% vs 39%); they were younger (65% vs 37% aged ≥ 65 years); fewer had renal involvement (5% vs 17%); fewer had tumors with high-risk cytogenetics features (del(17), t(4;14), and t(14;16); 18% vs 25%); and the frequency of patients with relapsed-only (non-refractory) MM was higher (93% vs 70%). Notably, the majority of 1LoT-SCT patients (71%) had received SCT ≥ 24 months prior to TOURMALINE-MM1 study entry, indicating that they had not relapsed shortly after transplantation.

3.6 | Gene expression analyses identified markers of early differentiation in 1LoT-SCT patients

We compared RNAseq data from 1LoT-SCT patients to pooled data from 1LoT-noSCT and 2/3LoT patients to evaluate differences at the molecular level. As shown in Table 2, the 10 most differentially expressed genes were the immunoglobulin heavy constant delta and epsilon genes, IGHD and IGHE (3.57 and 2.08 fold higher), components of the BCR complex (CD19, CD81, CD79A) (1.97, 1.51, and 1.54 fold higher), and genes involved in different steps of cell maturation (PAK5, VPREB1, IGL1) (1.54, 1.81, and 1.68 fold higher). All these genes were significantly upregulated in 1LoT-SCT patients, while c-MYC expression was significantly lower (0.65 fold lower).

3.7 | 1LoT-SCT patients demonstrated higher levels of CD19 and CD81 expression

Pairwise comparison of CD19 or CD81 expression showed statistically significantly higher expression of both markers in 1LoT-SCT patients. CD19 expression was significantly higher in 1LoT-SCT vs 1LoT-noSCT (P < .001) or 2/3LoT patients (P = .0079); no difference was observed between 1LoT-noSCT and 2/3LoT (P = .11) patients (Figure 3A). Similarly, CD81 expression was higher in 1LoT-SCT vs 1LoT-noSCT (P = .006) or 2/3LoT (P = .02) patients, with no difference between 1LoT-noSCT and 2/3LoT patients (P = .55) (Figure 3B). We also looked at CD138 expression levels and, while we did not see any significant difference between the three groups of patients (Figure S1), we observed lower CD138 expression among patients with tumors high with CD19 or CD81 expression (P = .00155) (Figure S2). Analyses of CD19 and CD81 expression in patients in the top and bottom quartiles of c-MYC expression, independent of line of therapy, confirmed the inverse correlation of these markers. CD19, CD81, and CD79A expression was significantly greater in tumors from patients in the lowest vs highest c-MYC expression quartile (P = .0164, P = .00114, and P = .0223, respectively), independent of the number or type of prior therapies (Figure 3C). These data suggest that tumors displaying these molecular characteristics are present throughout the RNAseq population but are more frequent in 1LoT-SCT patients.

3.8 | Lenalidomide and ixazomib appear to target tumors at different stages of maturation

To further understand how different molecular features influence the activity of IRd and placebo-Rd, we stratified patients based on median c-MYC, CD19, or CD81 expression and evaluated PFS with IRd and placebo-Rd in each subgroup. PFS with IRd appeared longer among patients with c-MYC-high vs c-MYC-low tumors (median not reached vs 20.6 months; HR 0.72; 95% CI, 0.43, 1.2; P > .05) and those with CD19-low vs CD19-high (median not reached vs 21.4 months; HR 0.85; 95% CI, 0.51, 1.4; P > .05) or CD81-low vs CD81-high (median not reached vs 18.5 months; HR 1.5; 95% CI, 0.87, 2.5; P > .05), although the differences were not statistically significant (Figure 4).

In contrast, PFS with placebo-Rd appeared shorter among c-MYC-high vs c-MYC-low patients (median 11.3 vs 16.6 months; HR 1.3; 95% CI, 0.9, 2.0; P > .05) and longer among CD19-high vs CD19-low (median 17.5 vs 11.1 months; HR 0.6; 95% CI, 0.4, 0.91; P = .05) and CD81-high vs CD81-low (median not reached vs 11.1 months; HR 0.51; 95% CI, 0.33, 0.7; P = .01) patients, although the difference between c-MYC groups was not significant (Figure 4). Taken together, these data suggest that IRd and placebo-Rd target tumors at different stages of maturation, with placebo-Rd performing better in less mature tumors (low levels of c-MYC and high levels of CD19 or CD81 expression). Conversely, IRd appeared more beneficial in more mature tumors (high levels of c-MYC and low levels of CD19 or CD81 expression).

4 | DISCUSSION

MM is a complex disease demonstrating clear evidence of substantial clonal heterogeneity, with different clones coexisting in the same patient.18 Next-generation sequencing studies have shown the complexity of MM at the genetic level, with changes in chromosome numbers, genetic translocations, and genetic mutations. However, evidence is emerging that the clonal cells that constitute the disease are not homogeneously mature plasma cells.3 The MYC signaling pathway is of great interest in MM.19 MYC abnormalities are typically characterized by complex rearrangement of the proto-oncogene c-MYC,20 which encodes a transcription factor that regulates cell proliferation, growth, protein translation, metabolism, and apoptosis.21 Several studies have also suggested an involvement of c-MYC during the expansion of committed progenitors in the adult hematopoietic system,12 thereby playing a role in the differentiation of B cells.22,23 During this process, c-MYC appears to control the expression of specific integrins, regulating the balance between self-renewal and differentiation by modulating the migration and/or adhesion of hematopoietic stem cells.12

Data from TOURMALINE-MM1 have shown a different magnitude of PFS benefit between 1LoT and 2/3LoT patients.7 Using the RNAseq dataset from the study, we uncovered molecular differences between tumors of patients who relapsed post-SCT vs those who relapsed after prior therapies not including SCT.
After excluding cereblon expression as responsible for the differential activity of IRd vs placebo-Rd, we focused our attention on c-MYC. The rationale for evaluating c-MYC expression in tumors was provided by observations that c-MYC plays a role in PI-induced cell death and by a prior analysis suggesting that patients with tumors expressing higher levels of c-MYC derive relatively greater benefit from PI therapy with bortezomib. We initially demonstrated that median c-MYC levels were significantly higher in 2/3LoT vs 1LoT patients and that within 1LoT patients the lowest levels were observed among 1LoT-SCT patients (Figure 1). This observation was strengthened by results from a multivariate analysis that showed the best predictor for c-MYC expression levels within 1LoT patients was placebo-Rd in 1LoT patients in TOURMALINE-MM1 might have been driven by the 1LoT-SCT subgroup (this group represented 65% of 1LoT patients). Additionally, while PFS with IRd appeared independent of c-MYC level (c-MYC-high vs c-MYC-low: median 21.4 vs 20.6 months in 1LoT-SCT, median 18.5 vs 17 months in 1LoT-noSCT, not reached vs not reached in 2/3LoT patients), we observed differences in PFS with placebo-Rd among 1LoT-noSCT (c-MYC-high vs c-MYC-low: median 9.69 vs 12.2 months) and 2/3LoT (median 11.3 vs 14.1 months) patients. Notably, the 1LoT-SCT subgroup appeared to have the longest PFS with placebo-Rd, independent of c-MYC expression, suggesting that the greater PFS benefit with IRd vs placebo-Rd in TOURMALINE-MM1 in 1LoT-noSCT and 2/3LoT patients might have been driven by reduced activity of placebo-Rd in these subgroups. This finding is supported by a recent report suggesting high c-MYC expression as a potential mechanism of resistance to lenalidomide activity in MM.

We next looked at the baseline characteristics of 1LoT-SCT patients compared to 1LoT-noSCT and 2/3LoT patients. We found that 1LoT-SCT patients had better prognostic features, including a lower proportion of elderly patients, better performance status, a lower rate of high-risk cytogenetics, and a higher rate of relapsed-only (non-relapsed-only) disease. A high proportion of 1LoT-SCT patients (71%) received SCT ≥ 24 months prior to TOURMALINE-MM1 study entry, suggesting that they did not have an early, aggressive relapse outcome. Patients with early relapse have more aggressive MM and shorter PFS and overall survival compared to those with relapse >1 year post-SCT, who have a more indolent disease biology.

Although high c-MYC levels are associated with increased tumor sensitivity to IRd, this did not appear to be the case in 1LoT-SCT patients, as PFS was similar with IRd and placebo-Rd (Figure 2B). We compared RNAseq data between 1LoT-SCT patients and 1LoT-noSCT and 2/3LoT patients, and the immunoglobulin heavy constant delta and epsilon genes, IGHD and IGHE, components of the BCR complex (CD19, CD81, CD79A), and genes involved in different pathways associated with cell maturation. Gene expression profile of 1LoT-SCT patients vs 1LoT-noSCT and 2/3LoT patients with RNAseq data, pooled across TOURMALINE-MM1 treatment arms.
steps of cell maturation (PAX5, VPREB1, IGLL1) were among the 10 most differentially expressed genes. These genes were significantly overexpressed in 1LoT-SCT patients’ tumors, while c-MYC was downregulated (Table 2). The presence of high CD19 levels in 1LoT-SCT patients was unexpected, although minor subsets of CD19-positive cells have been observed previously in a disease predominantly comprising CD19-negative mature plasma cells. These CD19-positive cells tend to be found in circulation; it is possible that these cells were collected with blood during screening bone marrow sampling. It has been reported that levels of CD19-positive cells in the peripheral blood are significantly higher in MM patients vs healthy donors. Cell numbers are low during induction and high-dose treatment but appear to increase post-SCT, when a simultaneous rise in CD19 cells and CD19 mRNA is detected. Interestingly, these cells have less differentiated phenotypes and appear to make up a drug-resistant, clonogenic disease reservoir.

In our dataset, CD19 and CD81 expression was inversely correlated with CD138 expression, a marker of B cell maturity (Figure S2), further supporting the hypothesis of the presence of immature tumor cells in 1LoT-SCT patients. While CD19, CD81, and CD79A were significantly overexpressed in 1LoT-SCT patients’ tumors, no differences were observed between 1LoT-noSCT and 2/3LoT patients (Figure 3A/B). Importantly, CD19 and CD81 expression appeared to be inversely correlated with c-MYC expression across all subgroups analyzed (Figure 3C). This observation suggests that subsets of these less mature, CD19-positive and CD81-positive tumors might be present in all MM patients, independent of prior therapy, but that the size of these subsets depends on prior therapy received, with the largest proportion observed in 1LoT-SCT patients.

Analyses of PFS with IRd and placebo-Rd activity according to c-MYC, CD19, or CD81 expression levels (Figure 4) showed prolonged PFS with placebo-Rd in patients with tumors with higher...
CD19 and CD81 expression, as well as low c-MYC expression. We could therefore hypothesize that in TOURMALINE-MM1 the Rd component of therapy targeted tumors with a less mature phenotype, while ixazomib targeted clonal populations with a more mature/differentiated phenotype, consistent with higher proteasomal stress.

The differential distribution of less mature tumor cells among patients with different prior therapies might in part explain the differential magnitude of PFS benefit with IRd vs placebo-Rd in TOURMALINE-MM1. Interestingly, previous reports have alluded to a preferential targeting of plasma cell precursors by lenalidomide, suggesting that resistance could be associated with the selection of mature plasma cell subclones. Similarly, it has been suggested that bortezomib preferentially kills mature cells and that resistance is associated with the emergence of dedifferentiated MM cells.

It has been reported that immunoglobulin secretion increases with the differentiation of plasma cells, while proteasome activity decreases. Following differentiation, cells have higher levels of ubiquitin-conjugated proteins and lower free ubiquitin levels, suggesting that differentiated, more mature MM cells are under proteasomal stress as a consequence of the imbalance between greater workload and lower proteasomal capacity. This imbalance is thought to explain the exquisite sensitivity of MM cells to PIs. In our study, a trend toward lower M-protein levels in 1LoT-SCT patients was observed (data not shown), supporting prior observations of less mature tumors in this subgroup.

While these data provide some explanations for the differential PFS benefit of IRd vs placebo-Rd in TOURMALINE-MM1, we recognize that our analyses have some limitations, including the small numbers of patients in each of the subgroups. Although we conducted a multivariate analysis incorporating various baseline disease characteristics, our analysis did not account for all the molecular subtypes, as these data were not collected in TOURMALINE-MM1; for example, c-MYC expression has been shown to be associated with hyperdiploidy. In addition, in the context of clonal evolution in MM, it would have been of value to incorporate data from bone marrow aspirates taken at diagnosis or earlier in the disease course; however, these data were not available. Thus, while our analyses have enabled the development of hypotheses to help understand TOURMALINE-MM1 findings, it is important to emphasize that more detailed prospective studies, with larger sample sizes and longer follow-up (to capture clonal changes), and addressing additional parameters of potential differential prognostic relevance, are required to validate and extend the clinical relevance of our findings.

**ACKNOWLEDGEMENTS**

The authors would like to thank all patients and their families, physicians, research nurses, study coordinators, and research staff participating in this study. The authors acknowledge Laura Webb, PhD, and Steve Hill, PhD, of FireKite, an Ashfield company, part of UDG Healthcare plc, for writing assistance during the development of this manuscript, which was funded by Millennium Pharmaceuticals Inc, and complied with Good Publication Practice 3 ethical guidelines (Battisti WP, et al Ann Intern Med 2015;163:461-4), and Renda Ferrari, PhD, of Millennium Pharmaceuticals, Inc, Cambridge, MA, USA, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited, for editorial support. This study was sponsored by Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited.
CONFLICT OF INTEREST

ADB: Employment, Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. NJB: Research funding: Celgene; Honoraria: Janssen, Celgene, Amgen. NCM: Consultancy: Celgene, Novartis, Takeda; Scientific founder: Oncopep. HA-L: Research funding: Amgen, Celgene, Sanofi, Takeda; Membership of advisory committees: Abbvie, Amgen, Celgene, Janssen, Sanofi, Takeda. TM: Advisory board member, Janssen-Cilag, Novartis, Takeda, Bristol-Myers Squibb, Abbvie, Pfizer. LV: None. LP: None. PG: None. MC: Consultancy, member, Janssen-Cilag, Novartis, Takeda, Bristol-Myers Squibb. SKK: Institutional research funding for clinical trials: Celgene, Takeda, Janssen, BMS, Sanofi, KITE, Merck, Abbvie, Medimmune, Novartis, Roche-Genentech, Amgen; Consultancy/Advisory board participation (no personal payments): Celgene, Takeda, Janssen, KITE, Merck, Abbvie, Medimmune, Genentech, Amgen; Consultancy/Advisory board participation (with personal payment): Oncopeptides, Adaptive; Honorarium for educational event: Dr Reddys Lab, Ono Pharmaceuticals. SVR: Institutional research funding for clinical trials: Takeda. JJK: None. DB: Employment, Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. JL: Employment, Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. BL: Employment, Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. SB: Employment, Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. LS: Employment, Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. JZ: Employment, Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. D-LE: Employment (at time of study/analyses), Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. KL: Employment, previously Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited, currently TESARO, Waltham, MA. HvDV: Employment (at time of study/analyses), Millennium Pharmaceuticals, Inc, a wholly owned subsidiary of Takeda Pharmaceutical Company Limited. PGR: Advisory Committee Member: Karyopharm, Oncopeptides, Celgene, Takeda, Janssen, Sanofi. Research support: Oncopeptides, Celgene, Takeda, BMS. PM: Advisory board member and honoraria: Takeda, Celgene.

AUTHOR CONTRIBUTIONS

ADB, NJB, NCM, HA-L designed the analyses. ADB, DB, D-LE, PGR, PM contributed to the design of the overall study. ADB, JL, BL, SB, LS, JZ performed the analyses. NJB, NCM, HA-L, TM, LV, LP, PG, MC, CL, SKK, SVR, JJK, PGR, PM provided patient samples and/or experimental tools for the analyses. ADB, NJB, NCM, HA-L, D-LE, KL, HvDV analyzed and interpreted the data. ADB, KL, HvDV wrote the manuscript. All authors reviewed the manuscript and approved the final draft for submission.

DATA AVAILABILITY STATEMENT

Takeda makes patient-level, de-identified data sets and associated documents available after applicable marketing approvals and commercial availability have been received, an opportunity for the primary publication of the research has been allowed, and other criteria have been met as set forth in Takeda's Data Sharing Policy (see https://www.takedaclinicaltrials.com for details). To obtain access, researchers must submit a legitimate academic research proposal for adjudication by an independent review panel, who will review the scientific merit of the research and the requestor's qualifications and conflict of interest that can result in potential bias. Once approved, qualified researchers who sign a data sharing agreement are provided access to these data in a secure research environment.

ORCID

Alessandra Di Bacco https://orcid.org/0000-0003-2072-8712
Philippine Moreau https://orcid.org/0000-0003-1780-8746

REFERENCES

1. Lawrence MS, Stojanov P, Polak P, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013;499(7457):214-218.
2. Manier S, Salem KZ, Park J, Landau DA, Getz G, Gohbrial IM. Genomic complexity of multiple myeloma and its clinical implications. Nat Rev Clin Oncol. 2017;14(2):100-113.
3. de Mel S, Lim SH, Tung ML, Chng W-J. Implications of heterogeneity in multiple myeloma. Biomed Res Int. 2014;2014:1-12.
4. Majumder MM, Silvennoinen R, Anttila P, et al. Identification of precision treatment strategies for relapsed/refractory multiple myeloma by functional drug sensitivity testing. Oncotarget. 2017;8(34):56338-56350.
5. Cornell RF, Kassim AA. Evolving paradigms in the treatment of relapsed/refractory multiple myeloma: increased options and increased complexity. Bone Marrow Transplant. 2016;51(4):479-491.
6. Moreau P, Masszi T, Grzasko N, et al. Oral ixazomib, lenalidomide, and dexamethasone for multiple myeloma. N Engl J Med. 2016;374(17):1621-1634.
7. Mateos MV, Masszi T, Grzasko N, et al. Impact of prior therapy on the efficacy and safety of oral ixazomib-lenalidomide-dexamethasone vs. placebo-lenalidomide-dexamethasone in patients with relapsed/refractory multiple myeloma in TOURMALINE-MM1. Haematologica. 2017;102(10):1767-1775.
8. Dang CV. MYC metabolism, cell growth, and tumorigenesis. Cold Spring Harb Perspect Med. 2013;3(8):a014217.
9. Kuehl WM, Bergsagel PL. Molecular pathogenesis of multiple myeloma and its premalignant precursor. J Clin Invest. 2012;122(10):3456-3463.
10. Glitza IC, Lu G, Shah R, et al. Chromosome 8q24.1/c-MYC abnormality: a marker for high-risk myeloma. Leuk Lymphoma. 2015;56(3):602-607.
11. Chng W-J, Huang GF, Chung TH, et al. Clinical and biological implications of MYC activation: a common difference between MGUS and newly diagnosed multiple myeloma. Leukemia. 2011;25(6):1026-1035.
12. Wilson A, Murphy MJ, Oskarsson T, et al. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev. 2004;18(22):2747-2763.
13. Vences-Catalán F, Kuo C-C, Sagi Y, et al. A mutation in the human tetraspanin CD81 gene is expressed as a truncated protein but...
does not enable CD19 maturation and cell surface expression. J Clin Immunol. 2015;35(3):254-263.

14. Avet-Loiseau H, Attal M, Moreau P, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. Blood. 2007;109(8):3489-3495.

15. Keats JJ, Chesi M, Egan JB, et al. Clonal competition with alternating dominance in multiple myeloma. Blood. 2012;120(5):1067-1076.

16. Walker BA, Wardell CP, Brioli A, et al. Translocations at 8q24 juxtapose MYC with genes that harbor superenhancers resulting in overexpression and poor prognosis in myeloma patients. Blood Cancer J. 2014;4:e191.

17. Weinhold N, Körn D, Seckinger A, et al. Concomitant gain of 1q21 and MYC translocation define a poor prognostic subgroup of hyperdiploid multiple myeloma. Haematologica. 2016;101(3):e116-e119.

18. Brioli A, Melchor L, Walker BA, Davies FE, Morgan GJ. Biology and treatment of myeloma. Clin Lymphoma Myeloma Leuk. 2014;14(Suppl):S65-S70.

19. Jovanovic KK, Roche-Lestienne C, Ghobrial IM, Facon T, Quesnel Murn J, Mlinaric-Rascan I, Vaigot P, Alibert O, Frouin V, Gidrol X. 2014;111(3):1543-1551.

20. Jovanovic KK, Roche-Lestienne C, Ghobrial IM, Facon T, Quesnel Murn J, Mlinaric-Rascan I, Vaigot P, Alibert O, Frouin V, Gidrol X. 2014;111(3):1543-1551.

21. Jovanovic KK, Roche-Lestienne C, Ghobrial IM, Facon T, Quesnel Murn J, Mlinaric-Rascan I, Vaigot P, Alibert O, Frouin V, Gidrol X. 2014;111(3):1543-1551.

22. Jovanovic KK, Roche-Lestienne C, Ghobrial IM, Facon T, Quesnel Murn J, Mlinaric-Rascan I, Vaigot P, Alibert O, Frouin V, Gidrol X. 2014;111(3):1543-1551.

23. Murn J, Mlinaric-Rascan I, Vaigot P, Alibert O, Frouin V, Gidrol X. 2014;111(3):1543-1551.

24. Murn J, Mlinaric-Rascan I, Vaigot P, Alibert O, Frouin V, Gidrol X. 2014;111(3):1543-1551.

25. Wirth M, Stojanovic N, Christian J, et al. MYC and EGR1 synergize in the regulation of c-Myc oncoprotein stress induction as defined by expression of CD45 isoforms and adhesion molecules. Haematologica. 2019;104(3):e116-e119.

26. Wu D, Fu J, et al. The proteasome inhibitor bortezomib depletes plasma cells and protects mice with lupus-like disease from nephritis. Eur J Haematol. 2007;78(4):349-356.

27. Franssen LE, Nijhof IS, Couto S, et al. Cereblon loss and up-regulation of c-myc are associated with lenalidomide resistance in multiple myeloma patients. Blood Cancer J. 2014;4:e191.

28. Ravi D, Beheshti A, Abermil N, et al. Proteasomal inhibition by ixazomib induces CHK1 and MYC-dependent cell death in T-cell and Hodgkin lymphoma. Cancer Res. 2016;76(11):3319-3331.

29. Lin Y, Wong K, Calame K. Repression of c-myc transcription by Blimp-1, an inducer of terminal B cell differentiation. Science. 1999;276(5312):596-599.

30. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

31. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

32. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

33. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

34. Lin Y, Wong K, Calame K. Repression of c-myc transcription by Blimp-1, an inducer of terminal B cell differentiation. Science. 1999;276(5312):596-599.

35. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

36. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

37. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

38. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

39. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

40. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

41. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

42. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

43. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

44. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

45. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

46. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

47. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

48. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

49. Murn J, Milinaric-Rascan I, Vaigot P, Albert B, Roustan L, Giraud X. 2018;111(3):1543-1551.

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Di Bacco A, Bahlis NJ, Munshi NC, et al. c-MYC expression and maturity phenotypes are associated with outcome benefit from addition of ixazomib to lenalidomide-dexamethasone in myeloma. Eur J Haematol. 2020;105:35-46. https://doi.org/10.1111/ejh.13405