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# Minimal Residual Disease by Flow Cytometry and ASO-RQ-PCR in Myeloma Patients Receiving Lenalidomide Maintenance: A Pooled Analysis

## MRD and ASO-RQ-PCR in MM patients

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## Abstract

**Background.** Minimal residual disease (MRD) is one of the most relevant prognostic factors in multiple myeloma (MM) patients; nevertheless, the impact of maintenance therapy on MRD levels is still unclear. We evaluated the role of MRD by multiparameter flow cytometry (MFC) and allelic-specific oligonucleotide real-time quantitative polymerase chain reaction (ASO-RQ-PCR) as predictor of progression-free survival (PFS) in newly diagnosed MM (NDMM) patients receiving lenalidomide maintenance until progression.

**Methods.** Seventy-three NDMM patients enrolled in the RV-MM-EMN-441 and in the RV-MM-COOP-0556 phase-III trials achieving  $\geq$ very good partial response after intensification/consolidation were included. Median age was 57 years (IQR:53-61) and all patients received Lenalidomide maintenance until progression. MRD was evaluated on bone marrow after intensification/consolidation, after 6 courses of maintenance and then every 6 months until clinical relapse, using both ASO-RQ-PCR (sensitivity of  $10^{-5}$ ) and MFC (sens.  $10^{-4}$ - $10^{-5}$ ).

**Results.** After intensification/consolidation, 33/72 (46%) patients achieved molecular-complete response (m-CR) and 44/70 (63%) patients flow-complete response (flow-CR). Almost 27% of MRD-positive patients after consolidation became MRD-negative during maintenance. After a median follow-up of 38 months, PFS was prolonged in patients achieving MRD negativity during maintenance, both by ASO-RQ-PCR (HR:0.29, 95%CI 0.14-0.62,  $p=0.0013$ ) and MFC (HR:0.19, 95%CI 0.09-0.41,  $p<0.001$ ). The impact of MRD negativity on PFS was similar in all subgroups (ASCT/no-ASCT, ISSI/II/III, High-/Standard-risk cytogenetics), and the two techniques were highly correlated.

**Conclusions.** MRD is a stronger predictor of PFS than standard risk factors and Lenalidomide maintenance further increases MRD negativity rate.

## Introduction

Multiple myeloma (MM) is a plasma cell malignancy, and its outcome has markedly improved during the last 10-15 years thanks to the introduction of novel agents and autologous stem cell transplantation (ASCT). Recently, consolidation and maintenance therapy increased rates and depth of response.<sup>1,2</sup> Because most of patients with complete response (CR) ultimately relapse, more sensitive methods are needed to detect and quantify minimal residual disease (MRD). Several techniques have been explored, such as quantitative allelic-specific oligonucleotide real-time quantitative polymerase chain reaction (ASO-RQ-PCR) and high-throughput next-generation sequencing (NGS) for molecular response evaluation; multiparameter flow cytometry (MFC) and next-generation flow (NGF) to evaluate bone marrow (BM) plasma cell residual disease. The lower is the MRD level with the higher sensitivity technique, the longer the progression-free survival (PFS).<sup>3</sup> A recent meta-analysis showed an advantage for MRD-negative over MRD-positive patients. In particular, in patients achieving conventional CR, MRD results were reported in five studies for PFS<sup>4-8</sup> and in six studies for OS.<sup>4-10</sup> MRD-negative patients had a significantly better PFS (HR 0.44,  $P < 0.0001$ ) and OS (HR 0.47,  $P < 0.0001$ ) compared with MRD-positive patients. Moreover, five studies evaluated MRD before and after ASCT, and found that patients achieving MRD negativity increased after ASCT.<sup>11-15</sup> Similarly, two studies found that maintenance therapy increased the proportion of patients achieving and maintaining MRD-negative status.<sup>11,12</sup> This meta-analysis also showed that patients with favorable cytogenetic profile who achieved MRD-negative status had the best OS; conversely, patients with high-risk cytogenetic profile who remained MRD-positive had the worst outcome. We evaluated MRD by ASO-RQ-PCR and MFC in patients who received Bortezomib or Lenalidomide-based front-line induction followed by ASCT/ no-ASCT consolidation and who started Lenalidomide maintenance. A pooled analysis was performed, due to the suitable

number of patients enrolled whose MRDs had been recorded at different time points during lenalidomide maintenance.

## **Methods**

### **Patients and study design**

We pooled 105 newly diagnosed MM (NDMM) patients enrolled in two phase III clinical trials: the RV-MM-EMN-441 (ClinicalTrials.gov identifier: NCT01091831)<sup>16</sup> and the RV-MM-COOP-0556 (ClinicalTrials.gov identifier: NCT01208766).<sup>17</sup> The two study designs are illustrated in the supplementary appendix (Figure S1). In the RV-MM-EMN-441 study, patients were randomized at enrollment in a 1:1:1:1 ratio to receive consolidation with six cycles of Cyclophosphamide-Lenalidomide-Dexamethasone (CRD) or Melphalan-conditioned Autologous Stem-Cell Transplantation (ASCT), and maintenance with either Lenalidomide or Lenalidomide-prednisone until relapse or intolerance. Patients received four cycles of Lenalidomide-Dexamethasone (RD) induction, followed by mobilization and stem cell collection. Subsequently, when eligibility for consolidation was confirmed, the treatment allocation was disclosed. In the RV-MM-COOP-0556, patients received 3-4 cycles of Bortezomib-Cyclophosphamide-Dexamethasone (VCD) induction, followed by mobilization and stem cell collection. Afterwards, patients were randomized to receive 4 cycles of Bortezomib-Melphalan-Prednisone (VMP) or 1-2 cycles of High-Dose-Melphalan (HDM) followed by ASCT. After intensification, patients were secondly randomized to receive 2 cycles of Bortezomib-Lenalidomide-Dexamethasone (VRD) consolidation or no consolidation, followed by Lenalidomide maintenance in both arms until progression or intolerance. Response to treatment was assessed according to the IMWG criteria.<sup>18</sup> Patients who achieved at least a VGPR after intensification/consolidation and had plasma cell infiltration  $\geq 5\%$  at

baseline were eligible for the MRD sub-study. This cut-off was chosen because of the technical issues in obtaining the molecular marker in patients with a lower plasma cell infiltration at baseline. MRD analysis was performed on BM aspirates collected at different time-points: after intensification/consolidation, after 6 courses of maintenance, and then every 6 months until clinical relapse. The MRD sub-study was conducted in accordance with the Declaration of Helsinki principles and was approved by the relevant Institutional Review Boards. Written informed consent was obtained from all patients.

### **MRD assessment by MFC**

MFC assays were performed on BM aspirates centralized to a single laboratory (Laboratory of Cytofluorimetry-University of Turin, Italy). BM processing was done within 24-48 hours from collection according to the EuroFlow guidelines.<sup>19,20</sup> In the RV-MM-EMN-441 trial, MRD was investigated using either two tubes with six-colors (tube 1: CD138FITC/CD56PE/CD20PerCp-Cy5.5/CD117APC/CD45APC-H7/CD38PE-Cy7; tube 2: cyKappaFITC/cyLambdaPE/CD19PerCp-Cy5.5/CD56APC/CD45APC-H7/CD38PE-Cy7).

Acquisition and analyses were performed using a FACSCanto II Flow Cytometer equipped with FACSDiva software (BD Biosciences, San José, CA) and a minimum of  $1 \times 10^6$  of events for each sample were acquired. In the RV-MM-COOP-0556 trial, we switched to a panel of two tubes with eight-color (tube 1: CD81F/CD27PE/CD138PC5.5/CD19PE-Cy7/CD20APC/CD38PB/CD45KO; tube 2: cyKappaFITC/cyLambdaPE/CD138PC5.5/CD19PE-Cy7/CD56APC/CD117APC-A750/CD38PB/CD45KO); acquisition and analyses were performed using a NAvios flow cytometer equipped with Kaluza software (Beckman Coulter, Brea, CA) and a minimum of  $2 \times 10^6$  of events for each sample were acquired. Flow-CR was defined as the detection of  $<20$  clonal

plasma cells among  $\geq 200.000$  nucleated cells at a sensitivity level of  $10^{-4}$  to  $10^{-5}$  in two consecutive evaluations.

### **MRD assessment by ASO-RQ-PCR**

Genomic DNA from BM samples was isolated using DNAzol reagent (Life Technologies-Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Patient-specific IgH rearrangements were amplified and directly sequenced from genomic DNA at diagnosis,<sup>21</sup> sequences were analyzed by IMGT/V-QUEST tool [<http://www.imgt.org>]<sup>22,23</sup> and patient-specific ASO-primers and consensus probes were designed as previously described.<sup>21</sup> IgH-based MRD detection by ASO-RQ-PCR was performed using an AbiPrism7900HT (Life Technologies-Applied Biosystems, Carlsbad, CA, USA) and MRD analysis was interpreted following the Euro-MRD guidelines.<sup>24</sup> Molecular-CR (m-CR) was defined as two consecutive negative MRD results by ASO-RQ-PCR with minimal sensitivity of  $10^{-5}$ . The molecular MRD kinetics analysis was performed using the observed marginal means of natural logarithms (ln) PCR values.

### **Cytogenetic characterization**

Fluorescent in situ hybridization (FISH) was performed on purified CD138+ cells obtained from BM at diagnosis following standard procedure. Patients were divided in two groups according to their FISH profile: high-risk, with at least one of del17p13 or t(4;14) or t(14;16); or standard-risk, without any of the previous chromosomal abnormalities. When such data were not available, patients were included in the missing data category.



## **Statistical analysis**

Data of the two trials were pooled together and analyzed. MRD population was defined as patients with an available MRD sample before and/or after starting maintenance. PFS was calculated from date of BM sampling before maintenance to the date of progression or death or the date the patient was last known to be in remission. OS was calculated from date of BM sampling before maintenance to the date of death or the date the patient was last known to be alive. Time-to-event data were analyzed using the Kaplan–Meier method, log-rank test was used to compare curves. Multivariate Cox proportional hazards models were used to estimate hazard ratios (HRs) and the 95% confidence intervals (CIs) for MRD status, gender, age, International Staging System (ISS), cytogenetic risk and intensification/consolidation therapy (ASCT and no ASCT). Subgroup analyses were performed to determine the consistency of effects of MRD-negative vs MRD-positive in the different subgroups, using interaction terms between MRD status and each of the covariate included in the Cox model. All HRs were estimated with their 95% confidence intervals (95%CI) and two-sided p-values. Pearson correlation coefficient (r) was used to compare methods for MRD analysis (ASO-RQ-PCR and MFC). Data were analyzed as of December, 2017 using R (Version 3.1.1).

## **Results**

### **Patient characteristics and MRD after intensification/consolidation**

Overall, 105 patients who achieved  $\geq$ VGPR and who had BM plasma cell infiltration  $\geq$ 5% at baseline were enrolled in the MRD sub-study. A total of 73 (70%) could be analyzed, 32 (30%) could not due to unsuccessful sequencing or lack of clonality (Figure S2). Median age was 57

years (IQR 53 to 61 years); 10 patients (14%) had ISS stage III and 24 (33%) had high-risk cytogenetic profile (Table 1). Thirty-five patients received ASCT intensification/consolidation (40% achieved a VGPR and 60% a CR or sCR), whereas 38 patients did not (39% achieved a VGPR and 61% a CR or sCR).

Before maintenance, 33/72 (46%) patients achieved m-CR (in 1 patient, sample was not collected at pre-maintenance) and 44/70 (63%) patients achieved flow-CR (in 1 patient, sample was not collected at pre-maintenance; in 2 patients, samples could not be evaluated due to low cellularity or hemodilution), with a higher proportion in the ASCT setting (Figure S3).

All 73 patients started maintenance with Lenalidomide. The achievement of MRD negativity after intensification/consolidation significantly improved PFS both by ASO-RQ-PCR (median not reached vs 37.1 months for MRD negative vs positive, respectively,  $p=0.01$ ) and MFC (median not reached vs 26 months, for MRD-negative vs MRD-positive, respectively,  $p=0.002$ ).

### **MRD status during maintenance**

At the time of data cut-off, the median duration of maintenance was 29.1 months (IQR 16.4-40.4), 9 patients discontinued therapy due to adverse events, including a second cancer (Figure S2). Lenalidomide-maintenance therapy further improved the rates of MRD negativity: among MRD-positive patients after intensification/consolidation, 12/39 (31%) obtained a m-CR and 6/26 (23%) obtained a flow-CR during maintenance: the higher negativity rate in the first group could partially explain the better PFS obtained in patients MRD positive by ASO-RQ-PCR after intensification/consolidation compared with those MRD positive by MFC.

In particular, 9 m-CR (4 after ASCT and 5 after CRD or VMP) and 5 flow-CR (2 after ASCT and 3 after CRD or VMP) were obtained after 6 months of starting maintenance, 1 flow-CR after 18 months, 2 m-CR after 18 months and 1 m-CR after 24 months. Both ASO-RQ-PCR and MFC showed that the achievement of MRD negativity during maintenance significantly improved PFS in all patients, with median PFS not reached with both techniques as compared with 26 months for persistent MRD-positive patients by ASO-RQ-PCR ( $p < 0.001$ ) and 19.5 months for persistent MRD-positive patients by MFC ( $p < 0.001$ ) (Figure 1). When compared with baseline prognostic factors for MM in a Cox model for PFS, MRD negativity was the most significant factor to reduce the risk of progression or death by using both methods (HR 0.29, 95%CI 0.14-0.62,  $p = 0.001$  by ASO-RQ-PCR; and HR 0.19, 95%CI 0.09-0.41,  $p < 0.001$  by MFC) (Figure 2).

By subgroup analyses, in MRD-negative patients, PFS was similar between ASCT vs no ASCT, as well as between patients with ISS I vs II/III (Figure 3). Importantly, MRD-negative patients with high-risk cytogenetic profile had a longer PFS (median not reached by both ASO-RQ-PCR and MFC) versus MRD-positive patients (median: 22.6 months by ASO-RQ-PCR and 15.4 by MFC) (Figure 4). Both MRD methods showed significantly prolonged OS for all MRD-negative patients, with 4-year OS of 84% and 80%, compared with 60% and 61% in ASO-RQ-PCR and MFC MRD-positive patients ( $p = 0.02$  and  $P = 0.06$ , respectively) (Figure 5).

Moreover, patients with persistent MRD negativity during maintenance had the best outcome compared with patients who became MRD-positive during maintenance or were MRD-positive during the whole treatment (Figure S4). In fact, as previously reported,<sup>25</sup> we identified three groups of patients with different molecular MRD kinetic patterns: i) 37 patients showed a persistent MRD response (51%) achieving a long-lasting m-CR until 36 months of maintenance, with a median tumor burden reduction of 10 ln-PCR, and in this group only 7 (19%) patients relapsed; ii); 14 patients experienced a transient MRD response (19%) achieving an initial MRD

response with a median tumor burden reduction of 8 ln-PCR; nevertheless, MRD subsequently reappeared, with a median increase of 4 ln-PCR, and in this group 9 patients relapsed (64%); iii) 22 patients showed a minimal MRD response (30%) with a median tumor burden reduction of 5 ln-PCR, and in this group 15 (68%) patients progressed. The same trend was observed in the MFC analysis (Figure S5). Finally, MRD relapse anticipated myeloma progression by a median of 8-9 months; 3 patients experienced extramedullary relapse and only 1/3 was previously MRD-positive.<sup>26</sup>

### **Comparison of MFC and ASO-RQ-PCR analyses**

Overall, MFC and ASO-RQ-PCR methods had a highly significant level of concordance for MRD analysis ( $r=0.9$ ,  $p<0.001$ ) (Figure 6). A total of 317 samples were analyzed for MRD detection both by MFC and by ASO-RQ-PCR. The two methods were concordant in 285 (90%) analyses, both MRD-positive in 34% or both MRD-negative in 56% analyses. Discordances between the two methods were found in 32 (10%) paired samples, in particular 27/203 (13%) MFC-negative samples were PCR-positive, while 5/114 (4%) MFC-positive samples were PCR-negative.

### **Discussion**

Depth of response is one of the most relevant clinical prognostic factors in MM. Thanks to effective drug-combinations and the positive results obtained with immunotherapy, deeper responses (CR) can now be achieved. Therefore, physicians are re-defining the goal of therapy, focusing on long-term control, quality of life and even cure.<sup>27</sup> MRD analysis is a valid tool to

better characterize response. Here, we assessed MRD both by ASO-RQ-PCR and MFC assays in NDMM patients eligible for high-dose therapy who received intensification/consolidation with Bortezomib and Lenalidomide-based strategies after front-line induction, followed by Lenalidomide maintenance. In a previous report<sup>26</sup> on a small cohort of the RV-MM-EMN-441 study, we found a significant impact of MRD on PFS. Yet, a correlation between the molecular and immunophenotypic techniques was not possible due to the small number of patients with detectable molecular marker. In this pooled analysis, we evaluated both techniques in a larger series and both methods confirmed the significant impact of MRD on PFS (HR 0.29,  $p=0.001$  and HR 0.19,  $p<0.001$ , respectively). Of note, sensitivity was slightly higher for ASO-RQ-PCR ( $10^{-5}$ ) but, moving from a 6-colour to a 2<sup>nd</sup> generation flow technique in the majority of our samples series (73%), we observed an improvement in the level of MRD sensitivity by MFC ( $10^{-4}$  to  $10^{-5}$ ).

In line with other studies,<sup>28,29</sup> we observed a high correlation between the two MRD techniques ( $r=0.9$ ,  $p<0.001$ ) and discordances only in 10% of paired samples. In addition, ASO-RQ-PCR was applicable only in 70% of samples (excluding samples with less than  $<5\%$  BM plasma cells), owing to the lack of clonality and unsuccessful sequencing; whereas MFC showed 97% of feasibility (9 samples out of a total of 327 were not evaluable due to low quality or hemodilution). Discordant results could be due to the half-life of M-components that disappear over a prolonged time compared with rapid induction of apoptosis of BM PCs.<sup>5</sup> Moreover, discordances could be attributed to different sensitivity and the different analyzed target. The newer techniques (NGS/NGF) can overcome these limits and are now accepted as standardized MRD assessment procedures. Moreover, the use of functional whole body imaging techniques such as PET/CT has highlighted the problem of spatial heterogeneity,<sup>30</sup> with negativity on BM analysis but with presence of skeletal lesion or extramedullary disease. In fact, we missed 2/3

extramedullary relapses by evaluating the BM compartment only. Therefore, these techniques may be considered complementary with sensitive immunophenotypic and molecular-based assays. Attempts to standardize results interpretation are ongoing.<sup>31</sup> Novel emerging technologies are evaluating the possibility to explore MRD in peripheral blood circulating tumor cells (CTCs) or by ultra-deep sequencing of cell-free DNA. Nevertheless, these methods can help to explore mutational profile and subclonal composition of MM cells rather than detecting MRD, due to the potential lack of sensitivity in peripheral blood after treatment.<sup>32-34</sup> We confirmed that MRD negativity is a strong prognostic factor in MM patients, together with baseline clinical and biological characteristics (ISS, cytogenetic risk, therapy). Importantly, MRD-negative patients with high-risk cytogenetics had prolonged PFS (median not reached by both ASO-RQ-PCR and MFC) versus MRD-positive patients (median 22.6 months by ASO-RQ-PCR and 15.4 by MFC). This observation is in line with data from a Spanish study, where the presence of baseline high-risk cytogenetic features and persistent MRD at day 100 post-transplant were associated with early relapse.<sup>6</sup> This is a very high-risk patient population and early intervention is mandatory, whereas the achievement of molecular- or flow-CR can overcome the poor outcome with high-risk cytogenetics.

One of the limitations of this pooled analysis was identified in the relatively small sample size, which limits the multivariate and subgroup analyses. In fact, in our Cox model, the impact of cytogenetics – as well as other well-known prognostic factors for MM – is not as significant as expected. Moreover, another aspect that affected the number of patients in the subgroup analysis is that many high-risk patients may not have reached the first MRD assessment point. Finally, another limitation is that we could not analyze the overall population of the two studies at the completion of consolidation because we did not assess all  $\geq$ VGPR patients by both techniques and, as a consequence, it was impossible to perform an intention-to-treat analysis

considering the missing patients as positive (there would be the chance to mistakenly include a lot of CR or VGPR patients). MRD may help define the success of a treatment and is a valid tool to compare different strategies, particularly in randomized trials. Here, we demonstrated that higher MRD negativity rates are achieved in the transplant arms (56-67% by ASO-RQ-PCR and MFC, respectively) vs no transplant (37%-59% by ASO-RQ-PCR and MFC, respectively); both source studies demonstrated a superior outcome with high-dose therapy vs CRD or VMP intensification.<sup>16,17</sup> Consistently, in the IFM/DFCI study, more patients achieved MRD negativity in the transplant arm, with no PFS difference in patients achieving MRD negativity according to treatment arm.<sup>35,36</sup> Similar results were reported in the MRC study comparing conventional chemotherapy to immunomodulatory drugs prior to transplant: although more patients achieved MRD negativity with immunomodulatory-based induction, no survival differences were seen according to type of induction.<sup>37</sup> Therefore, choosing a regimen that increases the chance of MRD negativity is crucial. Castor and Pollux studies, investigating the addition of Daratumumab to RD or VD in relapsed/refractory MM patients, also confirmed the better outcome of MRD-negative patients, regardless of previous therapies (Daratumumab-VD or RD vs VD or RD alone), although the experimental arms led to higher rates of MRD negativity.<sup>38-40</sup> We demonstrated that Lenalidomide continuous treatment can further improve quality of response or maintain it over time, suggesting that both MRD negativity and continuous therapy may be important in long-term outcome. In our analysis, almost one third of MRD-positive patients became MRD-negative during maintenance treatment and this substantially confirmed the similar trend of thalidomide maintenance in the analysis by Rawstron et al.<sup>41</sup> Of note, only a prospective study analyzing maintenance versus no maintenance could really confirm these data, since a delayed response to consolidation/intensification could blind the authentic maintenance response, especially because most were obtained in the first 6 months. Finally, on

the basis of these data, we could speculate that the earlier one achieves MRD negativity the better is the outcome, especially if it is persistent during the whole treatment.

Future randomized clinical trials based on MRD status at different time points will confirm such important results and will establish the role of delayed transplantation, consolidation vs no consolidation, and length of maintenance after front-line therapy.



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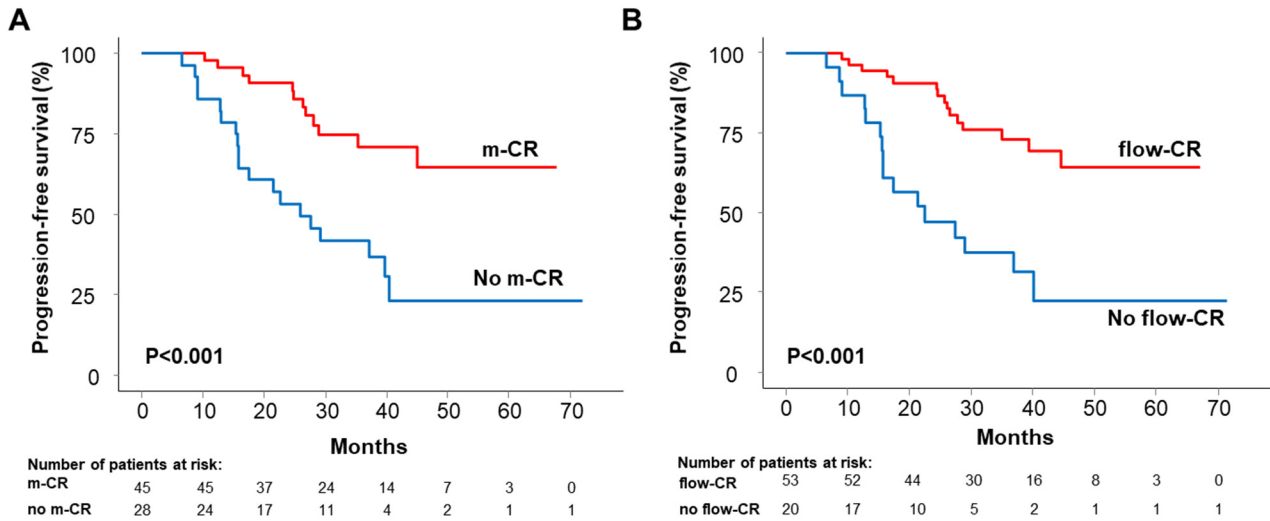
**Table 1. Patient characteristics**

<b>N=73</b>	
<b>Age (years)</b>	
Median (IQR)	57 (53 - 61)
<b>Gender N (%)</b>	
M	37 (51)
<b>ISS</b>	
I	30 (41)
II	33 (45)
III	10 (14)
<b>Creatinine (mg/dL)</b>	
Median (IQR)	0.9 (0.75 - 1.01)
<b>LDH (U/L)</b>	
Median (IQR)	302 (209 - 360)
Missing data N (%)	8 (11)
<b>Cytogenetic features N (%)</b>	
Deletion 17p	7 (10)
Translocation (4;14)	17 (23)
Translocation (14;16)	2 (3)
High risk	24 (33)
Missing data	6 (8)
<b>Trial</b>	
RV-MM-EMN-441	25 (34)
RV-MM-COOP-0556	48 (66)
<b>Random N (%)</b>	
no ASCT	38 (52)
ASCT	35 (48)

**IQR:** interquartile range, **ISS:** International Staging System, **LDH:** lactate dehydrogenase, **FISH:** fluorescence in situ hybridization, **ASCT:** autologous stem cell transplantation

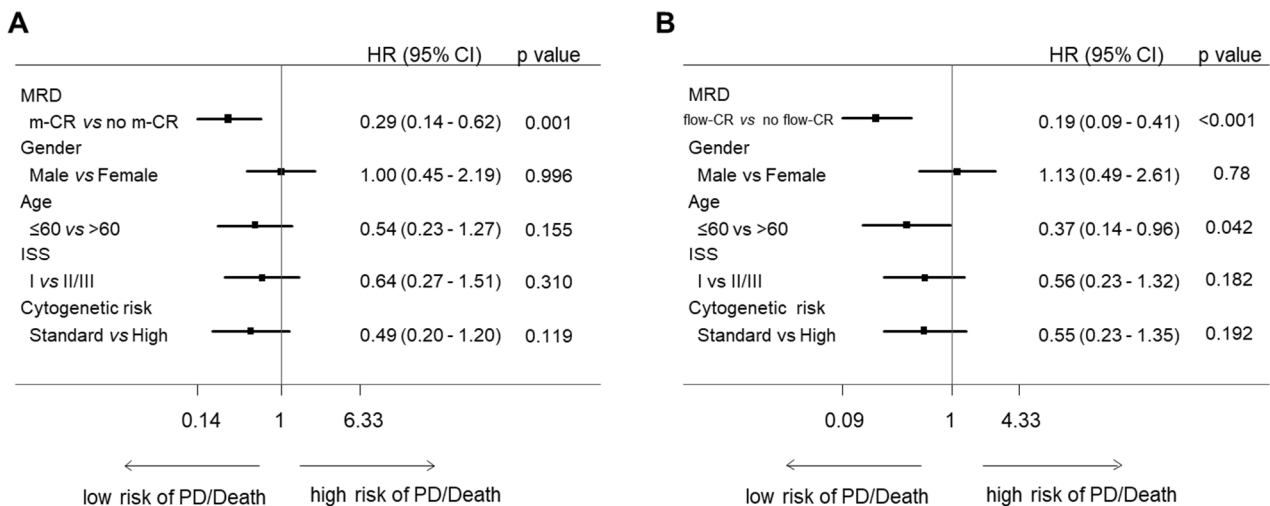
# Figures

**Figure 1. Kaplan-Meier estimates of PFS during maintenance**  
PFS analysis by A) ASO-RQ-PCR; B) MFC.



**Legend.** PFS, progression-free survival; ASO-RQ-PCR, allelic-specific oligonucleotide real-time quantitative polymerase chain reaction; MFC, multiparameter flow cytometry; m-CR, molecular-complete response; flow-CR, flow-complete response.

**Figure 2. Cox model for PFS during maintenance**  
Cox analysis by A) ASO-RQ-PCR; B) MFC.

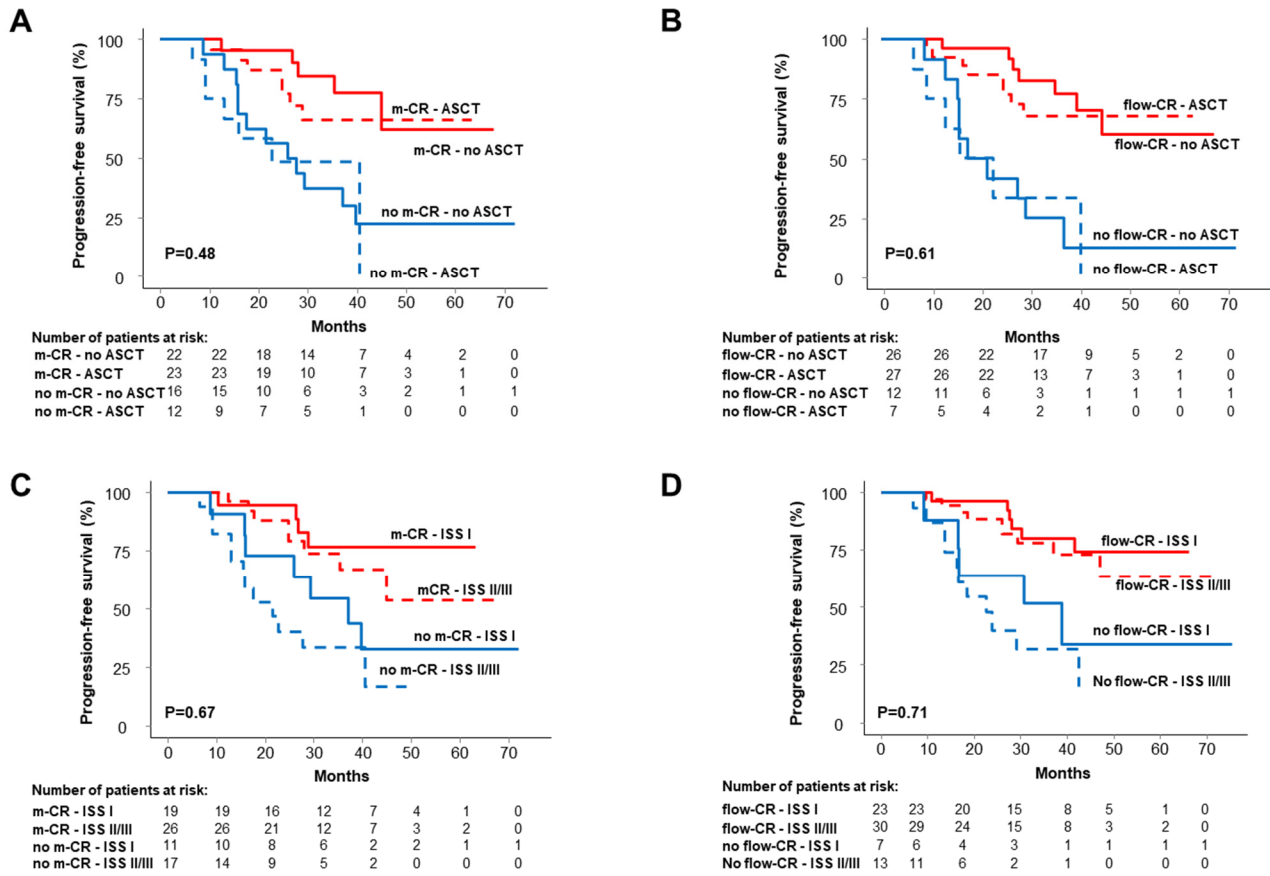


**Legend.** PFS, progression-free survival; ASO-RQ-PCR, allelic-specific oligonucleotide real-time quantitative polymerase chain reaction; MFC, multiparameter flow cytometry; m-CR, molecular-complete response; flow-CR, flow-complete response; MRD, minimal residual disease; PD, progressive disease; HR, hazard ratio; CI, confidence interval.

**Figure 3. Kaplan-Meier estimates of PFS during maintenance according to therapy and ISS**



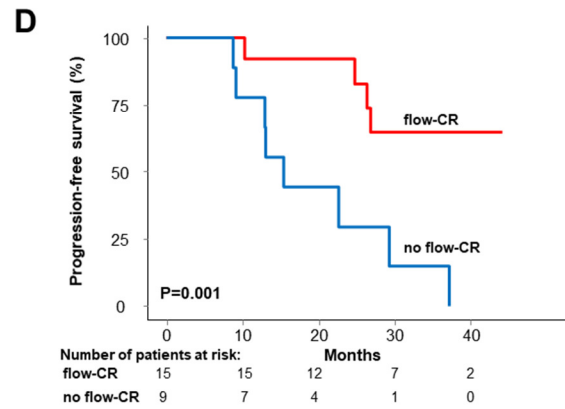
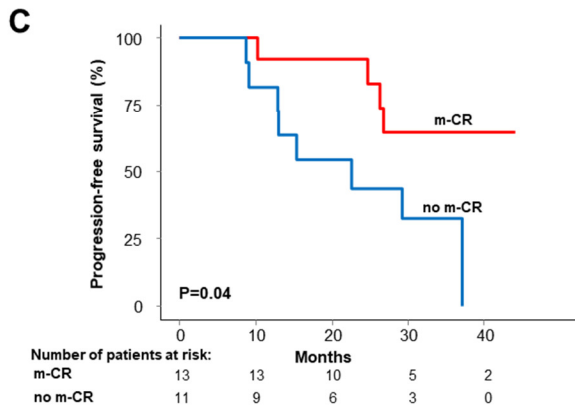
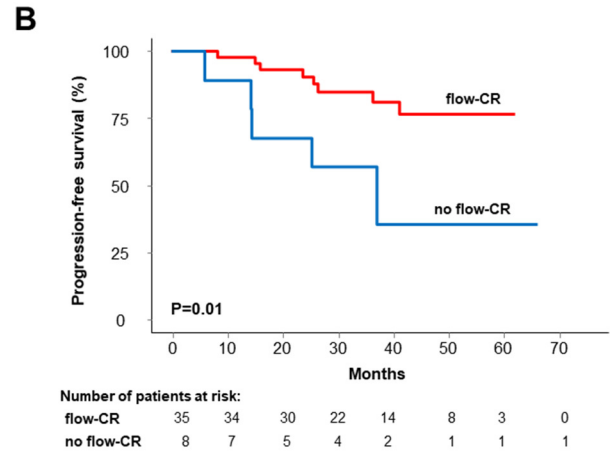
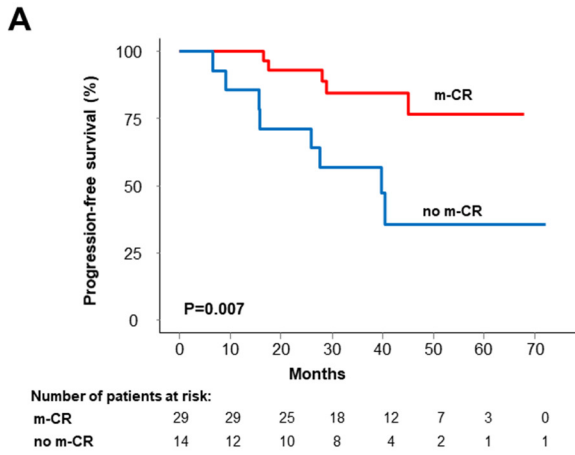
PFS analysis among A) ASCT vs no ASCT MM patients by ASO-RQ-PCR; B) ASCT vs no ASCT MM patients by MFC; C) ISS I vs ISS II/III MM patients by ASO-RQ-PCR and D) ISS I vs ISS II/III MM patients by MFC.



**Legend.** PFS, progression-free survival; ASO-RQ-PCR, allelic-specific oligonucleotide real-time quantitative polymerase chain reaction; MFC, multiparameter flow cytometry; m-CR, molecular-complete response; flow-CR, flow-complete response; ISS, International Staging System; MM, multiple myeloma; ASCT, autologous stem-cell transplantation.

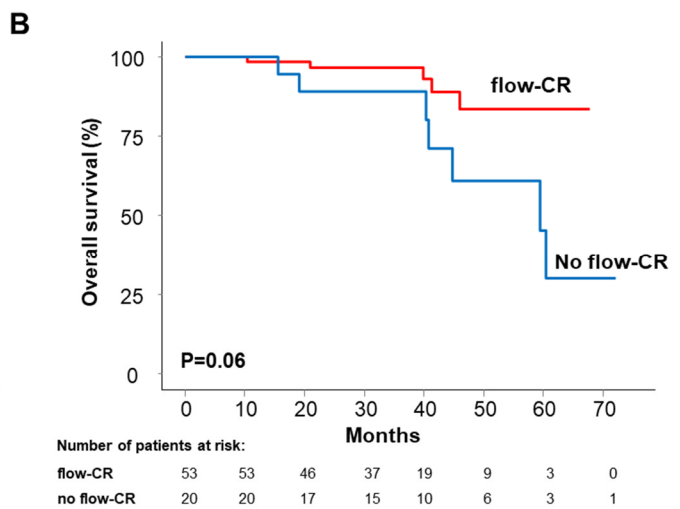
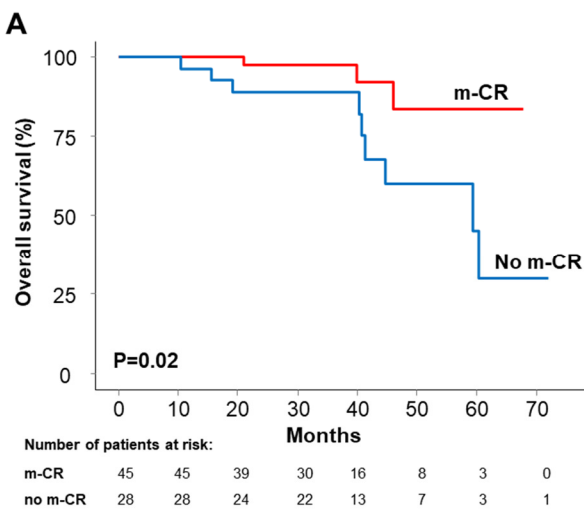
**Figure 4. Kaplan-Meier estimates of PFS during maintenance according to cytogenetic risk**

PFS analysis among patients with standard risk cytogenetic A) MRD negative vs MRD positive by ASO-RQ-PCR; B) MRD negative vs MRD positive by MFC; PFS analysis among patients with high risk cytogenetic; C) MRD negative vs MRD positive by ASO-RQ-PCR; D) MRD negative vs MRD positive by MFC.



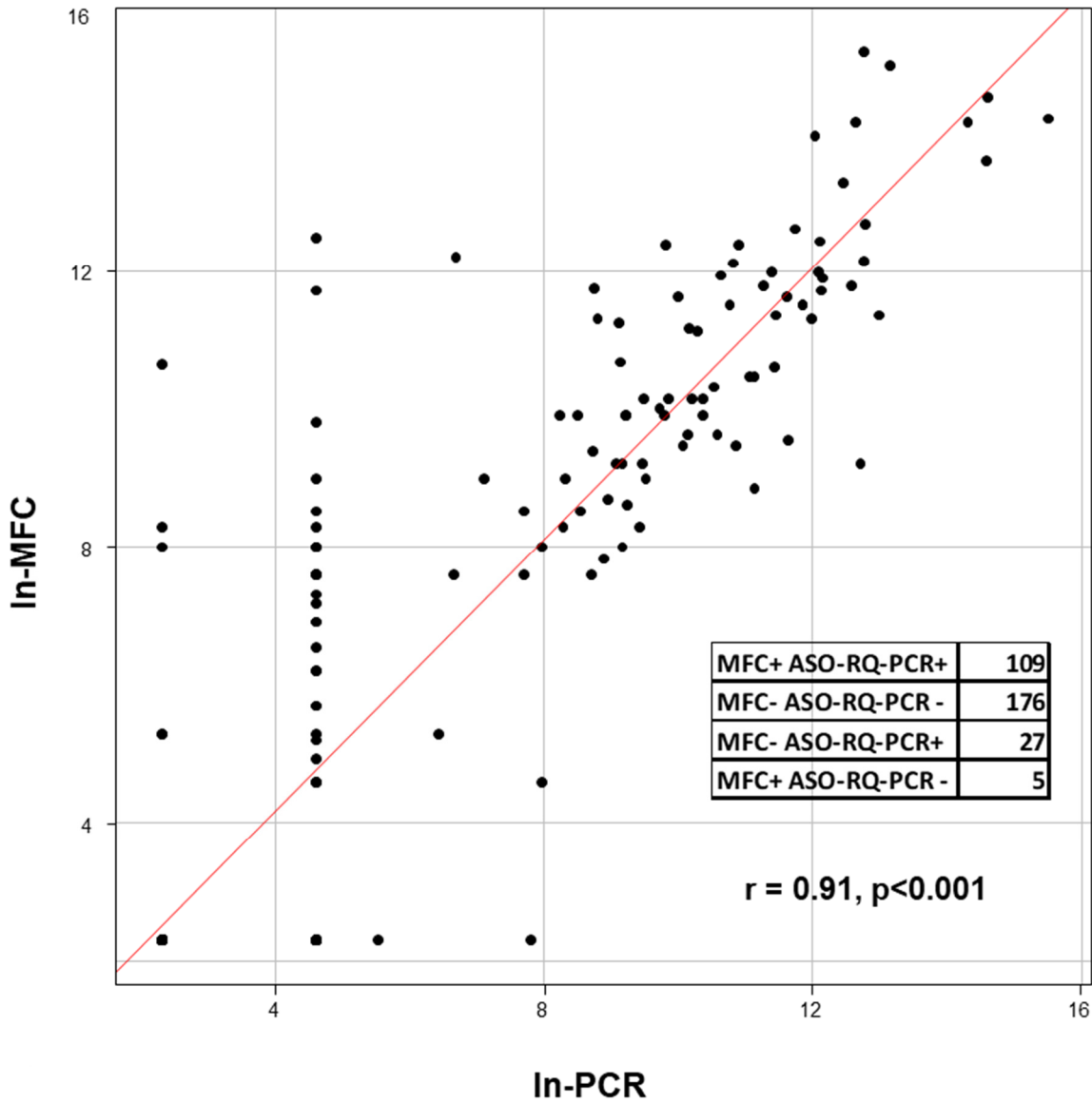
**Legend.** PFS, progression-free survival; ASO-RQ-PCR, allelic-specific oligonucleotide real-time quantitative polymerase chain reaction; MFC, multiparameter flow cytometry; m-CR, molecular-complete response; flow-CR, flow-complete response; MRD, minimal residual disease; ISS, International Staging System.

**Figure 5. Kaplan-Meier estimates of OS during maintenance**  
OS analysis by A) ASO-RQ-PCR; B) MFC.



**Legend.** OS, overall survival; ASO-RQ-PCR, allelic-specific oligonucleotide real-time quantitative polymerase chain reaction; MFC, multiparameter flow cytometry; m-CR, molecular-complete response; flow-CR, flow-complete response.

**Figure 6. Correlation between MRD results by ASO-RQ-PCR and by MFC**



**Legend.** ASO-RQ-PCR, allelic-specific oligonucleotide real-time quantitative polymerase chain reaction; MFC, multiparameter flow cytometry; MRD, minimal residual disease.

# Minimal Residual Disease by Flow Cytometry and ASO-RQ-PCR in Myeloma Patients Receiving Lenalidomide Maintenance A Pooled Analysis

## SUPPLEMENTARY APPENDIX

**Figure S1. Study designs:** (A) RV-MM-EMN-441; (B) RV-MM-COOP-0556

**Figure S2. Flow diagram**

**Figure S3. MRD status after intensification/consolidation. MRD status by** (A) ASO-RQ-PCR; (B) MFC

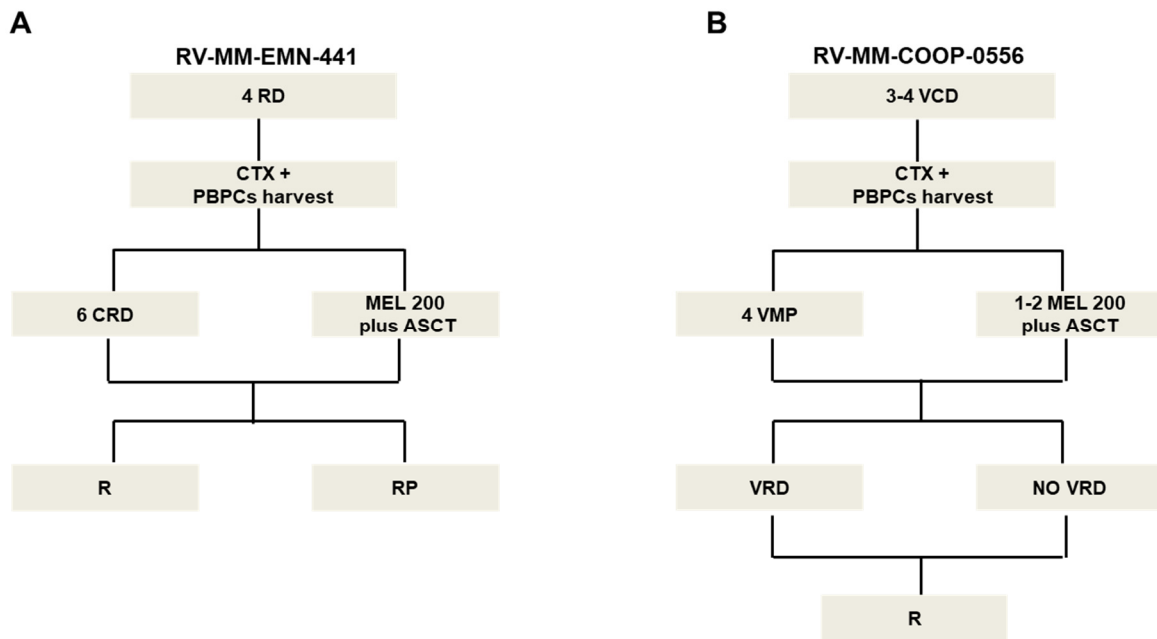
**Figure S4. Kaplan-Meier estimates of PFS and OS during maintenance**

PFS analysis among patients with persistent MRD negativity and patients who became MRD positive by (A) ASO-RQ-PCR, (B) MFC; OS analysis among patients with persistent MRD negativity and patients who became MRD positive by (C) ASO-RQ-PCR, (D) MFC.

**Figure S5. MRD kinetics: (A) ASO-RQ-PCR; (B) MFC**

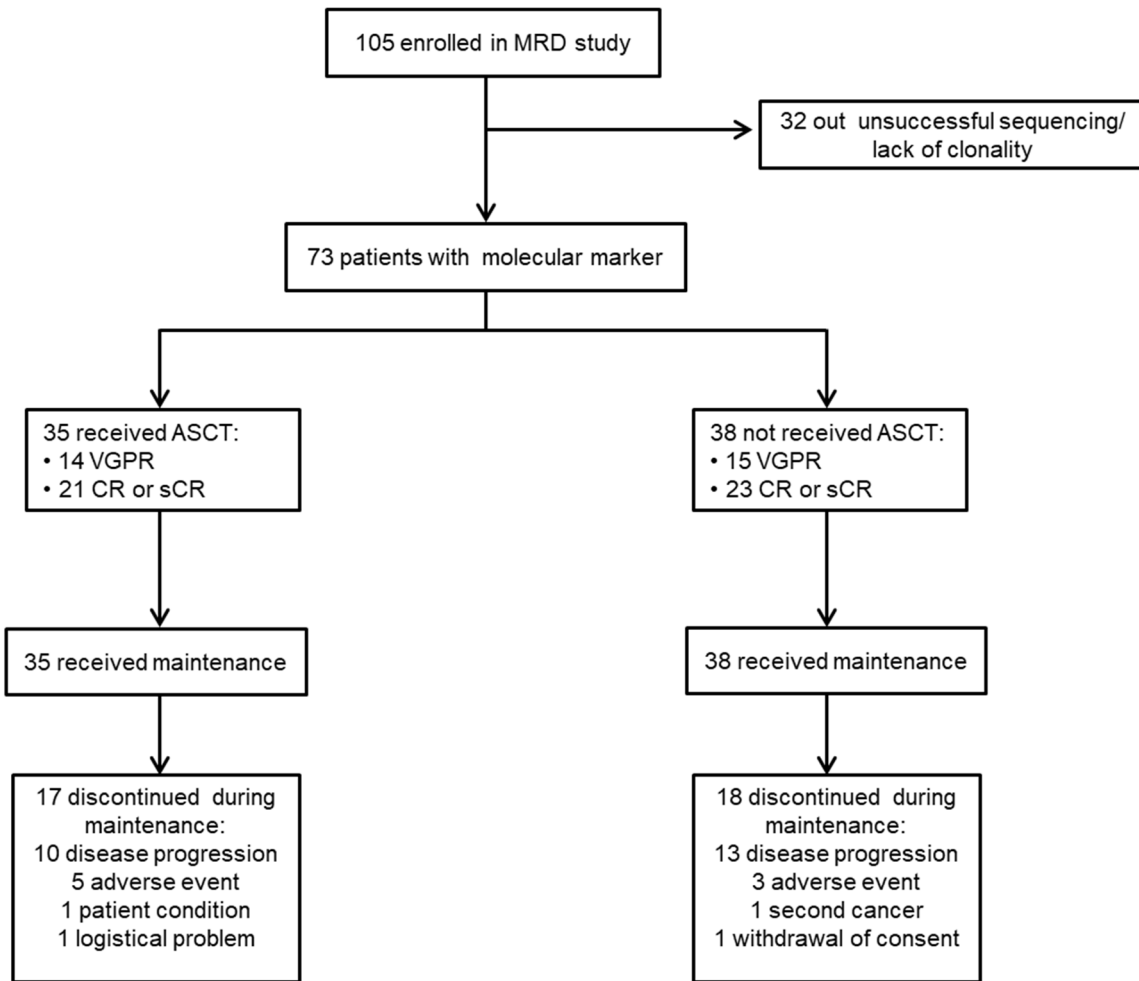
Blue line, patients with a stable MRD response; green line, patients with a transient MRD response; red line, patients with a minimal MRD response.

**Figure S1. Study designs: (A) RV-MM-EMN-441; (B) RV-MM-COOP-0556**



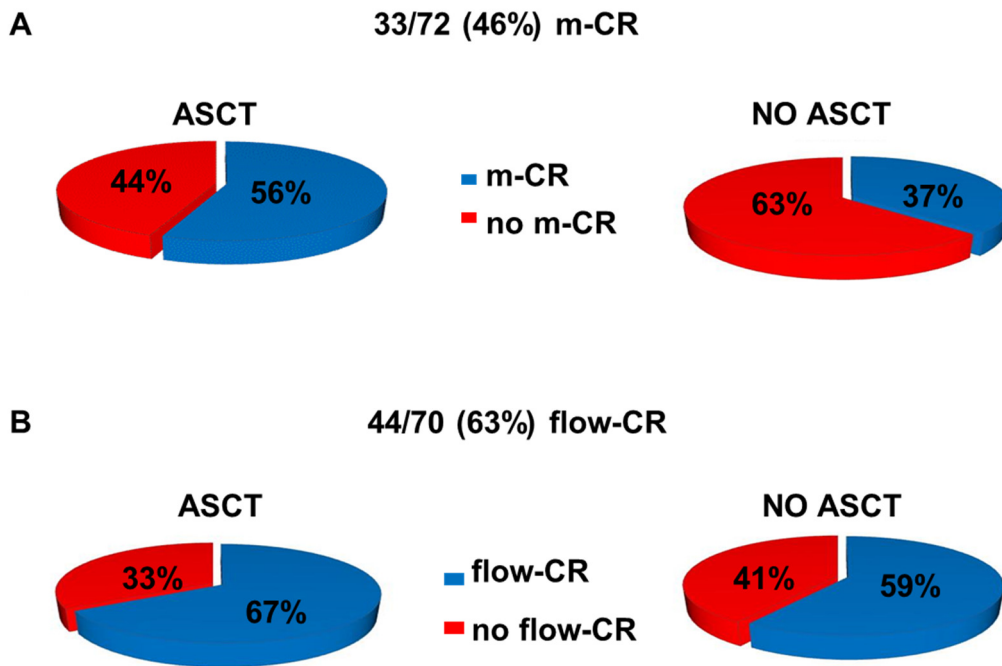
RD, Lenalidomide-Dexamethasone; CTX, Cyclophosphamide; PBPC, peripheral blood progenitor cells; CRD, Cyclophosphamide-Lenalidomide-Dexamethasone; MEL200, Melphalan 200/mg<sup>2</sup>; ASCT autologous stem-cell transplantation; R, Lenalidomide; RP, Lenalidomide-Prednisone; VCD, Bortezomib-Cyclophosphamide-Dexamethasone; VMP, Bortezomib-Melphalan-Prednisone; VRD, Bortezomib-Lenalidomide-Dexamethasone.

**Figure S2. Flow diagram**



VGPR, very good partial response; ASCT, autologous stem-cell transplantation; CR, complete response; sCR, stringent complete response.

**Figure S3. MRD status after intensification/consolidation. MRD status by (A) ASO-RQ-PCR; B) MFC**

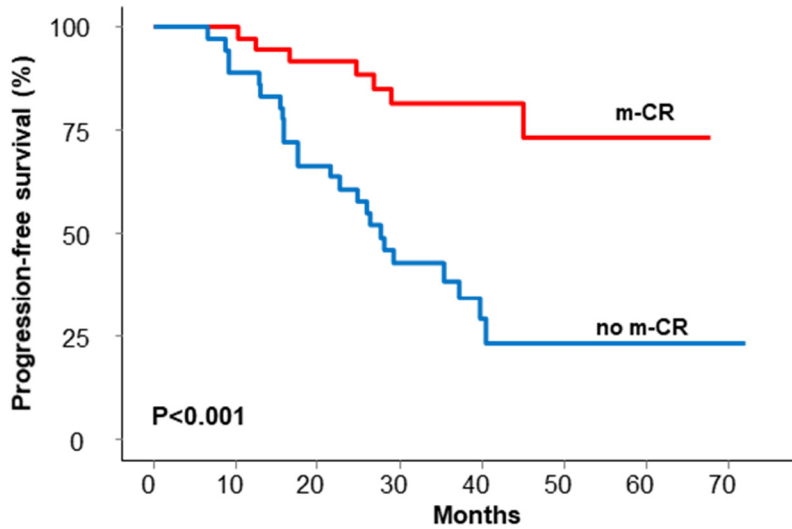


MRD, minimal residual disease; MFC, multiparameter flow cytometry; ASO-RQ-PCR, allelic-specific oligonucleotide real-time quantitative polymerase chain reaction; ASCT, autologous stem-cell transplantation; mCR, molecular-complete response; flow-CR, flow-complete response.

**Figure S4. Kaplan-Meier estimates of PFS and OS during maintenance**

PFS analysis among patients with persistent MRD negativity and patients who turned to MRD positive by (A) ASO-RQ-PCR, (B) MFC; OS analysis among patients with persistent MRD negativity and patients who turned to MRD positive by (C) ASO-RQ-PCR, (D) MFC.

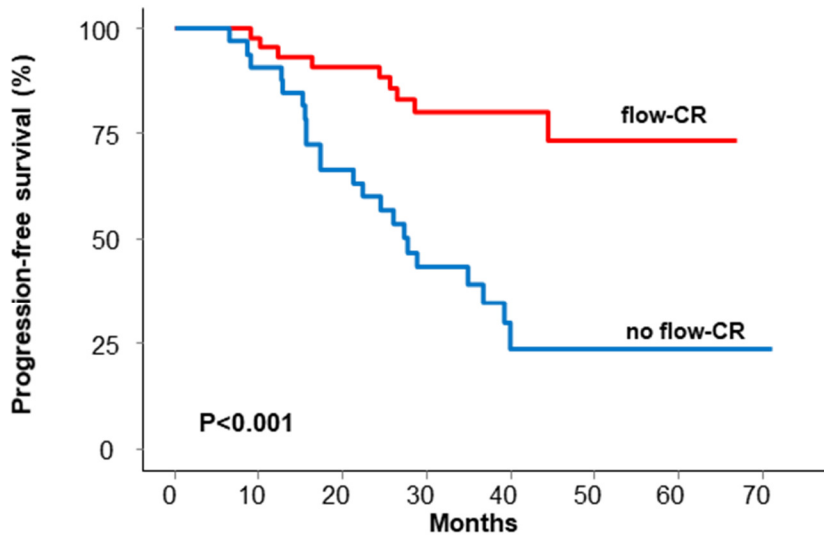
**A**



Number of patients at risk:

m-CR	37	37	30	21	13	6	3	0
no m-CR	36	32	24	14	5	3	1	1

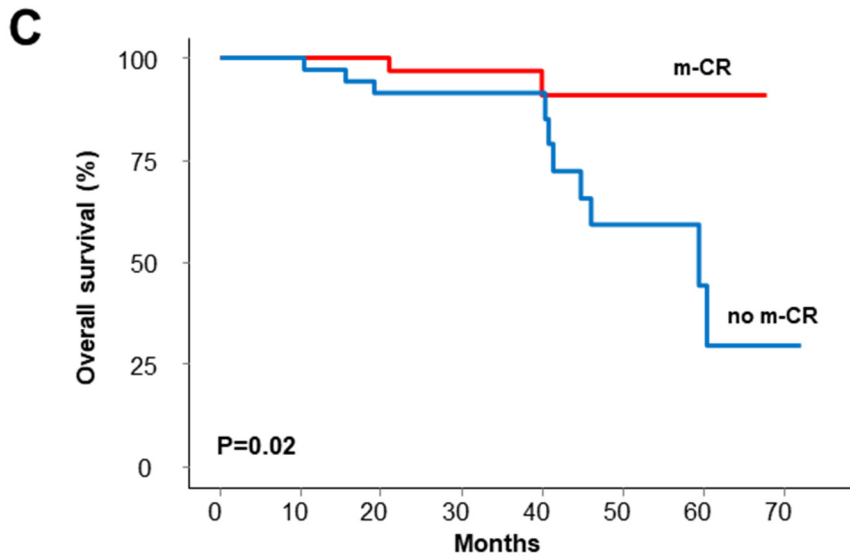
**B**



Number of patients at risk:

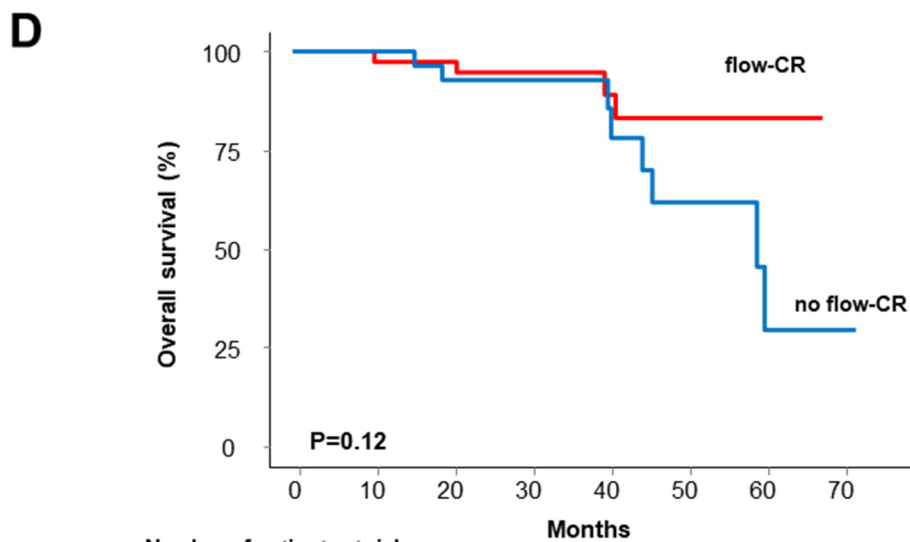
flow-CR	42	41	34	24	14	7	3	0
no flow-CR	31	28	20	11	4	2	1	1





Number of patients at risk:

m-CR	37	37	32	25	14	7	3	0
no m-CR	36	36	31	27	15	8	3	1



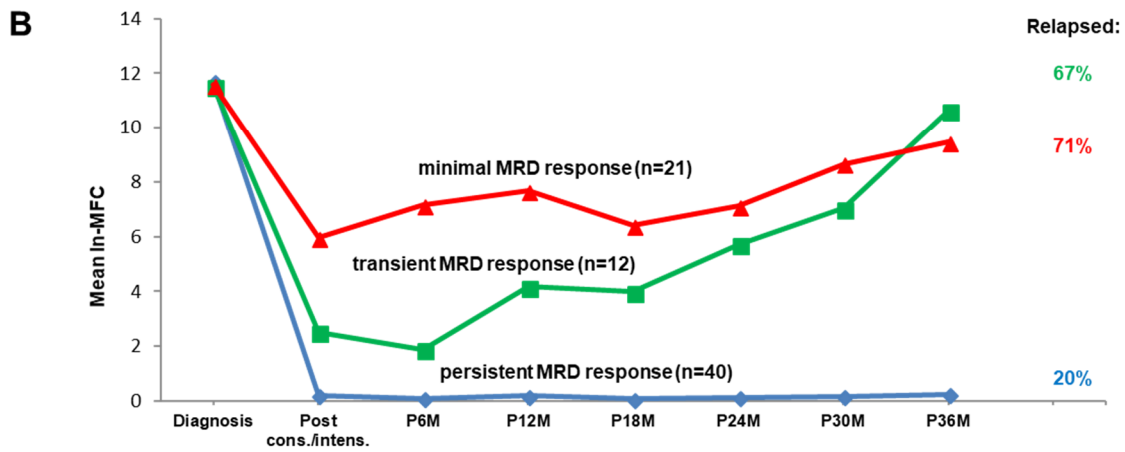
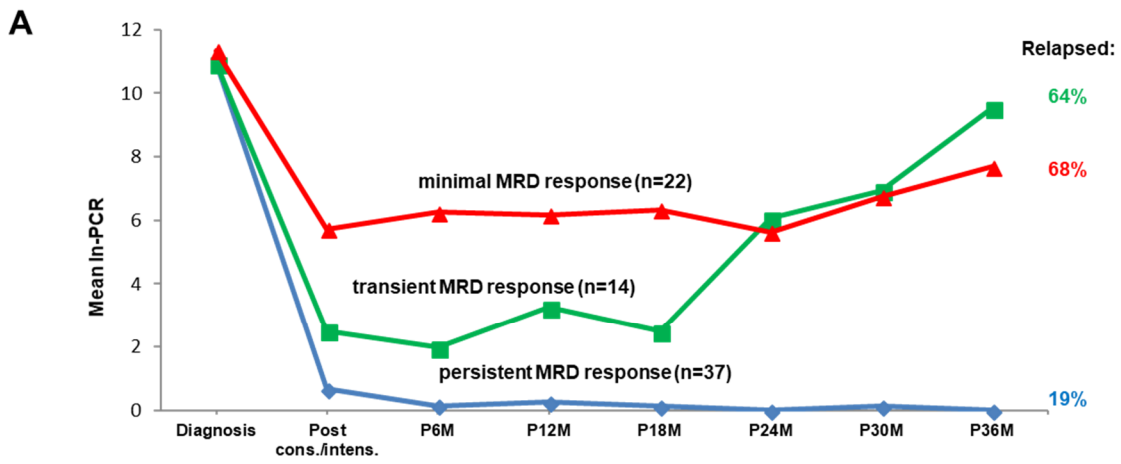
Number of patients at risk:

flow-CR	42	42	36	29	16	8	3	0
no flow-CR	31	31	27	23	13	7	3	1

MRD, minimal residual disease; PFS, progression-free survival; OS, overall survival; MFC, multiparameter flow cytometry; ASO-RQ-PCR, allelic-specific oligonucleotide real-time quantitative polymerase chain reaction; ASCT, autologous stem-cell transplantation; mCR, molecular-complete response; flow-CR, flow-complete response.

**Figure S5. MRD kinetics: (A) ASO-RQ-PCR; (B) MFC**

Blue line, patients with a stable MRD response; green line, patients with a transient MRD response; red line, patients with a minimal MRD response.



MRD, minimal residual disease; MFC, multiparameter flow cytometry; ASO-RQ-PCR, allelic-specific oligonucleotide real-time quantitative polymerase chain reaction.