Minimal residual disease monitoring and immune profiling using second generation flow cytometry in elderly multiple myeloma

Running head: 2nd generation flow MRD monitoring in elderly MM

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Key points:

- MRD monitoring is one of the most relevant prognostic factors in elderly MM irrespectively of patients age and cytogenetic risk
- Using 2nd generation MFC immune profiling concomitant to MRD monitoring also contributed to identify patients with different outcome

Abstract

The value of minimal residual disease (MRD) in multiple myeloma (MM) has been more frequently investigated in transplant-eligible than elderly patients. Since an optimal balance between treatment efficacy and toxicity is of utmost importance in elderly MM, sensitive MRD monitoring might be particularly valuable in this patient population. Here, we used 2nd generation 8-color multiparameter-flow-cytometry (MFC) to monitor MRD in 162 transplant-ineligible MM patients enrolled in the PETHEMA/GEM2010MAS65 study, The transition from 1st to 2nd generation MFC resulted in increased sensitivity, and allowed to identify three patient groups according to MRD levels: MRD-negative ($<10^{-5}$; n=54, 34%), MRD-positive between $<10^{-4}$ and $\geq10^{-5}$ (n=20, 12%), and MRD-positive $\geq10^{-4}$ (n=88, 54%). MRD status was an independent prognostic factor for time-to progression (-TTP- HR:2.7; *P*=.007) and overall survival (-OS- HR:3.1; *P*=.04) with significant benefit for MRD-negative patients (median TTP not reached, 70% OS at 3-years), and similar poorer outcomes for cases with MRD levels between $<10^{-4}$ and $\geq10^{-5}$ vs $\geq10^{-4}$ (both median TTP of 15 months; 63% and 55% OS at 3-years). Furthermore, MRD-negativity significantly improved TTP of patients >75-years (HR:4.8; *P*<.001), and those with high-risk cytogenetics (HR:12.6; *P*=.01). Using 2nd generation MFC, immune profiling concomitant to MRD monitoring also contributed to identify patients with poor, intermediate and favorable outcome (25%, 61% and 100% OS at 3-years; *P*=.01); the later patients being characterized by an increased compartment of mature B-cells. Our results show that similarly to transplant-candidates, MRD monitoring is one of the most relevant prognostic factors in elderly MM, irrespectively of patients' age and cytogenetic risk.

The trial is registered within ClinicalTrials.gov (number NCT01237249): https://clinicaltrials.gov/ct2/show/NCT01237249

Introduction

We are witnessing a remarkable progress in multiple myeloma (MM), with several new drugs being recently approved [1-5] and an armamentarium of emerging new agents with novel mechanisms of action showing promising efficacy [6], altogether resulting in a significant prolongation of patients' survival.[7] The increasing availability of drugs with well-balanced efficacy/toxicity profiles has led to the design of more complex and prolonged treatment strategies [8,9], but it has also raised the unmet need for surrogate markers to predict overall survival (OS) and accelerate the approval of new agents.[10] Thus, there is a growing body of evidence indicating that minimal residual disease (MRD) assessment can potentially be used as a biomarker to evaluate the efficacy of different treatment strategies and potentially act as surrogate for OS, particularly among transplant-eligible patients [11,12]; however, it is perhaps in elderly MM, the most common patient subgroup and for which an optimal balance between efficacy and toxicity is of utmost importance, that sensitive response assessment could help to avoid under- or over-treatment. Unfortunately, the value of MRD monitoring in elderly MM has only been investigated in two series of well-defined transplant-ineligible patients: the PETHEMA/GEM2005MAS65 study [13] and the non-intensive pathway of the MRC Myeloma IX clinical trial.[14] While the achievement of MRD negativity predicted for a significant prolongation in time-to progression (TTP) [15-17] and OS [18] in the PETHEMA/GEM2005MAS65 study, no statistically significant differences in survival were noted between MRD negative and positive patients in the non-intensive pathway of the MRC Myeloma IX clinical trial [19]; therefore, although recent studies indicate that high MRD negative rates can be achieved by a significant number of transplant-ineligible patients treated with optimized therapeutic combinations [20,21], the clinical significance of MRD monitoring in elderly MM remains an (important) open question.

Here, we investigated the role of MRD assessment in transplant-ineligible MM patients enrolled in the PETHEMA/GEM2010MAS65 clinical trial using a 2nd generation 8-color multiparameter flow cytometry (MFC) assay. Upon demonstrating the increased specificity and sensitivity of 2nd vs 1st generation MFC, and the clinical relevance of MRD detection at 10^{-5} levels, we show that MRD negativity in elderly MM predicts for prolonged survival, irrespectively of patients' cytogenetics and age. Moreover, by taking further advantage of the 2nd generation 8-color MFC assay, we also showed for the first time that immune profiling of MM during MRD monitoring after therapy, is prognostically relevant and allows the identification of patients with either poor survival or sustained disease control despite persistent MRD.

Patients and methods

Study design. The PETHEMA/GEM2010MAS65 is an open-label, phase 2 trial for newly-diagnosed elderly MM patients, randomized (1:1) into a sequential scheme consisting of 9 cycles of bortezomib, melphalan and prednisone (VMP) followed by 9 cycles of lenalidomide and low-dose dexamethasone (Rd), or the same regimens in an alternating scheme (one cycle of VMP alternating with one Rd, up to 18 cycles).[22] All samples were collected after informed consent was given by each patient, according to the local ethical committees and the Helsinki Declaration. The trial is registered within ClinicalTrials.gov (number NCT01237249).

Patients. Overall, 162/241 patients enrolled in the PETHEMA/GEM2010MAS65 had bone marrow (BM) aspirates monitored for MRD. Patient selection for MRD testing was based on the presence of M-component response; accordingly, 80% of the patients with BM aspirates centralized for MRD assessment were in very good partial response or better, and 50% were in CR as defined by the International Myeloma Working Group response criteria.[23] The distribution of patients between treatment arms was well balanced (n=78 and n=84 for the sequential and alternating arms, respectively) (Figure 1). Median follow-up after enrollment of the 162 patients under study was of 36 months (and 30 months in the whole series of 241 patients [22]). At 36 months, 79/162 (49%) of patients had progressed and 34/162 (21%) had died.

2nd generation multiparameter flow cytometry (MFC). A single 8-color antibody combination (CD45-PacB, CD138-OC515, CD38-FITC, CD56-PE, CD27-PerCPCy5.5, CD19-PECy7, CD117-APC, CD81-APCH7) was used to discriminate between phenotypically aberrant and normal PCs, and MRD negativity was defined when <20 clonal plasma cells (PCs) were detected among ≥2x10^6 leukocytes (<0.001%; limit of detection: 10^{-5}). Briefly, phenotypically aberrant PCs were identified according to under-expression of CD19, CD27, CD38, CD45, and/or CD81, overexpression of CD56 and asynchronous expression of CD117. A minimum of two aberrant phenotypes (e.g.: co-expression of CD56 and CD117) were required to define a cluster of clonal PCs. Six of the 225 (3%) patients had, according to their diagnostic immunophenotyping (following EuroFlow guidelines [24]) during enrollment in the PETHEMA/GEM2010MAS65 study, light-chain restricted clonal PCs lacking aberrant phenotypes for all markers tested. Since light-chains were not assessed with the 2nd generation MFC assay, MFC-based MRD monitoring was considered not applicable for these six cases; therefore, the applicability of the 2nd generation MFC assay was of 97%. The 8-color combination also allowed for the enumeration of erythroid (CD117$^+$, CD38$^{-/dim}$, CD45$^{-/dim}$, SSC^{lo}) and myeloid (CD117$^+$, CD38$^+$, CD45^{dim}, SSC^{hi}) hematopoietic progenitors, erythroblasts (CD45$^-$, CD38$^-$, SSC^{lo}), mast cells (CD117^{bright}, CD45^{dim}), eosinophils (CD45^{bright},

CD81^{bright}, SSC^{hi}), basophils (CD38^{+}, CD81^{-}, CD45^{dim}), monocytes (CD45^{+}, CD38^{+}, CD81^{+}, SSC^{int}), neutrophils (CD45^{dim}, CD81^{-}, SSC^{hi}), B-lymphocytes and their respective precursor (CD19^{+}, CD45^{dim}, CD38^{bright}, CD27^{-}), naïve (CD19^{+}, CD45^{+}, CD38$^{-/dim}$, CD27^{-}) and memory (CD19^{+}, CD45^{+}, CD38$^{-/dim}$, CD27^{+}) subsets, as well as TNK-plus NK-cells (CD45^{+}, CD56^{+}, CD19^{-}, SSC^{lo}) and remaining T-lymphocytes (CD45^{+}, CD56^{-}, CD19^{-}, SSC^{lo}); such data was used to generate individual patient' immune profiles for 146 patients. Briefly, principal component analysis (PCA) based on the 13 cell subsets enumerated was performed using the automated population separator (APS; principal component 1 vs. principal component 2) graphical representation and multivariate analysis tool of the Infinicyt software (Cytognos SL, Salamanca, Spain), as described elsewhere.[25] BM samples were acquired in a FACSCantoII flow cytometer using the FACSDiva software program (Becton Dickinson Bioscience, San Jose, CA), and data was analyzed with the Infinicyt software. MRD assessment was centralized in three PETHEMA/GEM laboratory-cores, cytometrists were blinded to all clinical data, and results were prospectively uploaded into a locked intranet dataset.

Cytogenetic Characterization. Interphase fluorescence-in-situ-hybridization (FISH) was performed at diagnosis on immunomagnetic-enriched PCs from 132/162 cases with MRD assessment after therapy. Patients were tested for *IGH* translocations, +1q, and del(17p13); those cases displaying a t(4;14), t(14;16) and/or del(17p13) were classified as having high-risk disease (n=26) and all other cases as standard-risk (n=106).

Statistical analyses. The Kruskal-Wallis test was used to estimate the statistical significance of differences observed between groups. Survival curves were plotted by the Kaplan-Meier method, and compared by the two-sided log-rank test. Time-to progression (TTP) was defined as the time from MRD assessment to disease progression, and overall survival (OS) as time from MRD assessment to death from any cause. A multivariate Cox proportional hazard model was developed to explore the independent value of variables with significant impact on the univariate analysis, and variables were retained in the model for levels of significance P <.05.The SPSS software (version 20.0; IBM, Chicago, IL, USA) was used for all statistical analyses.

Results

2nd generation MFC-based MRD monitoring. In a first step, we determined the differences in specificity and sensitivity between the 2nd generation 8-color MFC assay and the 1st generation test, based in only 4-markers (CD19, CD38, CD45 and CD56) and the evaluation of $2x10^5$ cells. For this purpose, we created a reference database consisting of normal and clonal PCs in order to determine, by PCA, the individual contribution of the novel markers to discriminate between both PC populations (Figure 2A). CD56 ranked as the most significant marker followed by CD19, CD81, CD27, CD117, CD45, CD38, and CD138; thus, up to three new markers (i.e.: CD81, CD27 and CD117) ranked higher than CD45 and CD38. Afterward, we focused on 50 randomly selected MRD-positive patients enrolled in this study to compare, according to the reference database, the performance of 4- vs. 8-color discrimination between clonal and normal PCs. PCA of 4-color data showed MRD cells from 9/50 patients to be located in the overlapping area between 1 and 2 standard deviations (SD) of the normal and clonal PCs references (82% accuracy; Figure 2B); by contrast, in PCA of 8-colors data all but two patients were accurately located in the clonal PC reference, outside 1 or 2 SD curves of the normal PC reference (96% accuracy; Figure 2C). To investigate the potential increment in sensitivity introduced by 2nd vs 1st generation MFC, we used the Infinicyt software to reduce the total number of analyzed cells from $2x10^6$ (2nd generation) to $2x10^5$ (1st generation) in the same 50 MRD-positive patients described above. Noteworthy, when only $2x10^5$ cells (1st generation) were analyzed up to 15/50 MRD-positive cases (30%) were wrongly classified as being MRD-negative because clonal PCs became undetectable or insufficient to define an MRD cluster (Supplementary Figure 1). Furthermore, we showed that detecting persistent MRD with a sensitivity of 10^{-5} was clinically meaningful, since only MRD-negative cases ($<10^{-5}$) had significantly longer survival, while patients with MRD levels between 10^{-4} and 10^{-5} had similar outcome to that of cases with MRD levels $\geq 10^{-4}$, (Figures 2D and 2E).

Clinical significance of MRD negativity in elderly MM. We first assessed the impact of the first 9 cycles of chemotherapy in the patients' MRD status. Twenty-five of 127 (20%) cases monitored at cycle 9 were MRD-negative, without significant differences between the sequential vs alternating regimens (20% vs 19%; *P* =.97). MRD-based stratification resulted in marked differences in outcome, with MRD-negative patients at cycle 9 showing significantly prolonged TTP and OS vs patients in CR but MRD positive and to those in less than CR (Figures 3A and 3B, respectively). In fact, no significant differences were observed between MRD-positive patients in in CR vs less than CR.

To understand the kinetics of MRD response with sequential vs alternating 18 cycles of treatment, we analyzed 83 patients with paired MRD assessments at cycles 9 and 18. Sixteen (19%) MRD-positive cases at cycle 9 became MRD-negative at cycle 18, with no significant differences between rates of MRD negativity after sequential vs alternating regimens (23% vs 15%, respectively; *P* =.28). No MRD-negative cases at cycle 9 turned to MRD positive at cycle 18. The overall MRD-negative rate at cycle 18 were slightly higher (but not significantly) in patients randomized to the sequential vs alternating schema (46% vs 33%; *P* =.16). Noteworthy, the design of the PETHEMA/GEM2010MAS65 trial allowed to investigate the immediate impact in patients' outcome according to their MRD status without additional therapy. Thus, the median TTP from the moment of MRD assessment (cycle 18) was of only 12 months for patients in less than CR, 20 months for cases in CR but MRD-positive, and not reached for the MRD-negative group (Figure 3C).

Afterward, we investigated the impact of MRD-negativity among the cytogenetically defined standard- and high-risk subgroups. Noteworthy, high-risk patients attaining MRD-negativity had significantly prolonged TTP vs MRD positive patients and similar TTP to MRD-negative standard-risk cases; by contrast, MRD-positive patients albeit standard-risk cytogenetics had significantly inferior TTP, although superior to high-risk MRD-positive cases (Figure 4A). We also investigated whether the impact of attaining MRD-negativity was equally beneficial according to patients' age. Interestingly, while median TTP from MRD assessment was not reached for patients with both 65-75 and >75 years who reached MRD-negativity, it became remarkably shorter for MRD-positive patients, irrespectively of age (Figure 4B). These findings were similarly noted when patients' MRD status was analysed separately at cycles 9 and 18. Multivariate analysis including prognostic factors such as patients' age, ISS, FISH cytogenetics, CR and MRD response, showed that only cytogenetics and MRD monitoring retained independent prognostic value for both TTP and OS (Table 1).

Prognostic value of immune profiling during MRD monitoring. To evaluate whether the BM immune profile of individual patients at the time of MRD assessment could also be predictive of outcome, we developed individual patient' immune signatures (n=146) based on the unsupervised BM distribution of 13 immune cell populations identified with the 2nd generation MFC assay (n=58 at cycle 9, n=88 at cycle 18). This approach revealed the existence of 3 patient clusters (Figure 5A) - A (n=16), B (n=117) and C (n=13) – that were segregated by progressively increasing numbers of erythroblasts and B-cell precursors, together with progressively decreasing numbers of mature naïve and memory B-cells (Figure 5B). There were no significant differences in cluster frequency according to treatment schema, nor according to

baseline ISS or FISH risk-stratification. When compared to patients in clusters C and B, cases that clustered in group A had a trend toward a longer TTP (Figure 5C) and significantly superior OS (Figure 5D). Although the numbers preclude a definitive conclusion, a similar trend in patients' outcome according to their immune profile was observed when separately analyzed at cycles 9 and 18. Noteworthy, there were no significant differences according to patients' MRD status across the three clusters; thus, even among MRD-positive patients immune profiling continued to show an impact in patients survival, with 3-year OS rates of 100%, 65% and 0% for clusters A, B and C, respectively; *P*=.003).

Discussion

Over the last decade, different groups have shown the added value of MRD assessment over conventional response criteria in transplant-eligible MM patients.[16,17,19,26-28] MRD clearance is also achievable in elderly MM in the era of novel and more effective treatment strategies [15-17,19-21], but because its prognostic value has only been sporadically investigated in well-selected transplant-ineligible patients [15,19], its potential role as a biomarker to predict survival remains less clear in elderly MM. Herein we show that on intention to treat, up to 22% (n=54/241) of transplant-ineligible patients enrolled in the PETHEMA/GEM2012MAS65 study reached MRD-negativity, which resulted in a significant prolongation in TTP and OS. Similarly to what has been previously postulated for transplant-candidates [11,28], MRD response emerged here as one of the most relevant prognostic factors also in elderly MM patients.

The available data on the prognostic value of flow-based MRD assessment was mostly obtained using conventional, "1st generation" MFC based in 4- or 6-color combinations, with a limit of detection of 10^{-4}.[29] More sophisticated ("2nd generation") MFC has been progressively introduced [21,26] which is expected to improve the sensitivity and specificity of MRD monitoring, but the extent of such improvement has never been investigated. Here, we used the cytometric software developed by the EuroFlow Consortium [24,30] to show that the transition from 1st generation 4-color to a 2nd generation 8-color MFC assay that measured ten-times more cells, resulted in a significantly increased specificity and sensitivity. Noteworthy, we showed that by applying the limit of detection reached with 1st generation MFC (i.e.: 10^{-4}), up to 30% of patients with persistent MRD detectable by 2nd generation MFC would had been wrongly classified as MRD-negative. We also showed that the ability to monitor MRD up to the 10^{-5} sensitivity level is clinically relevant, since this level identifies a subset of patients (those between 10^{-4} and 10^{-5}) with inferior survival than MRD-negative ($<10^{-5}$) cases, and similar to that of MRD-positive patients at the $\geq 10^{-4}$ levels. Our results extent on recent data reported by Korde et al [21], in which the prognostic value of MRD monitoring using novel 8-color MFC compares well to that of next-generation sequencing (NGS) (1 relapse among MRD negative cases by MFC vs 0 relapses among MRD negative cases by NGS), and shows superior intention-to-treat applicability [98% vs 80% for MFC vs NGS, respectively].[21] That notwithstanding, the advent of even more sensitive "next-generation" MFC will likely outperform the method used in the present study [31] and therefore, the ability of MFC to monitor MRD and predict survival will continue to increase in MM. The same applies for the advent of more sensitive and applicable NGS as compared to former molecular methods.[17,21] Accordingly, the recent development and availability of two highly-sensitive and

potentially standardized next-generation methods envisions that MRD monitoring and patient prognostication will be even more powerful in the future.

In the present study, we have shown that sensitive MRD assessment after the first 9 cycles of chemotherapy allowed to discriminate patients with remarkable different outcomes; thus, only 16% of MRD-negative patients at cycle 9 have progressed so far, whereas more than half (54%) of MRD-positive cases have relapsed albeit receiving further chemotherapy. Noteworthy, no significant differences were observed between MRD-positive patients in CR vs less than CR, suggesting that current response categories fail to identify patients with different outcome if MRD persists. Even among patients in CR plus a normal serum free light chain ratio, the persistence of MRD predicted significantly inferior TTP (data not shown). Furthermore, since MRD-positive cases at cycle 9 had identical dismal outcomes irrespectively of receiving 9 additional cycles of Rd or VMP/Rd (sequential or alternating scheme, respectively; data not shown), they might be considered as candidates to receive novel agents with alternative mechanisms of action (e.g.: monoclonal antibodies).[1,2] It should be noted that in contrast to previous studies in which MRD assessment was performed at intermediate stages of patients' treatment (e.g.: before maintenance) [15], the design of the PETHEMA/GEM2010MAS65 trial allowed to investigate the immediate impact on patients' outcome according to their MRD status without additional therapy. Thus, we report here new data showing that MRD-positive patients at cycle 18 (i.e.: without further therapy) had a TTP after MRD assessment of approximately 1.5 years without statistically significant differences according to conventional response criteria (i.e.: CR vs less than CR). The clinical significance of our results is two-fold: 1) MRD-positive patients should be considered as candidates for further (alternative) therapies in order to control chemoresistant PCs, and 2) the definition of CR would benefit also in elderly patients from incorporating MRD assessment into the response criteria.[11] In this regard, sequential MRD monitoring would be particularly attractive to identify patients with sustained MRD-negativity; accordingly, herein the best outcome was noted amongst the eighteen MRD-negative cases at both cycles 9 and 18, fifteen (i.e.: 83%) remain progression-free and seventeen (i.e.: 94%) alive, albeit no additional therapy.

Due to their poor prognosis and the unmet need for novel agents, patients with high-risk cytogenetics are ideal candidates to investigate the role of MRD monitoring both as a clinical end-point for novel treatment modalities and a surrogate biomarker for survival. Here, we show that patients with high-risk FISH abnormalities reaching MRD-negativity may experience a TTP similar to that of MRD-negative cases and standard-risk cytogenetics; by contrast, TTP of standard-risk MRD-positive patients was slightly but significant (P =.02) superior to that of high-risk MRD-positive cases,

highlighting the independent and complementary role of cytogenetic and MRD risk stratification in elderly MM. Another interesting finding here reported is the fact that reaching MRD-negativity equally benefited elderly patients aged over 75. These observations suggest that eradication of MRD might be considered as a clinical end-point for all elderly patients, providing the tolerability of the proposed treatment strategy.

Recently, Barlogie *et al.* have shown that the vast majority of CR patients achieving long-term survival (10-years relapse-free), were also MRD-negative.[7] However, attaining deep-remission is not a pre-requisite in order to achieve long-term disease control [7,32], at least in specific cases, and more accurate identification of such patients should also become a research priority. Here, we show for the first time that immune profiling in MM after therapy, in parallel to MRD monitoring, might be prognostically relevant by allowing the identification of patients with either poor survival or sustained disease control. Accordingly, flow-based MRD monitoring offers complementary information to the quantification of MRD levels, and may contribute to identify a subset of patients that albeit being MRD-positive can still experience prolonged survival due to a unique immune signature specifically characterized by a more prominent regeneration of mature B-lymphocytes. In fact, a similar immune signature was previously found in both MRD negative and positive MM patients reaching long-term disease control.[33]

In summary, here we show that 2nd generation MFC supersedes previous flow-based MRD monitoring by identifying patients with lower MRD levels ($<10^{-4}$) and poor outcome, as well as MRD-positive cases with prolonged survival associated with a unique immune profiling at the time of response assessment. We also revealed that similarly to transplant-candidates, MRD monitoring is one of the most relevant prognostic factors in elderly MM, complimentary to the cytogenetic risk and superior to conventional response criteria; thus, patients with standard-risk MM and those in CR but remaining MRD-positive experience poor outcomes, and warrant potential treatment individualization to improve their survival. The availability of highly effective therapies for elderly MM patients urges the need to address if response-driven (i.e.: MRD based) treatment decisions can reduce the difference in survival between transplant-eligible vs elderly patients (or even standard- vs high-risk MM); this requires a cooperative effort towards novel clinical trials design in which patients are accurately stratified according to sensitive MRD monitoring prior to alternative treatment strategies, or even randomized into different therapeutic approaches according to their MRD status. Such clinical trials are needed to establish the exact role of MRD testing in elderly MM.

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Writing, review and/or revision of the manuscript: BP and JFSM wrote the manuscript. All authors reviewed the manuscript.

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Table 1. Multivariate analyses including baseline and post-treatment disease features with significant effect on time-to progression (TTP) and/or overall survival (OS) in univariate analysis.

	TTP		OS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (<75 vs. ≥75 years)	-	-	1.7 (0.8 – 3.7)	.16
ISS (stage I vs II&III)	-	-	2.0 (0.6 – 6.8)	.28
Interphase FISH cytogenetics (standard- vs high-risk)	2.0 (1.1 – 3.4)	.02	4.3 (2.0 – 9.2)	<.001
Depth of response (CR vs <CR)	1.7 (0.9 – 3.4)	.07	1.2 (0.5 – 2.8)	.63
MRD (negative vs positive)	2.7 (1.3 – 5.5)	.007	3.1 (1.1 – 8.8)	.04

TTP: time-to progression; OS: overall survival; CI: confidence interval; ISS: International Staging System; FISH: Fluorescence In Situ hybridization; High-risk FISH: t(4;14), t(14;16) and/or del(17p13).

MRD status (negative vs positive) was determined at cycle 18 for the 118 out of the 162 patients with MRD assessment (Figure 1). Thus, the for the remaining 44 cases the MRD status was determined at cycle 9 since no bone marrow aspirates from these patients were centralized at cycle 18, typically because of disease progression (32%), toxicity (20%), withdrawal of the informed consent (9%), or death (5%).

Figure 1. PETHEMA/GEM2010MAS65 minimal residual disease (MRD) study consort diagram. Two-hundred twenty-five patients were immunophenotyped at diagnosis, and six (3%) were excluded from further MRD monitoring due to the lack of aberrant phenotypes. One-hundred twenty-seven patients had MRD assessed at cycle 9 after consecutive cycles of VMP (n=60) or alternating VMP/Rd (n=67). One-hundred eighteen patients had MRD assessed at cycle 18 after sequential VMP followed by Rd (n=61) or alternating VMP/Rd (n=57); eighty-three of them with MRD data on cycles 9 and 18. Thus, 162 patients enrolled in the PETHEMA/GEM2010MAS65 had at least one MRD study. VMP: bortezomib, melphalan, prednisone; Rd: lenalidomide, low-dose dexamethasone

Figure 2. Improved specificity of minimal residual disease (MRD) monitoring in multiple myeloma (MM) of 2nd vs 1st generation multiparameter flow cytometry (MFC). (A) Principal component analysis (PCA) model for the phenotypic-based discrimination between normal (n=17) bone marrow (BM) plasma cells (PCs) from healthy individuals and BM clonal PCs (n=71) from MM patients. In the 2-D PCA plots, every healthy individual and patient is represented by a single dot, and normal or MM reference PC groups by 1 (dashed lines) and 2 (solid lines) standard deviation curves. Phenotypic makers are ordered according to their higher versus lower significance to discriminate between normal vs. clonal PCs. **(B&C)** Phenotypically selected clonal PCs from 50 MRD+ MM patients (blue dots) were plotted against the PCA model based on all 8 phenotypic markers available with 2nd generation MFC (CD38, CD138, CD19, CD27, CD45, CD56, CD81 and CD117) vs the PCA model based on 4 phenotypic markers only, available with 1st generation MFC (CD38, CD19 CD45 and CD56). **(D)** Time-to progression and **(E)** overall survival according to the MRD status by 2nd generation MFC (n=162). Fifty-four patients had undetectable MRD or MRD levels below 0.001% (MRD-ve <10^{-5}); twenty cases had detectable MRD in between 0.001% and 0.02% (MRD+ve ≥10^{-5} to <10^{-4}); the remaining eighty-eight patients had detectable MRD at 0.01% or higher levels (MRD+ve ≥10^{-4}).

Figure 3. Time-to progression (TTP) and overall survival (OS) according to the depth of response of multiple myeloma patients at cycle 9 (Panels A and B, respectively; n=127) and cycle 18 (Panels C and D, respectively; n=118).

Figure 4. Impact of reaching MRD negativity on time-to progression (TTP) of according to patients' cytogenetic risk (n=132) and age (Panel B; n=162).

Figure 5. In Panel A, 2-dimensional principal component analysis (PCA) plot of the patients' immune profiles defined by the distribution of 13 immune cell populations (excluding normal and clonal PCs) in the bone marrow at the time of MRD assessment (n=146). The distribution of the 13 immune cell populations in Clusters A (n=16), B (n=116) and C (n=13) is shown in Panel B, whereas in Panel C time-to progression (TTP) and overall survival (OS) according to the patients' immune-profiles clusters is shown.