Comparative analysis of minimal residual disease detection by multiparameter flow cytometry and enhanced ASO RQ-PCR in multiple myeloma

R Silvennoinen1−7, T Lundan3, V Kairisto3, T-T Pelliniemi6,5, M Putkonen6, P Anttila7, V Huotari8,9, P Mäntymaa10, S Siitonen11, L Uotila11, T-L Penttilä12, V Juvonen3, T Selander13 and K Remes6

Multiparameter flow cytometry (MFC) and allele-specific oligonucleotide real-time quantitative PCR (ASO RQ-PCR) are the two most sensitive methods to detect minimal residual disease (MRD) in multiple myeloma (MM). We compared these methods in 129 paired post-therapy samples from 22 unselected, consecutive MM patients in complete/near complete remission. Appropriate immunophenotypic and ASO RQ-PCR-MRD targets could be detected and MRD analyses constructed for all patients. The high PCR coverage could be achieved by gradual widening of the primer sets used for clonality detection. In addition, for 13 (55%) of the patients, reverse orientation of the ASO primer and individual design of the TaqMan probe improved the sensitivity and specificity of ASO RQ-PCR analysis. A significant nonlinear correlation prevailed between MFC-MRD and PCR-MRD when both were positive. Discordance between the methods was found in 32 (35%) paired samples, which were negative by MFC-MRD, but positive by ASO RQ-PCR. The findings suggest that with the described technique, ASO RQ-PCR can be constructed for all patients with MM. ASO RQ-PCR is slightly more sensitive in MRD detection than 6−10-color flow cytometry. Owing to technical demands ASO RQ-PCR could be reserved for patients in immunophenotypic remission, especially in efficacy comparisons between different drugs and treatment modalities.

**INTRODUCTION**

The importance of attaining complete remission (CR) with first-line myeloma treatment has become apparent.1,2 Accordingly, the development of new effective treatment strategies presents the challenge of finding the most precise means of comparing the efficacy of different therapies. In 2006, the International Myeloma Working Group published the consensus criteria for assessing response in multiple myeloma (MM), and categories of stringent CR, near CR (nCR) and very good partial remission were established.3 International Myeloma Working Group updated the response and progression criteria in 2011, and the terms stringent CR, immunophenotypic remission (IR) and molecular remission (MoR) have been proposed for uniform reporting of clinical trials.4

So far, the various response assessment methods have been compared with only a few published trials.5 In a study by Sarasquete et al.6 of 32 MM patients in CR, multiparameter flow cytometry (MFC) proved applicable for minimal residual disease (MRD) measurement in 90% and ASO RQ-PCR in 75% of samples. ASO RQ-PCR showed better sensitivity in MRD detection. For the samples positive by both methods the correlation in MRD levels measured was good (r=0.861).

Puig et al.7 recently published an extensive study of 170 MM patients who had achieved at least partial response. Lack of clonality detection and unsuccessful sequencing or suboptimal ASO performance limited the applicability of PCR to only 42% of all cases. By adding data from a previous study they could, however, compare four-color MFC and ASO RQ-PCR in MRD detection in a cohort of 103 MM patients. All in all, persistent MRD could be identified in 46% of cases by MFC and in 54% of cases by ASO RQ-PCR. They found a significant correlation in MRD quantification by both techniques (r=0.881, P<0.001).

In summary of the findings of previous studies, ASO RQ-PCR is the most sensitive method available for MRD detection, the major drawback being, however, that it has proved applicable to only 42−75% of patients. MFC has been described as having higher applicability, but MRD analysis by these means has not reached the highest sensitivities attainable by ASO RQ-PCR. There is a need for a sensitive, well-standardized method with high applicability and feasible costs for routine laboratory use. The EuroFlow Consortium has published antibody panels for standardized n-dimensional flow cytometric immunophenotyping of plasma cell disorders in 2012 for more accurate identification of normal and aberrant plasma cells at diagnosis and follow-up samples.8 Recently, a next-generation sequencing (NGS) method has been compared with ASO RQ-PCR and MFC in MRD detection in MM and B-cell disorders and has shown excellent concordance with...
PCR-MRD and MFC-MRD in 79.6%–85% and 83%, respectively.9,10 This new method does not require patient-specific reagents because it utilizes standard consensus primers and also has standardized sensitivity and quantifiable ranges. NGS methods may not only offer a more sensitive approach for tumor load measurement, but also information about mutations for personalized treatment. Although NGS was not able to identify an immunoglobulin (Ig)H clonotype in 2/10 MM patients owing to somatic hypermutation of IgH variable region,11 a suitable clone for MRD follow-up was identified in 91% of MM patients.10

We applied an enhanced ASO RQ-PCR approach for MRD evaluation among consecutive patients reaching CR or nCR in a prospective study by the Finnish Leukemia Group. In years 2009–2013, as previously reported,11 in the present study, we compared MFC with ASO RQ-PCR in all the 129 paired follow-up samples currently available from these patients for MRD evaluation. In addition, the response assessment by MFC and ASO RQ-PCR is compared with immunofixation electrophoresis (IFE) and free light chain (FLC) ratio.

PATIENTS AND METHODS

Patients

Forty-seven patients with previously untreated, symptomatic MM, aged between 40 and 65 years were initially treated by induction therapy comprising four 3-week cycles of bortezomib 1.3 mg/m² intravenously on days 1, 4, 8 and 11 plus dexamethasone 40 mg on days 1–4 (all cycles) and on days 9–12 (cycles 1–2). Cyclophosphamide at a dose of 2 g/m² and granulocyte colony-stimulating factor were used for stem cell mobilization. Autologous stem cell transplantation (ASCT) was performed 24–48 h after conditioning with high-dose melphalan (200 mg/m²). This study includes all 22 patients who achieved nCR or CR. Samples were collected after informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committees of the participating hospitals and by the Finnish Medicines Agency, and is registered with ClinicalTrials.gov, number NCT00861250. The design of the study has been published elsewhere.11

Clinical and laboratory evaluations were made at the time of registration, before each cycle, before mobilization and ASCT. Follow-up visits were scheduled at 3-month intervals during the first year, at 4 months during the second year and thereafter until progression. International uniform criteria were used to confirm progressive disease.5,12

Response assessment by protein analyses

Responses were assessed according to the international uniform criteria together with nCR response.5,12 Response evaluation was based on serum and urine protein electrophoresis and immunofixation or on serum FLC levels when serum and urine paraprotein were not measurable. nCR was defined as an electrophoresis-negative but immunofixation-positive status together with bone marrow plasma cells < 5%. Semi-automated agarose IFE was performed with Hydrasys LC or Hydrasys Isofocusing Systems and Hydragel 4 IF immunofixation gels (Sebia, Evry, France) as previously described.13 Analyses were decentralized to five laboratories in the university hospitals of Finland. All laboratories participate regularly in IFE quality control survey.

FLC assay

The FLC assay was centralized in TUKYSLAB (Turku University Hospital Laboratories, Turku, Finland). The assays for kappa- and lambda-free light chains in serum were performed turbidimetrically in a Roche Modular P Analyzer (Mannheim, Germany) using reagents from The Binding Site Ltd (Birmingham, UK) (Freelite Human kappa Free Kit for Hitachi/Modular P and Freelite Human Lambda Free Kit for Hitachi/Modular P). Reference intervals were determined in HUSLAB by analyzing the kappa and lambda light chains of 124 Finnish blood donors (60 men and 64 women, aged 26–72 years) with normal results in serum capillary electrophoresis (albumin, alpha-1, alpha-2, beta-1, beta-2 and gamma fractions as well as total protein; all within their respective reference ranges, with no abnormal fractions). The reference intervals produced, representing 2.5–97.5% percentiles in the reference distribution, were as follows: serum kappa-free light chain 6.9–25.6 mg/l, serum lambda-free light chain 8.6–26.5 mg/l and free kappa/lambda ratio 0.52–1.40. In the clinical interpretation, ratios outside the reference interval were considered aberrant.

Response assessment by MFC

The MFC assay was standardized and decentralized to five laboratories in the university hospitals of Finland. Bone marrow samples were washed with PBS, stained with antibodies and analyzed with FacsCanto (Becton Dickinson, CA, USA), FacsCanto II (Becton Dickinson, CA, USA) flow cytometers for 6-, 8- and 10-color protocols, respectively. The percentages of 6-, 8- and 10-color protocols were 35%, 36% and 29% of samples, respectively. The expressions of CD138, CD38, CD45, CD19, CD56, CD27, CD28, CD117 and intracytoplasmic kappa/lambda were assessed in all samples, CD45 in 91% and CD20, CD81 and CD200 in 70% of samples. The sample was considered suitable for MRD analysis if plasma cells, normal or neoplastic, were detectable. Plasma cells were gated for analysis with CD138 and CD38 expressions and light-scatter properties. Myelomatous plasma cells and normal plasma cells were identified with different expressions of the above mentioned antigens according to European Myeloma Network guidelines.14 Myeloma cells either expressed antigens not normally present on plasma cells or lacked antigens normally present on plasma cells. The most common immunophenotypic aberrancies observed in this study were frequency in parenthesis); CD19+ (100%), CD56+ (77%), CD200+ (80%), CD81+ (62%), CD27+ (59%), CD117+ (41%), CD28+ (36%), CD20+ (27%) and 55% were clonal for λ. For MFC-MRD level was reported as a percentage of myeloma cells of total number of nucleated cells. The median number of events for MFC analyses was 790 000 (range, 95 831 – 2 963 089). At least 50 immunophenotypically abnormal plasma cell events were needed for MRD. MFC-MRD was assessed by patient-specific panels, and the sensitivity of the method was calculated by dividing 50 with the number of nucleated cells acquired, the median sensitivity being thus 0.00064% (range, 0.0007–0.032%). All five laboratories had concordant results in a national quality control survey.

Detection of clonal Ig gene rearrangements and ASO primer design

The molecular and ASO RQ-PCR assays were centralized in TUKYSLAB (Turku University Hospital Laboratories, Turku, Finland). For molecular analysis unfractionated bone marrow samples were used for DNA extraction. The pretreatment bone marrow DNA samples were screened for clonal IgH, IgK and IgL rearrangements using BIOMED-2 multiplex primer sets and heteroduplex analysis on polyacrylamide gels.15 Samples negative by the multiplex primers were further analyzed by singleplex PCR reactions with family-specific consensus primers.16,17 Pooled buffy coat DNA from healthy blood donors was used as polyclonal control.

The clonal PCR products were cut from the polyacrylamide gel and sequenced in both directions by automated capillary electrophoresis sequencing (Applied Biosystems 3500xL Series Genetic Analyzer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). The sequence analysis included identification of the V-, D- and J-genes, and (V) DJ junction analysis to design the ASO primer at the hypervariable complementarily determining region 3. For oligonucleotide melting temperature (Tm) calculations an algorithm in the PrimerExpress (Applied Biosystems) was used. The initially designed ASO primer was first used in a preliminary real-time PCR together with the appropriate germline J-gene TaqMan probe and reverse primer.18 In case where there was no amplification product or the amplification was not sufficiently specific compared to eventual amplification from polyclonal control, a patient-specific TaqMan probe for the V-gene and a reverse-oriented ASO primer were designed and used for MRD analysis.

Response assessment by ASO RQ-PCR

The pretreatment sample served as quantification standard for the follow-up samples. The sensitivity and quantitative range of the analyses were determined from according to EuroMRD guidelines. However, putative low infiltration of clonal cells in pretreatment sample cause further challenge in MRD analysis of MM. Briefly, the pretreatment sample was diluted with a polyclonal buffy coat DNA in a log-linear manner down to the level of 10⁻¹. The achieved sensitivity determined from the dilution series was further adjusted by taking into account the percentage of myeloma cells in the pretreatment sample assessed by flow cytometry, and the detection limit was specified individually for each patient and sample (Table 1).
The albumin gene was used for normalization of the ASO RQ-PCR results. 0.5 μg of follow-up sample DNA was used in each of the three replicate analyses. The maximum sensitivity achievable was thus ~0.0004% (4E−6) based on the total amount of 1.5 μg of DNA, corresponding to ~240,000 nucleated cells. Molecular MRD analyses were carried out according to the EuroMRD guidelines both in the PCR setup and in interpretation of data.19 Molecular progression was defined as an at least 10-fold (1 log) increase in MRD in ASO RQ-PCR between consecutive bone marrow analyses.

Statistics
Statistical analyses for the relationship between MFC-MRD and PCR-MRD were performed with R statistical software (R Core Team 2013, R Foundation for Statistical Computing, Vienna, Austria), version 2.15.2. The exponential growth function was used to calculate the value of nonlinear correlation between MFC-MRD and PCR-MRD results. The possible predictive impact of IFE and FLC ratio for IR and MolR was analyzed using the logistic regression model with random effects. Progression-free survival (PFS) was analyzed by the Kaplan–Meier method, and the differences for significance between curves were tested with the two-sided log-rank test. PFS was measured from the ASCT date to the date of progression or death. For PFS analyses SPSS software (version 19.0; SPSS, Chicago, IL, USA) was used.

RESULTS
PCR amplification and sequencing of Ig genes
Results of the amplification of Ig genes are summarized in Table 1. Ig clonality could be detected in the pretreatment sample in all of the 22 patients who achieved nCR or CR. Where the initially recognized IgH clonality could be sequenced and a fully functioning ASO primer designed, no further clonality screening was undertaken. If necessary, further analyses included Igk and IgL multiplex PCRs and IgH singleplex PCRs.

The proportion of clonal plasma cells in the bone marrow aspirate collected and stored before treatment varied between 0.16 and 26% as evaluated by flow cytometry. In the sample with the lowest proportion of clonal cells (0.16%) all multiplex PCRs with Biomed primer sets yielded negative results. A specific clonality product could only be identified by the class-specific IgH singleplex PCR. This also applied to another sample with a low (2.1%) proportion of clonal plasma cells. Singleplex PCRs were performed for an additional six patients in order to produce better quality samples for sequencing, but for all of these the clonal product was also identifiable in multiplex PCR analyses.

At least one clonal Ig gene rearrangement could be successfully sequenced for each patient. ASO primers were designed for both rearrangements in cases where both heavy chain and light chain rearrangements were sequenced. The primer giving the best sensitivity was then chosen for follow-up analyses. For 20 patients IgH was chosen as the ASO target, for 1 patient each Igk and IgL. More detailed data on the gene segment repertoire by rearrangement are given in Table 2.

ASO primer design and testing
For nine patients the standardized method (originally designed for acute lymphoblastic leukemia) with one specific forward primer and a J-gene-specific consensus TaqMan probe yielded an assay sufficiently sensitive and specific for MRD assessment. For 13 patients the standard assay was either not sensitive enough or did not function at all. For all of these patients an alternative approach with a reverse-oriented allele-specific primer on the junctional sequence, individually designed TaqMan probe and forward consensus primer on the V-gene, yielded an assay sufficiently sensitive and specific for MRD assessment. One patient (patient no. 22) did not wish to have any follow-up bone marrow specimens drawn, and no molecular follow-up was undertaken.

| Table 1. Detection of clonal Ig rearrangements in the pretreatment sample |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Patient  |
| no.     | Clonal cells in the aspirate (%) | IgH multiplex | Igk multiplex | IgL multiplex | IgH singleplex | Selected ASO target | Individual TaqMan probe | Sensitivity of ASO RQ-PCR analysis (%) |
|---------|-----------------|----------------|----------------|----------------|----------------|----------------------|----------------------|----------------------|
| 1       | 2.1             | −              | −              | −              | +              | VDJ (IgH)            | +                    | 0.0004               |
| 2       | 10.0            | +              | +              | +              | n.d.           | VDJ (IgH)            | +                    | 0.001                |
| 3       | 3.5             | −              | +              | −              | n.d.           | VJ (IgK)             | −                    | 0.0007               |
| 4       | 1.7             | +              | n.d.           | n.d.           | n.d.           | VDJ (IgH)            | +                    | 0.001                |
| 5       | 1.39            | +              | n.d.           | n.d.           | −              | VDJ (IgH)            | +                    | 0.0007               |
| 6       | 21.5            | −              | +              | +              | −              | VDJ (IgH)            | +                    | 0.001                |
| 7       | 6.0             | +              | +              | +              | +              | VJ (IgK)             | +                    | 0.001                |
| 8       | 8.0             | +              | −              | −              | +              | DJ (IgH)             | −                    | 0.0004               |
| 9       | 20.0            | +              | n.d.           | n.d.           | +              | DJ (IgH)             | −                    | 0.001                |
| 10      | 20.0            | −              | +              | +              | +              | VJ (IgL)             | −                    | 0.0007               |
| 11      | 8.4             | +              | n.d.           | n.d.           | −              | VDJ (IgH)            | +                    | 0.001                |
| 12      | 4.3             | +              | +              | n.d.           | +              | DJ (IgH)             | −                    | 0.0004               |
| 13      | 9.1             | +              | n.d.           | n.d.           | n.d.           | DJ (IgH)             | −                    | 0.001                |
| 14      | 26.0            | +              | n.d.           | n.d.           | n.d.           | DJ (IgH)             | −                    | 0.003                |
| 15      | 0.6             | +              | −              | −              | n.d.           | VDJ (IgH)            | +                    | 0.0006               |
| 16      | 5.0             | +              | +              | +              | n.d.           | VDJ (IgH)            | −                    | 0.003                |
| 17      | 5.0             | +              | +              | +              | n.d.           | VDJ (IgH)            | +                    | 0.001                |
| 18      | 6.0             | +              | +              | +              | +              | DJ (IgH)             | −                    | 0.0006               |
| 19      | 0.16            | −              | −              | −              | +              | DJ (IgH)             | −                    | 0.0004               |
| 20      | 7.5             | +              | −              | −              | n.d.           | VDJ (IgH)            | +                    | 0.0004               |
| 21      | 11.0            | +              | n.d.           | n.d.           | +              | VDJ (IgH)            | +                    | 0.001                |
| 22      | 0.6             | +              | n.d.           | n.d.           | −              | VDJ (IgH)            | +                    | 0.0006               |
| Summary | 0.16 − 26%      | 17/22 (77%)    | 9/15 (60%)     | 7/13 (54%)     | 9/14 (64%)     | 13/22 (55%)          | 0.0004 − 0.0007      |
Comparison of ASO RQ-PCR, MFC, IFE and FLC in response evaluation

Thirty (59%) of the 22 patients with nCR/CR achieved MolR on intention-to-treat analysis and 4 more patients (7%) showed MFC-MRD negativity, but were PCR-positive. In these patients a total of 129 consecutive samples from the time point of transplantation were collected and analyzed with a median follow-up of 16 (range, 6–28) months. MFC and PCR were concordant in 75% of analyses, both positive in 29% or both negative in 46% of analyses. In 35% (32/91) of MFC-negative samples MFC was negative with a median sensitivity of <0.006% (range, 0.05–0.001%), but PCR detected MRD with a median level of 0.003% (range, 0.02–0.0004%). Only one sample was MFC positive, 0.005%, but PCR negative (sensitivity <0.001%). A comparison with positive and negative PCR and MFC results of all 129 samples is given in Table 3. The relationship of all MFC-MRD and PCR-MRD results are illustrated in Figure 1, where a coefficient of determination \( r^2 = 0.72 \) (\( P < 0.001 \)) is seen.

Neither negative serum nor urine IFE nor normal FLC ratio was statistically significant predictors for MolR (\( P = 0.44 \) and \( P = 0.06 \), respectively). Their predictive value for IR was even lower. The distribution of MFC- and PCR-MRD results between IFE-negative and-positive samples is illustrated in Figure 2, where also the numbers of abnormal FLC ratios in each group are given. Serological concordance between IFE and FLC ratio was 63%.

Prognostic value of MRD monitoring by ASO RQ-PCR and MFC

We also updated survival by MRD load. Both MRD methods showed statistically significantly prolonged PFS for MRD-negative patients, with an estimated median PFS of 48 months in both groups, compared with a PFS of 16 months in PCR-positive (\( P = 0.004 \)) and to 13 months in MFC-positive (\( P = 0.011 \)) patients (Figure 3). The prognostic value of our ASO RQ-PCR method was also analyzed in the group of 16/22 (73%) patients in CR, and showed statistically significant PFS for MRD-negative patients (Figure 4).

**DISCUSSION**

Several ongoing studies on different myeloma treatments indicate promising impact on future treatment strategies. Assessment of responses calls for a practical and reliable method to detect MRD, reflecting the depth of response. Both MFC and PCR have been investigated in this context. According to earlier observations, there is a correlation between the two methods, but PCR seems to be more sensitive by roughly one logarithm. Nonetheless, a clone-specific primer is mandatory for PCR assays, and the success rate in designing such clone-specific and sensitive assays has varied from 42 to 100%. Puig et al.\(^7\) discuss the reasons for the low feasibility rate of ASO RQ-PCR in MM. They point out that feasibility studies should include unselected MM patients, and the universally accepted EuroMRD guidelines for interpretation of ASO RQ-PCR should be followed. Somatic hypermutations are characteristic of MM and they may hamper clonality detection, sequencing success and

---

### Table 2. Gene segment repertoire by rearrangement

| \( IGHV-J \) | \( IGHD-J \) | \( IGKDEL \) |
|---|---|---|
| \( IGHV \) | \( IGHD \) | \( IGHJ \) | \( IGHD \) | \( IGHJ \) |
| 1 | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | Biallelic | 1 |
| 2 | 1 | 2 | 3 | 2 | 1 | 2 | 3 | 2 | Intron-RSS | 3 |
| 3 | 9 | 3 | 8 | 3 | 3 | 3 | 3 | 3 | IGKV1 | 1 |
| 4 | 5 | 4 | 1 | 4 | 8 | 4 | 4 | 4 | IGK2 | 1 |
| 5 | — | 5 | — | 5 | 3 | 5 | 3 | 5 | — |
| 6 | — | 6 | 4 | 6 | 2 | 6 | 2 | 6 | — |
| 7 | — | 7 | — | — | — | — | — | — | — |

In addition to rearrangements described in the table IgK KV-JK rearrangement was detected in four patients (two IGKV3-JK5, one IGKV3-JK1 and one IGKV2-JK2 rearrangement). IgL rearrangement was detected in two patients (one IGLV3-JL2/3 and one IGLV2-JL2/3).

---

### Table 3. The comparison of all PCR-pos/neg and MFC-pos/neg samples from patients who reached nCR/CR

| PCR/MFC | MFC neg | MFC pos | Total N = 129(|%|) |
|---|---|---|---|
| PCR neg | N = 91(|%) | 1 | 60 (47)| |
| PCR pos | 32 (35) | 37 (97) | 69 (53) | |

**Abbreviations:** CR, complete remission; MFC, multiparameter flow cytometry; neg, negative; nCR, near CR; pos, positive.

---

**Figure 1.** The relationship between PCR-MRD (%) and MFC-MRD (%) results \( n = 122 \), positive or negative, of 22 patients in nCR/CR from ASCT to the median follow-up of 16 (range, 6–28) months, \( r^2 = 0.72 \) (\( P < 0.001 \)). The relationship between PCR-MRD (%) and MFC-MRD (%)—positive results was \( r^2 = 0.74 \) (\( P < 0.001 \)).
ASO performance. The authors used the standardized method of ASO RQ-PCR designed for acute lymphoblastic leukemia using only one specific forward primer. However, they suggest that alternate approaches for assay design might improve the feasibility rate.

As highlighted in a study by Martinez-Lopez et al., clonal rearrangement can actually be detected at the molecular level in a very high percentage (91.5%) of all MM patients. This percentage should be even higher if Ig light chain rearrangements are considered, in addition to IgH and Kde rearrangements. By sequencing all these rearrangements and using alternate locations for consensus primers and, if necessary, even individualized TaqMan probes to cover cases with somatic mutations in the V-gene, it should be possible to attain a much higher feasibility rate. We have taken three steps to enhance the feasibility of ASO RQ-PCR in our patient cohort of consecutive and unselected symptomatic MM patients in nCR/CR, namely (1) use of singleplex Ig consensus primers in addition to the multiplex primer approach, (2) inclusion of the light chain rearrangements for clonality detection and (3) if necessary, use of a reverse-oriented allele-specific primer and individually designed TaqMan probe in the ASO RQ-PCR analysis.

With applicability rates of ASO RQ-PCR and MFC proving roughly equal, the remaining question concerns the sensitivity of these two methods. Improvement in the sensitivity of MFC should be possible by increasing the number of events under analysis. Even if the quality or available amount of bone marrow sample imposes limitations it should be possible to improve the sensitivity by increasing the number of simultaneous fluorescent colors in the analysis. Protocols and equipment are available for 6, 8 or even 10 colors, whereas most studies published so far have been made on 4-color protocols. Further, the sensitivity of ASO RQ-PCR can be improved by increasing the amount of DNA analyzed (for example, by adding replicate PCR reactions). Molecular analysis by PCR requires that DNA from the pretreatment sample has been stored for eventual later construction of the ASO RQ-PCR analysis. One distinct advantage of PCR-based analysis in comparison with flow cytometry is that long-term storage and later centralized analysis of samples can be arranged in multicenter studies. The same applies to the NGS method, in which the best achievable sensitivity (10^-6) can also be limited by an insufficient amount of DNA.

The methodological efforts of three-step ASO RQ-PCR increase the costs of analysis by ~30%. This method would be suitable especially for multicenter myeloma studies to study deep remission status among patients in CR. It is possible that the sensitivity of MFC of <10^-6 could also be improved to <10^-5.10^-6.
by using 8–10-color MFC and analysis of 0.5–1 × 10^7 cells, but theoretically the sensitivity of MFC will not reach the sensitivity and specificity of ASO RQ-PCR on any similar amount of sample materials analyzed. The fundamental difference between these two methods is that MFC usually requires 10–50 residual clonal cells for MRD detection, whereas an optimal ASO RQ-PCR and NGS method may detect even a single residual clonal cell. It may thus be predicted that on any amount of sample material analyzed ASO RQ-PCR or NGS will have about 10-fold higher sensitivity than MFC. For rearrangement detection, NGS may be the method of choice as it offers the advantage of clone quantification in the pretreatment sample and wide screening of possible rearrangements.

The weakness of our MFC method could be decentralizing, which, however, was mandatory for use of fresh samples. This study was performed before the publication of EuroFlow standardization for MFC in plasma cell disease,8 but MFC was performed by the guidelines of European Myeloma Network14 in all five centers. The authenticity of MFC-negative results could be demonstrated with the PFS difference between MFC-positive and -negative patients (Figure 3). Thus, novel standardized MFC should be compared with our enhanced ASO RQ-PCR regarding applicability, costs and sensitivity, and reflected on the outcome of patients.

The FLC ratio was not useful for response estimation in the present study. Patients in nCR/CR IFE and FLC assays are utilized patients.

In conclusion, with our enhanced ASO RQ-PCR we were able to offer PCR follow-up for all patients by the protocol with a good sensitivity of MRD. ASO RQ-PCR was slightly more sensitive than our standardized, 6–10-color MFC. However, immunophenotypic response showed a significant nonlinear correlation with molecular response, r^2 = 0.72 (P < 0.001). MRD evaluation by ASO RQ-PCR thus served as a better biological indicator of treatment efficacy among these very favorably responding patients, but this needs to be confirmed in larger studies comparing this enhanced ASO RQ-PCR with new MFC methods. This enhanced three-step ASO RQ-PCR will probably not be implemented in daily practice owing to the laborious process, but an MRD method with superior sensitivity and applicability is still needed in clinical trials when declaring any novel treatment more efficient than the other.

CONFLICT OF INTEREST
RS has received honoraria from Genzyme, Sanofi, Celgene and Janssen-Cilag, and research funding from Janssen-Cilag and Celgene; KR has received honoraria from Novartis and an educational grant from Novartis and Celgene; TR has received educational grants from Celgene, Bristol-Myers-Squibb and Novartis; TL has received an educational grant from Bristol-Myers-Squibb; VJ has received an educational grant from Novartis and the remaining authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This study was financially supported by the competitive Research Funding of Temple University Hospital (Grant 9M0097) and by a grant from Janssen-Cilag. Study bortezomb was provided by Janssen-Cilag. The authors take full responsibility for the content of the paper.

AUTHOR CONTRIBUTIONS
RS, KR, VK, TTP designed the study, VK, TL, VJ performed the ASO RQ-PCR analytics, TTP, TLP, VH, PM, SS performed the MFC analyses, LU standardized the FLC assay, PA, MP, KR, RS provided study material or patients, TS and RS performed statistical analyses, RS wrote the manuscript, VK and KR revised the manuscript and all authors edited and approved the manuscript.

REFERENCES
1 Van de Velde HJK, Liu X, Chen G, Akaana A, Deraedt W, Bayssas M. Complete response correlates with long-term survival and progression-free survival in high-dose therapy in multiple myeloma. Haematologica 2007; 92: 1399–1406.
2 Harousseau JL, Attal M, Avet-Loiseau H. The role of complete response in multiple myeloma. Blood 2009; 114: 3139–3146.
3 Durie BG, Harousseau JL, Miguel JS, Blade J, Barlogie B, Anderson K et al. International uniform response criteria for multiple myeloma. Leukemia 2006; 20: 1467–1473.
4 Rajkumar SV, Harousseau JL, Durie B, Anderson KC, Dimopoulos M, Kyle R et al. Consensus recommendations for the uniform reporting of clinical trials: report of the International Myeloma Workshop Consensus Panel 1. Blood 2011; 117: 4691–4695.
5 Hart AJ, Jagasia MH, Kim AS, Mosse CA, Savani BN, Kassim A. Minimal residual disease in myeloma: are we there yet?. Biol Blood Marrow Transplant 2012; 18: 1790–1799.
6 Sarasaquite ME, Garcia-Sanz R, Gonzalez D, Martinez J, Mateo G, Martinez P et al. Minimal residual disease monitoring in multiple myeloma: a comparison between allelic-specific oligonucleotide real-time quantitative polymerase chain reaction and flow cytometry. Haematologica 2005; 90: 1365–1372.
7 Puig N, Sarasaquite ME, Balanzategui A, Martinez J, Paja F, Garcia H et al. Critical evaluation of ASO RQ-PCR for minimal residual disease evaluation in multiple myeloma. A comparative analysis with flow cytometry. Leukemia 2014; 28: 391–397.
8 van Dongen JM, Lhermitte L, Böttcher S, Almeida J, van der Velden VHJ, Flores-Montero J et al. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia 2013; 26: 1908–1957.
9 Ladetto M, Brüggemann M, Montilillo L, Ferrero S, Pepin F, Drandi D et al. Next-generation sequencing and real-time quantitative PCR for minimal residual disease detection in B-cell disorders. Leukemia 2014; 28: 1299–1307.
10 Martinez-Lopez J, Lahuerta JJ, Pepin F, Gonzalez M, Barrio S, Ayala R et al. Prognostic value of deep sequencing method for minimal residual disease detection in multiple myeloma. Blood 2014; 123: 3073–3079.
11 Silvennoinen R, Kairisto V, Pelliniemi T-T, Putkonen M, Anttila P, Säily M et al. Assessment of molecular remission rate after bortezomib plus dexamethasone induction treatment and autologous stem cell transplantation in newly diagnosed multiple myeloma patients. Br J Haematol 2013; 160: 561–564.
12 Kyle RA, Rajkumar SV. Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma. Leukemia 2009; 23: 3–9.
13 Putkonen M, Kairisto V, Junvenon V, Pelliniemi TT, Rauhala A, Itälä-Remes M et al. Depth of response assessed by quantitative ASO-PCR predicts the outcome after stem cell transplantation in multiple myeloma. Eur J Haematol 2010; 85: 416–423.
14 Rawstron AC, Orfao A, Bekscak M, Bezdicikova L, Broosmans RA, Bumbea H et al. Response of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders. Haematologica 2008; 93: 431–438.
15 Van Dongen JIM, Langerak AW, Brüggemann M, Evans PAS, Hummel M, Lavender FL. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003; 17: 2257–2317.
16 Aubin J, Davi F, Nguyen-Salomon F, Leboeuf D, Debert C, Taher M et al. Description of a novel FLI1 IgH PCR strategy and its comparison with three other strategies for the detection of clonality in B cell malignancies. Leukemia 1995; 9: 471–479.
17 Szczepanikos T, Pongers-Willemsen MJ, Langerak AW, Harts WA, Wijikhuys AJ, van Weerden ER et al. Ig heavy chain gene rearrangements in T-cell acute lymphoblastic leukemia exhibit predominant DH6-19 and DHLT-27 gene usage, can result in complete V-D-J rearrangements, and are rare in T-cell receptor alpha beta lineage. Blood 1999; 93: 4079–4085.
18 Verhagen OJHM Willemsen MJ, Breunis WB, Wijikhuys AJM, Jacobs DCH, Joosten SA et al. Application of germline IgH probes in real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia. Leukemia 2000; 14: 1426–1435.
19 Van der Velden VH, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer ER et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia 2007; 21: 604–611.
20 Bakkus MHC, Bouko Y, Samson D, Apperley JF, Thielemans K, Van Camp B et al. Post-transplantation tumour load in bone marrow, as assessed by quantitative ASO-PCR, is a prognostic parameter in multiple myeloma. Br J Haematol 2004; 126: 665–674.

21 Martinez-Lopez J, Fernández-Redondo E, García-Sánz R, Montalbán MA, Martínez-Sánchez P, Pavia B et al. Clinical applicability and prognostic significance of molecular response assessed by fluorescent-PCR of immunoglobulin genes in multiple myeloma. Results from a GEM/PETHEMA study. Br J Haematol 2013; 163: 581–589.

22 Pedreida CE, Costa ES, Lecrevisse Q, van Dongen JIM, Orfao A. Overview of clinical flow cytometry data analysis: recent advances and future challenges. Trends Biotechnol 2013; 31: 415–425.

23 Dispenzieri A, Kyle R, Merlini G, Miguel JS, Ludwig H, Hajek R et al. International Myeloma Working Group guidelines for serum-free light chain analysis in multiple myeloma and related disorders. Leukemia 2009; 23: 215–224.

24 Mark T, Jayabala D, Coleman M, Pearse RN, Wang YL, Lent R et al. Atypical serum immunofixation patterns frequently emerge in immunomodulatory therapy and are associated with a high degree of response in multiple myeloma. Br J Haematol 2008; 143: 654–660.

25 de Larrea CF, Cibeira MT, Arostegiu JI, Rosiñol L, Montserrat E et al. Emergence of oligoclonal bands in patients with multiple myeloma in complete remission after induction chemotherapy: association with the use of novel agents. Haematologica 2011; 96: 171–173.

26 Paiva B, Martinez-Lopez J, Viduales M-B, Mateos MV, Montalban M-A, Fernandez-Redondo E et al. Comparison of immunofixation, serum free light chain, and immunophenotyping for response evaluation and prognostication in multiple myeloma. J Clin Oncol 2011; 29: 1–6.

27 de Larrea CF, Cibeira MT, Montserrat E, Arostegiu JI, Rosiñol L, Montserrat R et al. Abnormal serum free light chain ratio in patients with multiple myeloma in complete remission has strong association with the presence of oligoclonal bands: implications for stringent complete remission definition. Blood 2009; 114: 4954–4956.

28 Singhal V, Vickrey E, Krishnamurthy J, Singh V, Allen S, Mehta J. The relationship between the serum free light chain assay and serum immunofixation electrophoresis, and the definition of concordant and discordant free light chain ratios. Blood 2009; 114: 38–39.

29 Martinez-Lopez J, Paiva B, Mateos MV, Montalban MA, Saez-Gomez MA, Alonso L et al. Does stringent complete remission (sCR) criteria represent a deeper level of remission as compared to conventional CR? Clin Lymphoma Myeloma Leuk 2013; 415: S235.