**TITLE:** Critical evaluation of ASO RQ-PCR for minimal residual disease evaluation in multiple myeloma. A comparative analysis with flow cytometry.

**RUNNING HEAD:** Residual disease in myeloma: PCR vs. flow

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**KEYWORDS:** Multiple myeloma, multiparameter flow cytometry, ASO RQ-PCR, minimal residual disease.

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ABSTRACT

We have analyzed the applicability, sensitivity and prognostic value of Allele Specific Oligonucleotide Real time Quantitative Polymerase Chain Reaction (ASO RQ-PCR) as method for minimal residual disease (MRD) assessment in patients with multiple myeloma (MM), comparing the results with those of Multiparameter Flow Cytometry (MFC). 170 patients enrolled in 3 consecutive Spanish trials achieving at least partial response after treatment were included. Lack of clonality detection (n=31), unsuccessful sequencing (n=17), and suboptimal ASO performance (n=51) limited the applicability of PCR to 42% of cases. MRD was finally investigated in 103 patients (including 32 previously studied) with persistent disease identified by PCR and MFC in 54% and 46% of cases, respectively. A significant correlation in MRD quantitation by both techniques was noted (r=0.881, \( p<.001 \)) being reflective of treatment intensity. Patients with \(<10^{-4}\) residual tumor cells showed longer progression free survival compared to the rest (not reached vs. 31 months, \( p=0.002 \)), with similar results observed with MFC. Among complete responders (n=62), PCR discriminated two risk groups with different PFS (49 vs. 26 months, \( p=0.001 \)) and OS (NR vs. 60 months, \( p=0.008 \)). Thus, although less applicable than MFC, ASO RQ-PCR is a powerful technique to assess treatment efficacy and risk stratification in MM.

KEYWORDS: Multiple myeloma, multiparameter flow cytometry, ASO RQ-PCR, minimal residual disease.
1. INTRODUCTION

Significant progress has been made in the treatment of multiple myeloma (MM) leading to 30-50% complete response (CR) rates in transplant but also in non-transplant eligible patients,1-10 as well as increased progression free (PFS) and overall survival (OS). Thus, more stringent definitions of response and increasingly sensitive methods for monitoring treatment efficacy are needed.11

PCR is used for residual disease monitoring in CML, ALL and APL to determine prognosis and to guide therapy.12-14 In MM, PCR with allele-specific oligonucleotide (ASO) primers complementary to the immunoglobulin heavy chain variable sequence (ASO PCR) is the most sensitive approach for the detection of malignant plasma cells (PC), reaching up to 10⁻⁵.15 The clinical value of qualitative approaches has been modest because, due to its high sensitivity, most cases remain positive despite heterogeneous outcomes.16-18 As alternative, real-time quantitative PCR (ASO RQ-PCR) provides an accurate quantification of residual disease thus overcoming the problem. However, the hypermutated configuration of the *IGHV* genes in PC causes mismatches between the gene segments and the corresponding primers hampering minimal residual disease (MRD) assessment in MM.19 Despite this, several reports using quantitative PCR have been published in MM, showing effective outcome discrimination in the transplant setting.20-24 Thus, the term molecular response has been included in the IMWG criteria, claimed as the highest degree of response.11

Multiparameter flow cytometry (MFC) can distinguish between normal and malignant PC by the aberrant expression of cell surface markers in approximately 90% of patients and is sufficiently sensitive to detect as few as 10⁻⁴ atypical PC in a normal bone marrow (BM).25-27 Recent studies conducted by the Spanish and UK groups have shown that negative MRD by MFC is predictive for prolonged PFS and OS, even in patients in CR.25-27 These data support the concept of immunophenotypic response recently defined by the IMWG.11

ASO RQ-PCR and MFC have only been compared in few studies based on small number of patients.20,28,29 Such a comparison is however crucial in order to determine their relative applicability, sensitivity and prognostic value and to guide clinical groups to define the optimal approach for prospective clinical trials. Here, we have performed an analysis in depth with a rigorous approach using the Euro-MRD consortium guidelines of the real applicability and potential pitfalls of the ASO RQ-PCR assay as analytic method for MRD quantification in a large series of patients with MM.15 We have also compared ASO RQ-PCR and MFC as two different approaches for MRD assessment in patients with MM treated with and without ASCT in the era of novel anti-myeloma agents.
2. METHODS

2.1. Patients and treatments

All patients under study were included in Spanish PETHEMA/GEM trials, namely the GEM2000 (NCT00560053; VBMCP/VBADx6 followed by ASCT), GEM05MENOS65 (NCT00461747; either VBMCP/VBAD plus bortezomib in the last two cycles, thalidomide/dexamethasone or bortezomib/thalidomide/dexamethasone) for transplant-eligible patients, whereas elderly patients were treated according to the GEM05MAS65 study (NCT00443235; six induction cycles with bortezomib/melphalan/prednisone or bortezomib/thalidomide/prednisone). Drug dosage and schedule have been extensively described elsewhere.\textsuperscript{30, 31}

Patients achieving at least partial response either at day +100 after ASCT (if included in the GEM2000 and GEM05MENOS65 protocols) or after induction therapy (if enrolled in the GEM05MAS65 protocol) were referred for MRD investigations. Response to treatment was assessed according to the European Bone Marrow Transplantation group, slightly modified to include the near CR category.\textsuperscript{32} Samples were collected after informed consent was obtained in accordance with the Declaration of Helsinki and with approval from the ethics committees of all participating institutions.

2.2. Sampling, DNA extraction, PCR amplification and sequencing of IGH and IGK genes

A total of 170 patients achieving at least a partial response had BM samples available both at diagnosis and after treatment. Genomic DNA was extracted using standard methods. PCR amplifications of complete IGHV-J, incomplete IGHD-J and IGKDEL rearrangements were performed according to the BIOMED-2 Concerted Action.\textsuperscript{33} The clonal population was identified by fragment analysis in an ABI3130 DNA Sequencer (Applied Biosystems, Foster City, CA) according to well-established procedures.\textsuperscript{34}

Clonal products were directly sequenced twice using an automated ABI3130 DNA Sequencer using Big-Dye terminators. Germline IGHV, IGHD, IGHJ, IGHKDEL and intron-RSS genes were identified using the ImMunoGeneTics (IMGT)(http://www.imgt.org/) and BLAST (accession number EMB/X97051, http://www.ncbi.nlm.nih.gov/blast/) public databases.\textsuperscript{35,36} Once the segments were identified, the N-region/s were highlighted for ASO-primer design.
2.3. **ASO-primer design and Real-time Quantitative PCR (RQ-PCR)**

All ASO primers were designed using the OLIGO 6.1 software (Molecular Biology Insights, Cascade, CO) complementary to the corresponding junction region/s following previously published recommendations. 37

ASO-primer specificity testing was done with DNA from diagnosis and a buffy coat from healthy donors as positive and negative controls, respectively. If available, IGHD-J rearrangements were preferred as a target, followed by IGHV-J and IGKDEL.38

For IGHV-J and IGHD-J rearrangements, the method established by Verhagen’s et al was used for RQ-PCR.39 For IGKDEL rearrangements, we used the germline IGKDEL probe and reverse primer described by van der Velden et al, together with the forward ASO designed at the same strand as the germline probe.40 All reactions were carried out according to the EuroMRD guidelines recommendations.15

DNA from baseline samples was serially diluted into the buffy coat pool from healthy individuals from $10^{-1}$ to $10^{-5}$, and standard curves were performed with appropriate dilutions. Calculations were made to allow amplification of 1.5 µg of MRD samples. RQ-PCR data were interpreted according to van der Velden et al.15

2.4. **Minimal residual disease assessment by multiparameter flow cytometry**

Erythrocyte-lysed whole BM samples were immunophenotyped using a four-color direct immunofluorescence technique. The phenotypic aberrancies detected at diagnosis were used as patient-specific probes for MRD assessment.25,41 For MRD analysis we used a two-step acquisition procedure: first, information from 2-5x10⁴ events corresponding to whole sample cellularity was stored; then data about CD38 hi gated events were stored, between 2x10⁵ – 2x10⁶ leukocytes per tube. The multiparameter strategy used to differentiate normal and pathological PC has been previously described.25,41 MRD negative patients were classified as those showing absence of phenotypically aberrant PC with a sensitivity between 10⁻⁴–10⁻⁵.

2.5. **Statistical methods**

To estimate the statistical significance of the differences observed between means, the Mann-Whitney U and Kruskal-Wallis tests were employed. The $\chi^2$ test was used for comparison of dichotomous variables between groups. The relationship between the percentage of PC detected by ASO RQ-PCR and MFC was evaluated through Pearson correlation. Survival was analyzed by the Kaplan-Meier method, and differences between curves were tested for statistical significance with the two-sided log-rank test. PFS was measured from the start
of treatment to the date of progression or death. OS was measured from the start of treatment to the date of death or last visit. For all statistical analyses, SPSS software (version 15.0; SPSS, Chicago, IL) was used.

3. RESULTS

3.1. PCR amplification of IGH and IGK genes

Results of the amplification of IGH (V-J and D-J) and IGKDEL genes as per BIOMED 2 guidelines are summarized in Tables 1 and 2. In 31 out of the 170 (18%) patients included in the study none of the 3 markers tested could be amplified and thus, these cases were excluded from further analysis. Comparing the characteristics of these false negative cases versus those found to be clonal, we observed that the percentage of pathological PC and the concentration of tumor DNA were both significantly lower in the former group. From the remaining 139 cases, one marker could be amplified in 69 cases (50%) (IGHV-J in 44, IGHD-J in 16 and IGKDEL in 9) and two markers in 54 (39%) (IGHV-J + IGHD-J in 37, IGHV-J + IGKDEL in 16, and IGHD-J + IGKDEL in 1 case). In 16 samples (11%) all the 3 markers tested were amplified. A flow diagram depicting samples evolution can be found in the Supplemental section (Figure 1).

3.2. Sequencing of IGH and IGK genes

Out of the 139 cases found to be clonal, in 17 cases (14%), no successful sequencing was obtained. Comparing the characteristics of the samples successfully sequenced and the failures, again tumor DNA concentration was found to be significantly lower in the unsuccessfully sequenced group (Table 2). From the remaining 122 samples, one marker was sequenced in 77 cases (63%; 48 IGHV-J, 17 IGHD-J and 12 IGKDEL), 2 markers in 34 cases (28%; IGHV-J + IGHD-J in 19, IGHV-J + IGKDEL in 13 and IGHD-J + IGKDEL in 2 cases) and all 3 markers could be identified in 11 samples (9%). Results of the gene segments identified by rearrangement are detailed in Table 3.

3.3. ASO-primer design and testing

The 122 samples with an available target were used for primer design. A total of 154 ASO-primers were designed: 91 in IGHV-J, 42 in IGHD-J and 16 in IGKV/intron-RSS-IGKDEL region. Five primers were designed in the corresponding IGHJ region. In 71 samples one primer was designed whereas two, three and four primers were designed in 23, 11 and one samples, respectively. Out of the 154 ASO-primers tested, 96 (62%)
were considered suitable for further analysis, whereas 58 had to be discarded. Among the 91 primers designed in IGHV-J, 38 (42%) performed successfully, 35/42 (83%) of those designed in IGHD-J and 12/16 in IGKDEL (75%).

3.4. ASO RQ-PCR vs. multiparameter flow cytometry

Out of the 170 cases initially included in the study, a total of 71 fulfilled Euro-MRD criteria and were considered suitable for MRD assessment, thus conferring a final applicability to ASO RQ-PCR for MRD monitoring in MM of 42%. Nevertheless this percentage would increase to 70% if we exclude the 48 failures (31 patients in which clonality could not be detected plus the 17 cases with no successful sequencing) attributable to pre-analytical problems. In order to obtain a larger series allowing us to draw stronger conclusions, for the subsequent MRD analyses we added the data from 32 additional patients previously studied by our group. These 32 cases mentioned were obtained from a total of 71 initially analyzed, therefore with a similar applicability than the present series (45%). However, since analyzed before the Euro-MRD guidelines were available, we have not included them in the analysis of applicability and pitfalls of the ASO RQ-PCR technique based on the Euro-MRD guidelines. All these samples had both a molecular marker and a patient-specific immunophenotypic profile available for MRD assessment by ASO RQ-PCR and MFC, respectively.

A comparison between the sensitivities of ASO RQ-PCR and MFC for MRD detection in the 103 follow-up samples obtained after treatment showed that ASO RQ-PCR detected clonotypic cells in 55 (54%) cases, whereas phenotypically aberrant PC were identified by MFC in 47 (46%) cases. The mean (SD) percentage of tumor cells detected by ASO RQ-PCR and MFC was 0.31 (1.26) and 0.39 (1.36), respectively. A significantly high correlation between the MRD levels obtained by both techniques was observed (Figure 1; r=0.881, p<0.001), despite 18 (17.5%) discordant cases (11 with positive MRD by ASO RQ-PCR but negative by MFC, and 7 negative by ASO RQ-PCR but MRD positive by MFC). Grouping patients by treatment protocol, the quantity of tumor cells detected by both techniques correlated with the intensity of the treatment received, although not reaching statistical significance. Thus, the mean (SD) of tumor cells detected by ASO-RQ PCR was 0.60 (2.08), 0.35 (0.56) and 0.037 (0.11) for GEM2005MAS65, GEM2000, and GEM2005MENOS65 protocols, respectively [similar for MFC: 0.70 (2.20), 0.34 (0.52), and 0.14 (0.54)].
3.5. Prognostic value of MRD monitoring by ASO RQ-PCR and MFC

We have investigated the predictive prognostic value of different MRD thresholds (10^{-2}, 10^{-3} and 10^{-4}) on PFS. All of them discriminated two different risk categories, although 10^{-4} was the most significant cut-off value. Thus, both techniques segregated two cohorts with significantly different PFS, both in intensively treated patients (PCR: 54 vs. 27 months, \(p=0.001\); MFC: 45 vs. 27 months, \(p=0.02\)) and in non-intensively treated patients (PCR: NR vs. 31 months, \(p=0.029\); MFC: NR vs. 27 months \(p=0.002\), Figure 2). The differences in OS have not reached statistically significant differences yet. Then, we combined the results obtained by both techniques to define 4 risk groups as follows: PCR+/MFC+, PCR-/MFC-, PCR-/MFC+ and PCR+/MFC-. No differences in PFS were observed between the two groups of patients with discordant results by both techniques who also showed a similar outcome to that of double negative patients (median PFS of 39, 45 and 48 months for PCR-/MFC+, PCR+/MFC- and PCR-/MFC-, respectively); by contrast, double positive patients showed a significantly shorter PFS (26 months, \(p<0.001\)).

Finally, we focused only on patients in CR (n=62). The MRD threshold of 10^{-4} discriminated two groups of patients with different PFS (PCR: 49 vs. 26 months, \(p=0.001\); MFC: 45 vs. 25 months, \(p=0.001\)), and also different OS (PCR: NR vs. 60 months, \(p=0.008\); MFC: 72 vs. 45 months, \(p=0.014\)). These results are shown in Figure 3.

4. DISCUSSION

In the era of new treatment strategies in MM, with patients achieving unprecedentedly high CR rates, a new and yet unmet need has emerged: redefinition of response criteria. MFC has proven to be useful and clinically relevant but it requires experience and fully standardization and it is not broadly employed. As alternative, ASO RQ-PCR is a well standardized technique for MRD monitoring in ALL, AML, CML and also in MM, but it is time and labor-consuming which explains why patient series in MM are small and its clinical value remains to be established. This is counterbalanced by its high sensitivity for detection of MRD as well as the capacity to analyze not only the PC compartment but all clonal B cells.

Here, we report on a large series of MM patients investigated for MRD assessment by ASO RQ-PCR. First, we analyzed in depth the real applicability of this technique in MM, aiming to identify potential pitfalls. Our results show that ASO RQ-PCR yielded a limited applicability of 42%, which is significantly lower than that of MFC (>90%).25-27 Exploring potential differences between the samples successfully analyzed and the
failures, we found the former group having a significantly higher percentage of pathological PC and tumor DNA concentration, thus highlighting the quality of the samples as an essential prerequisite to perform this type of studies. There were some cases with a successful PCR performance and low plasma cell infiltration that would not completely fit with this explanation, but they were a minority and further, our group has recently reported that the use of CD138+ selected samples can improve the percentage of successfully sequenced samples from 60% to 96%. Most samples (88%) were referred from external centers but the analysis was performed within the first 24 hours from collection and thus the reliability of the studies by flow is preserved: molecular studies are not significantly affected by late sample arrival, which is of benefit in these type of centralized studies. Excluding pre-analytical problems, our study could show an applicability of 70% despite having used additional molecular markers, such as IGHD-J or IGKV-KDE/intron-RSS known to increase the applicability of the procedure in 10% and 9% additional cases, respectively. We attribute these further failures to somatic hypermutations (SHM), characteristic of MM, that surely hampered clonality detection, sequencing success and ASO performance. Thus, despite various attempts of improvement, we observed a lower applicability rate than expected. This could be due to the following reasons: 1) our study is based on an unselected sample population; 2) we have strictly followed the Euro-MRD guidelines for interpretation of real-time quantitative PCR data and 3) we have used the standardized method designed for ALL, using only one specific forward primer, in order to test a method potentially applicable to the routine practice. However, applicability could be increased if two specific primers and a probe are also used. The reported rate of ASO RQ-PCR applicability rates is highly variable, ranging from 28 to 84%. These heterogenous results are probably due to the use of different methods, including the use of specific primers and probes (mostly resulting in the higher applicability rates) as well as the use of selected cases without information regarding pre-analytical problems. As alternative, the use of the new high throughput sequencing strategies is opening new possibilities still under early evaluation.

ASO-RQ PCR and MFC have been directly compared in two small studies, one of them performed by our own group, and none of the techniques could be considered definitively superior to the other. In the present study, the mean percentage of residual tumor cells detected by ASO RQ-PCR and MFC was not significantly different (0.31 vs 0.39, respectively). However, residual clonotypic cells could be detected by ASO-RQ-PCR in 53% of cases while in 46% of patients using a 4-color MFC approach, thus suggesting a higher sensitivity of the former technique and further confirming the results of previous dilution experiments. Nevertheless, the sensitivity of MFC could be increased with the use of ≥8 colors, moreover, the correlation among the results obtained by both techniques was very high (r=0.881), and in fact discordances among both techniques were
relatively low (17.5%) and could be attributed to specific factors, such as the different sensitivity of both methods as well as the different targets analyzed. Thus, ASO RQ-PCR has the theoretical advantage of being able to identify all clonotypic cells, not only PC but also potential precursor clonal B cells, counterbalanced however by the fact that clonality is not equivalent to malignancy or poor outcome; this could explain the lack of differences in outcome noted between discordant cases. Regarding costs, we have estimated the prize of ASO RQ-PCR in Spain in 350 euros per diagnostic sample and in 100 euro for follow-ups, excluding human costs; in contrast, a 4-color flow cytometry test would cost approximately 50 euros per diagnostic sample and a similar quantity in follow-ups. However, prizes are greatly variable among countries and these numbers should be considered merely orientative.

We have also investigated the role of ASO RQ-PCR to evaluate treatment efficacy. The percentage of patients with undetectable disease by PCR noted in our study confirms the high efficacy of current treatment approaches in MM and highlights the efficacy of new drugs. Further, in patients with persistent disease, the residual tumor load correlates with the intensity of the treatment received. Thus, the lowest MRD level was detected in patients treated with novel agents plus ASCT, followed by conventional agents with ASCT, while the highest MRD level corresponded to patients treated with novel agents but not transplanted. It was also remarkable to see the parallelism of MFC and ASO RQ-PCR, showing both comparable results in terms of quantification of residual tumor cells in these three therapeutic subgroups. These results confirm the utility of MRD assessment to monitor treatment efficacy, as a valuable tool in the era of new drugs, particularly for the design of risk adapted consolidation and maintenance therapies. 49

Finally, we have compared the ability of these 2 methods to predict outcome in the era of new treatment strategies and drugs in MM. As shown in Figure 2, both methods offered almost superimposable survival curves with a very high predictive value, both when used for MRD assessment in intensively treated and in non-intensively treated patients. This was demonstrated upon using different MRD thresholds (10^-2, 10^-3, and 10^-4). Importantly, among patients in CR two cohorts with different PFS but also OS segregated using 10^-4 as MRD threshold, thus highlighting the heterogeneity of conventional responses and further confirming the significant value of both methods to predict outcome. Despite these findings, it is important to note that extramedullary relapses remain elusive for both methods. 50

Overall, our results show that ASO RQ-PCR and MFC are two valid methods to monitor treatment efficacy, highly predictive of outcome both in transplanted and non-transplanted patients. ASO RQ-PCR is fa-

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vored by a slightly higher sensitivity whereas MFC is significantly more applicable. Therefore, MFC should be considered the method of choice for MRD assessment in MM, while molecular methods can be deemed as a complementary tool until a substantial comparative advantage is demonstrated.
5. **ACKNOWLEDGEMENTS**

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6. **DISCLOSURE OF CONFLICTS OF INTEREST**

Authors have no conflicting financial interests.

Supplemental information is available at Leukemia’s website.
7. **REFERENCE LIST**


## TABLES

**Table 1.** Results of the amplification of IGH and IGK genes as per BIOMED2 guidelines

<table>
<thead>
<tr>
<th></th>
<th>VH-JH</th>
<th>DH-JH</th>
<th>Kde$^1$</th>
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<tr>
<td>Clonal</td>
<td>111 (65%)</td>
<td>70 (41%)</td>
<td>43 (46%)</td>
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<tr>
<td>Polyclonal</td>
<td>32 (19%)</td>
<td>27 (16%)</td>
<td>25 (26%)</td>
</tr>
<tr>
<td>Nonspecific /no amplification</td>
<td>27 (16%)</td>
<td>73 (43%)</td>
<td>26 (28%)</td>
</tr>
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</table>

$^1$Performed in 94 cases
Table 2. Comparison of the characteristics of the samples between the successfully analyzed group and the rest

<table>
<thead>
<tr>
<th>PPC: pathological plasma cells</th>
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<tr>
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<tr>
<td>PPC (%) (median, range)</td>
</tr>
<tr>
<td>Successful</td>
</tr>
<tr>
<td>Unsuccessful</td>
</tr>
<tr>
<td>p</td>
</tr>
<tr>
<td>Successful</td>
</tr>
<tr>
<td>Unsuccessful</td>
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<tr>
<td>p</td>
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<table>
<thead>
<tr>
<th>PPC (%) (median, range)</th>
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<th>Unsuccessful</th>
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<tr>
<td>7.32</td>
<td>7.80</td>
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<td>(0.02-95)</td>
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<td>7.73</td>
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<td>(0.02-69)</td>
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<th>[DNA] (ng/µL; median, range)</th>
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<th>Unsuccessful</th>
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<td>51.10</td>
<td>58.20</td>
<td>29.60</td>
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<td>57.90</td>
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<td>(2.10-556.3)</td>
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<td>(0.03-505.6)</td>
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<td>18.33</td>
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<td>(0.01-505.68)</td>
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Table 3. Gene segments repertoire by rearrangement

<table>
<thead>
<tr>
<th>IGHV-J (n=91)</th>
<th>IGHD-J (n=48)</th>
<th>IGKDEL rearrangements (n=37)</th>
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<tr>
<td>IGHV</td>
<td>IGHD</td>
<td>IGHJ</td>
</tr>
<tr>
<td>1</td>
<td>11 (12%)</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>6 (6%)</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>57 (63%)</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>15 (16%)</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>2 (2%)</td>
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</tr>
<tr>
<td>6</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>1 (1%)</td>
<td>7</td>
</tr>
</tbody>
</table>

1IGHD-J segment could not be identified in 2 cases
8. FIGURE LEGENDS

**Figure 1.** Correlation between the results of MRD quantitation by ASO-RQ PCR and MFC. Cases with discordant results (n=18) have been highlighted.

**Figure 2.** PFS according to MRD by ASO-PCR or MFC in; A) all patients; B) transplanted patients and; C) non-transplanted patients.

**Figure 3.** Progression Free and Overall Survival of Complete Responders according to MRD by ASO RQ-PCR