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Monoclonal Gammopathies

# Polymerase chain reaction-based detection of minimal residual disease in multiple myeloma patients receiving allogeneic stem cell transplantation

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ABSTRACT

***Background and Objectives.*** **Recent advances in the treatment of multiple myeloma (MM) include use of high-dose chemoradiotherapy followed by allografting. Although allografting with bone marrow (BM) or peripheral blood stem cells (PBSC) seems to improve clinical outcome and lengthen survival, only about 50% of patients reach stringently defined complete remission (CR), and most subsequently relapse. We assessed the clinical relevance of minimal residual disease (MRD) in 14 MM patients in CR after allografting with PBSC (6 patients) or BM (8 patients).**

***Design and Methods.*** **Among the 30 out of 72 MM patients in our Institute who achieved CR after allografting, 14 had a molecular marker suitable for allospecific polymerase chain reaction (PCR) analysis. Stringent molecular monitoring was done using clonal markers based upon rearranged immunoglobulin heavy-chain genes. Molecular remission (MCR) was defined as two consecutive negative PCR results.**

***Results.*** **Seven of 14 (50%) molecularly monitored patients, achieved MCR and did not relapse after a median molecular follow-up of 60 months (range 36-120). Median time to obtain first PCR negativity was 12 (BM group) and 6 months (PBSC group), respectively. Of the seven patients (50%) who never achieved MCR, one relapsed.**

***Interpretation and Conclusions.*** **In conclusion, 50% of the MM patients in CR studied by us also achieved stringently-defined MCR. MCR was associated with a very low rate of clinical relapse.**


Key words: multiple myeloma, allogeneic bone marrow transplantation, minimal residual disease, PCR

Multiple myeloma (MM) is an incurable B-cell malignancy that affects terminally differentiated plasma cells localized in the bone marrow.[1] The immunoglobulin heavy chain (IgH) genes that are clonally rearranged in MM are somatically hypermutated,[2] and there is no evidence that clonal evolution occurs during the course of the disease.[3] Recently, several advances in the treatment of MM patients have been reported, including the use of high-dose chemoradiotherapy followed by autologous[4-9] or allogeneic transplantation of hematopoietic stem cells.[5, 10-12] In most series so far reported, autotransplants yielded stringently defined complete remission (CR) rates of 30% to 50%[8] but there was no evidence of cure.[4,6,7] Re-infusion of myeloma cells contaminating the autologous graft may contribute to disease recurrence, as has been demonstrated in other hematologic malignancies and solid tumors.[13] This notion has recently led to phase I-II clinical trials aimed at exploring the role of purging techniques.[14,15] The advantages of allogeneic stem cell transplantation include the use of a tumor-free source of hematopoietic cells and the existence of a graft-versus-myeloma effect,[16-18] and ultimately result in prolonged relapse-free survival in comparison to autotransplants.[19] Moreover, although transplant-related mortality is 40-50%, the relapse rate of patients in CR is lower than after autografting.[20,21]

In MM, the rearranged immunoglobulin heavy chain (IgH) gene is a sensitive tumor marker and has been used to detect residual myeloma cells below the limits of detection by conventionally employed techniques, particularly after autologous bone marrow transplantation procedures.[22-26] Little is yet known about the molecular status of MM patients following allogeneic transplantation.[24,25] In particular, the significance of molecular remission (MCR) in patients achieving CR is currently unclear.

In the present study, we performed polymerase chain reaction (PCR) analysis of minimal residual disease (MRD) in a relatively large series of patients in CR after allogeneic transplantation, in order to determine the MCR rate and to assess its clinical relevance in terms of survival and relapse rate. In this regard, the rearranged variable region (VDJ) of the IgH gene was identified using two primers designed from the patient-specific IgH complementarity determining regions (CDR) II and III.[26]

## Design and Methods

### *Patient population*

Between 1984 and 1998, 72 patients with a confirmed diagnosis of active MM received allogeneic stem cell transplantation from HLA-identical sibling donors at the Institute of Hematology and Medical Oncology "Seràgnoli", University of Bologna. The

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**Table 1. Clinical characteristics of patients obtaining complete remission after allogeneic transplantation.**

| | *BM-allograft* | *PBSC-allograft* | *Total* |
|---|---|---|---|
| N. of patients | 52 | 20 | 72 |
| CR/TOT (%) | 16/52 (30.7%) | 10/16 (62.5%) | 26/68(38.2%) |
| Follow-up of CR molecularly studied patients (months; median, range) | 72 (24-160) | 24 (3-36) | 36 (3-160) |
| REL/CR | 6/16 (37.5%) | 2/10 (20%) | 8/26 (31%) |
| Age (yr.) of CR molecularly studied patients (median, range) | 39.5 (30-53) | 47 (30-54) | 38 (30-54) |
| $\beta_2$M (mg/L) of CR molecularly studied (median, range) | 1.6 (0.6-5.7) | 1.61 (1.4-2.4) | 1.59 (0.6-5.7) |
| No. of CR molecularly studied patients with stage III disease | 8 | 4 | 12 |
| Male/female (of CR pts molecularly studied) | 5/11 | 5/5 | 10/16 |
| Molecularly studied | 9/16 (56%) | 5/10 (50%) | 14/26 (53%) |
| Time to obtain first PCR– (months; median, range) | 12 (6-48) | 6 (3-6) | 12 (3-48) |
| Patients who relapsed after achieving MCR | 0/5 | 0/2 | 0/7 |
| MCR/TOT | 5/9 (55%) | 2/5 (40%) | 7/14 (50%) |

*Table 1 shows the clinical characteristics, MCR and relapse rates in patients who underwent allogeneic transplantation. The time to obtain first PCR negativity is expressed in months. Mol. anal. = rate of patients with molecular marker analyzed during the follow-up; MCR = molecular complete remission; BM = allografted patients who received donor bone marrow and PBSC = allografted patients who received donor peripheral blood stem cells as the source of their graft, respectively.*

source of hematopoietic stem cells was bone marrow (BM) in 52 patients and peripheral blood in the remaining 20. Preparations for engraftment included total body irradation (TBI) plus chemotherapy in 48 patients and chemotherapy alone with busulphan (Bu) and cyclophosphamide (Cy) in the remaining 24.[12,20] Graft-versus-host disease prevention was performed using either cyclosporin A (CsA) ± methotrexate (35 patients) or T-cell depletion ± CsA (37 patients). Four patients had a short follow-up after bone marrow transplantation (BMT), and were excluded from the study.

### *Selection of patients for molecular monitoring*

Only patients who attained stringently defined CR according to recently recommended criteria of MRD[21] by a PCR-based assay were included in the present study. Among the prerequisites for enrollment in the study was the availability of BM samples or smears, taken either at diagnosis or before transplant and at regular time points after transplantation. Some clinical characteristics of patients in CR are presented in Table 1. Statistical analysis was performed with Fisher's exact test.

### *Nucleic acid extraction and cDNA synthesis*

BM samples or smears were obtained after informed consent during standard diagnostic procedures. DNA was obtained by cell lysis and salting-out procedures, as reported elsewhere;[28] DNA from smears was obtained by lysing scraped cells and by phenol extraction and ethanol precipitation. DNA was spectrophotometrically quantified. RNA was isolated as reported.[28] One microgram of total RNA was reverse transcribed using 20 pmol of random primers. A 50 µL reaction was carried out in 10 mM dithiotreitol, 1 mM dNTPs (Pharmacia LKB Biotechnology, Uppsala, Sweden) and 1X reverse transcriptase buffer (50 mM Tris-HCl, 6 mM $MgCl_2$ and 40 mM KCl) with an additional 20 U of ribonuclease inhibitor (RNAsin, Boehringer) and 200 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Gibco BRL, Gaithersburg, MD, USA). The reaction was incubated at 37°C for 1 h.

### *Identification of VDJ gene rearrangement*

VDJs were amplified starting from genomic DNA or total cDNA depending on sample availability. Amplifications were performed as previously described.[26] Briefly, 1 µg of genomic DNA or 1 µL of total cDNA (1/50 of RT reaction) was amplified using a set of 7 consensus primers derived from framework-1 (FR1) region, or 6 consensus primers derived from the IgH leader[22] as sense primers, and a consensus primer derived from the joining region as the antisense primer.[26] The reaction was carried out for 30 cycles (denaturation 94°C for 30 sec, annealing 61°C for 40 sec and extension 72° for 50 sec) with a final extension of 7 min, as previously reported.[26] PCR products were analyzed by electrophoresis on 3% agarose gel. An approximately 300 bp band, corresponding to the VDJ gene rearrangement, was gel-purified and directly sequenced, using the VH primer corresponding to the VH family employed in the VDJ rearrangement. Sequencing analysis was performed using the FASTA program at the EMBL web site *http://www2.ebi.ac.uk/fasta3/*. CDRII and CDRIII regions were identified and a couple of sequence-specific primers was designed (sense on CDRII and antisense on the CDRIII region).[29] Primers were synthesized with a 391 PCR-MATE EP, DNA synthesizer (Applied Biosystem, Foster City, CA, USA) on a 0.2 mmol scale, according to the user's manual.

### *Detection of residual myeloma cells*

Samples were evaluated for the presence of residual myeloma cells after auto- or allo-grafting, every 3 months for the first 6 months, every 6 months for the rest of the first two years, and annually thereafter. One microgram of DNA or 10 µL of cDNA (1/5 of RT reaction) was amplified using patient-specific primers (50 pmol each) as previously described.[26] Thirty percent of PCR product was analyzed by 3% agarose gel electrophoresis and the expected band (approx. 150-160 bp) was compared with the diagnostic sample.[26] All PCR-negative results were repeated at least five times in order to avoid false negatives due to low amounts of PCR target DNA or cDNA in the sample to be analyzed. MCR was defined as PCR-negative results in at least two successive evaluations.

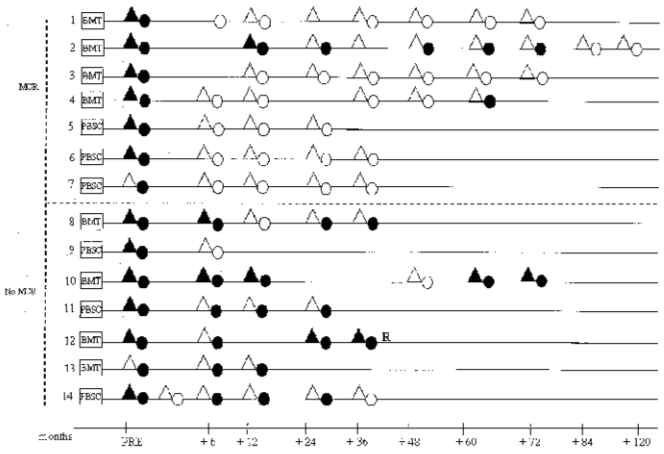


**Figure 1. Molecular monitoring of residual myeloma cells after allografting.**

*R = relapse, ○ = PCR-negative; ● = PCR-positive, △ = immunofixation negative, ▲ = immunofixation positive.*

## Results

### *VDJ gene rearrangement*

Fourteen patients who fulfilled the criteria for entering the retrospective molecular study were evaluated for the presence of MRD by a PCR-based strategy. In order to identify the VH family used in VDJ gene rearrangement, the VDJ region was amplified with a set of 7 VH family-specific primers, or 6 consensus primers derived from the IgH leader, together with a JH-consensus primer.

To obtain patient-specific markers, two patient-specific primers were designed for each sequence: a sense primer on CDRII region and an antisense primer on CDRIII. The sensitivity and specificity of the PCR-based assay was tested as previously described:[26] we obtained a median sensitivity of 1 tumor cell in $10^5$ normal marrow cells (range $10^4$-$10^7$) and no false positive results were obtained when DNA of B-chronic lymphocytic leukemia patients was used for negative controls.

### *Clinical and molecular outcome*

Twenty-six (36%) patients achieved CR after transplantation. Of these, 14 had a molecular marker (Figure 1 and Table 1), and 7 achieved MCR. In 6 patients in MCR, BM samples were persistently PCR-negative up to the latest analysis which was performed 24, 24, 26, 36, 72, 72 and 120 months after transplantation. In the remaining patient (#4), PCR-positivity emerged 60 months after transplantation. All these 7 patients remained in continuous CR for a median of 72 months (range 36 to 160 months) after transplantation.

Seven (50%) of the 14 patients with a molecular marker never achieved MCR: of these, one (#12) relapsed 36 months after transplantation, while the other six are in CR 36, 6, 72, 24, 12, and 12 months after transplantation. Whereas there was a significantly higher CR rate (63% vs. 31%, $p$=0.01) among patients transplanted with peripheral blood stem cells (PBSC) (as opposed to BM), no significant difference was found in the MCR rates between the two groups (31% vs. 20%, n.s.). Irrespectively of the cell source used for allografting, disease-free survival was much longer among patients who achieved CR and/or MCR. At the time of writing, no significant difference in relapse rate is observable between patients who achieved MCR (as well as CR) with respect to those who did not (Table 1).

## Discussion

A recent study on a large series of MM patients reported that high-dose chemotherapy plus allografting induced a stringently defined CR rate of 41%.[27] Nevertheless, the percentage of patients who obtain molecular remission is far lower, with only a few such cases having so far been reported.[24-26] So it is not yet really clear whether MM can be molecularly eradicated, and whether or not this is of clinical relevance. The present study addresses this issue in the context of the largest reported series of MM patients in CR after allografting. Fourteen patients for whom a suitable marker was available were monitored for MRD by a highly sensitive and specific PCR-based assay.

In our series, 7/14 (50%) of the patients who achieved CR after allografting also obtained MCR. None of the patients who achieved MCR relapsed, even though two of them reverted to molecular positivity. Whether MCR does actually represent a goal of MM therapy[30-32] remains to be demonstrated. However, it seems likely that, as in other hematologic malignancies,[33-37] in MM too, MCR might be a first step in improving the clinical outcome. It is also remarkable that only one patient who achieved CR but not MCR relapsed. Although MM is universally considered an incurable disease, allografting allowed at least 13 of our patients to achieve prolonged control of the disease, suggesting the possibility of an eventual cure. Our current results strongly suggest that the achievement of CR after allografting provides a relatively good prognosis, independently of specific molecular status.

Our study also illustrates the possibility of applying an efficient, specific molecular test to help gain a better understanding of the degree of tumor burden reduction obtained after allografting. We were unable to find any significant reduction in relapse rate among patients who achieved MCR with respect

to those who only obtained CR. However, our follow-up may be too short to allow any prognostic relevance of this molecular assay to emerge. Furthermore, molecular analysis was not possible in twelve of the twenty-six patients who achieved CR. Serial analysis of newly transplanted MM patients should provide more powerful indications regarding the possible prognostic value of molecular monitoring.

In the present study, 7 (58%) of the patients for whom molecular analysis was not possible relapsed, whereas the relapse rate among the 14 patients with available material for molecular analysis was very low (7%), regardless of whether or not they achieved MCR. This discrepancy may be due either to the increased use of PBSC for allografting in recent years (since 1995) or to improvements in post-transplan tation clinical management.

As far as the possible prognostic value of molecular evaluation of MRD is concerned, it should also be noted that our stringent definition of MCR as *two consecutive PCR negative results* coupled with the high sensitivity and specificity of our PCR assay[26] reduced the effective MCR rate among our patients (7/14; 50%). Despite this, we found that 86% of patients who either never achieved MCR or failed to maintain it actually remained in CR for at least one year anyway. This underlines the prognostic value of CR, independently of MCR. Prospective studies employing a quantitative assay[38] such as real-time PCR should investigate whether a threshold exists beneath which there is no significant risk of clinical relapse.

Finally, as regards the influence of different stem cell sources (PBSC vs. BM) for allografting on clinical status, we found that the CR rate was higher among patients transplanted with PBSC. However, this superiority was not reflected in the MCR rates, which were roughly equivalent with either cell source. Another explanation could be that the majority of patients transplanted with PBSC were submitted to allotransplantation procedures in the early phase of disease. The observation that relapse rates after allografting were low in both groups could be due either to the use of a tumor-free source of hematopoietic cells or to the existence of a graft-versus-myeloma effect, both ultimately resulting in a longer duration of disease control. Our preliminary data could support the hypothesis that the elevated level of donor lymphocytes in the PBSC-graft may be associated with an increased graft-versus-myeloma effect.[25] Nevertheless, as the two groups evaluated were not randomized, and some of the patients who achieved CR could not be molecularly monitored, these indications require confirmation in larger, randomized studies.

In conclusion, 50% of the MM patients in CR studied by us also achieved stringently-defined MCR (two consecutive negative PCR assays). Both CR and MCR were associated with a very low rate of clinical relapse. It is probably too early to assess whether MCR as defined by us bears any prognostic significance. Further studies using quantitative, real-time PCR methods should investigate whether a threshold exists beneath which allografted MM patients can be considered cured. Considered in the light of the reduced transplant-related mortality rate in allografted MM patients, our findings provide a rational basis for offering allografts to patients with an HLA-identical donor at an early stage in the disease.

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### Contributions and Acknowledgments

*GM and CT were the principal investigators: they designed the study. GM was responsible for ethical approval of the program, for funding and direct supervision. MC was responsible for the MM clinical trials, and clinical management of these patients. RML and NV were responsible for clinical response of the project. GB was responsible for clinical management of allotransplanted patients. PT, EZ, SR were responsible for collecting data and for molecular correlations. NT and EO were responsible for cytogenetic and molecular analysis. MA set up PCR procedures and drafted the paper. ST gave the final approval for submission. The order of authorship was made according to the substantial contribution given to the study. The authors are grateful to Robin M.T. Cooke for editing and to Maria Stella Zagarella for technical assistance.*

### Disclosures

*Conflict of interest: none.*

*Redundant publications: no substantial overlapping with previous papers.*

### Manuscript processing

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### Potential implications for clinical practice

- This study tries to find a correlation between MCR and clinical outcome. It shows the importance of molecular follow-up in the management of allotransplanted MM patients.

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