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CLINICAL IMPACT OF IMMUNOPHENOTYPIC REMISSION AFTER ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION IN MULTIPLE MYELOMA

Short title: Immunophenotipic remission post allografting

Luisa Giaccone MD,[1] Lucia Brunello MD, [1] Moreno Festuccia MD, [1] Milena Gilestro PhD, [1] Enrico Maffini MD, [1] Federica Ferrando MD, [1] Elisa Talamo, MD, [1] Roberto Passera PhD, [2] Mario Boccadoro MD, [1] Paola Omedè PhD, [1] Benedetto Bruno MD, PhD. [1]

[1] Division of Hematology, Azienda Ospedaliera Universitaria Città della Salute e della Scienza and Dipartimento di Biotecnologie Molecolari e Scienze per la Salute, University of Torino, Torino, Italy [2] Division of Nuclear Medicine, Azienda Ospedaliera Universitaria Città della Salute e della Scienza and University of Torino, Torino, Italy

Address correspondence to: Luisa Giaccone, M.D.

Division of Hematology

A.O. Città della Salute e della Scienza di Torino

Via Genova 3, 10126, TORINO, Italy

E-mail: luisa.giaccone@unito.it

Phone: +39-011-6334354

Fax: +39-011-6963737

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ABSTRACT

Immunophenotypic remission (IR) is a strong prognostic factor in myeloma patients. The combination of IR and conventional complete remission (CR) was retrospectively evaluated in 66 patients after allografting. IR was defined as absence of monoclonal plasma cells in bone marrow aspirates by multiparameter flow-cytometry. Conditioning was non-myeloablative in 55 patients; reduced-intensity in 10 and myeloablative in 1 patient. The allograft was given upfront in 35/66 (53%) patients. After a median follow-up of 7.1 years, 24 patients achieved both CR and IR (CR/IR group), 21 achieved IR but not CR with persistence of urine/serum M-component (noCR/IR group), and 21 did not achieve either CR or IR (noCR/noIR group). Median overall survival (OS) and event free survival (EFS) were "not reached" and 59 months in the CR/IR group; 64 and 16 months in the noCR/IR; and 36 and 6 months in the noCR/noIR respectively ($p<0.001$). Cumulative incidence of extra-medullary disease was 4,4 % in the CR/IR, 38,1% in the noCR/IR and 14,3% in the noCR/noIR groups respectively at 4 years ($p<0.001$). IR was a valid tool to monitor residual disease after allografting and allowed to define a cohort of patients at higher incidence of extra-medullary relapse.

INTRODUCTION

The increment in response rates of recent years, longer life expectancy and several treatment options in patients with multiple myeloma (MM) have drawn particular attention to the importance of an in-depth evaluation of ”complete remission” and the interest in the assessment of minimal residual disease (MRD) has been growing [1-3]. Two sensitive techniques are currently employed to evaluate MRD: qualitative and quantitative polymerase chain reaction (PCR)-based methods and multi-parameter flow-cytometry. PCR-based methods have been of great value in predicting clinical outcomes in MM patients following allografting [4-7], though expensive and labor-intensive, they are characterized by higher sensitivity. A patient-specific molecular marker is, however, detected in only 60% to 70% of patients. The evaluation of MRD through immunophenotyping is more broadly applicable in the MM patients population than PCR as it involves the identification of phenotypic aberrancies in myelomatous plasma cells, which are seen in more than 90% of MM patients. However, the antibody panels used for immunophenotype analysis consist of markers recommended by experts' opinions, and only recently attempts to validate and standardize them have been made [8].

MRD studies using flow-cytometry have so far been carried out on patients treated with autologous transplantation, conventional chemotherapy or new drugs [9-13]. MRD studies in the setting of allografting are however lacking. Here, we report an analysis on the achievement of immunophenotypic response (IR) after an allograft and its prognostic impact when combined with conventional complete remission (CR).

MATERIAL AND METHODS

Patients Between January 2000 and December 2011, 80 consecutive MM patients underwent an allograft at our Center. Sixty-nine out of 80 (median age 54 years, range 35-66), with a follow-up of at least 3 months were included in this study. Three were excluded from this analysis because of early treatment related death at 4 months post-transplant (no. 2), and because of incomplete data (no. 1). Overall, 66 patients were included and their characteristics are summarized in Table 1. Median time from diagnosis to allogeneic transplant was 13.2 months (range 6.6-101 months). Thirty-five out of 66 (43%) were treated at diagnosis according to a planned tandem "auto/allo" program and were also included in previously published prospective clinical trials [14-17]. All patients provided written informed consent to the proposed treatment and to the use of medical records for research purposes. The present study was approved by the Institutional Review Board of our Center and conducted according to the Declaration of Helsinki (NCT01440556).

Graft-vs.-host disease Acute graft-vs.-host disease (GVHD) was diagnosed according to the recent indications of the National Institute of Health [18]. Chronic GVHD was graded as previously described [19].

Response Assessment Disease response was assessed by urine and serum immune-fixation and bone marrow aspirates at 3, 6, 12, 18, 24 months after allografting, and yearly thereafter. Whole-body conventional radiography or magnetic resonance imaging were performed yearly or as clinically indicated (overt relapse or complaints of bone pain). Disease response and disease relapse were defined according to the European Bone Marrow Transplantation Group criteria [20]. Achievement of CR was defined as the absence of monoclonal component by immunofixation on both serum and urine, disappearance of any soft tissue plasmacytoma and less than 5% plasma cells in the bone marrow. The incidence of extra-medullary disease (EMD) in first relapse post allografting was monitored, and EMD was defined as previously described [21].

First pulls of bone marrow samples had to contain at least 13000 cells/uL for flow-cytometry MRD studies. Plasma cells quantification was obtained by 4 to 6-colour staining with the following monoclonal antibodies: CD38, CD138, CD56, CD19, CD45, cyKappa, cyLambda. A FACSCanto II

Flow-cytometer equipped with FACSDiva software (BD Biosciences, San Josè, CA) was used. A total of 1 x 106 events were acquired and analyzed for each sample, as previously reported [22]. Flow-cytometry analysis had a sensitivity of 10^{-4} cells [23]. IR was defined as less than 0.01% monoclonal plasma cells in the bone marrow sample.

Assessment of CR and IR was done at best response. According to the achievement of CR and/or IR, patients were divided into 4 groups: those who achieved CR and IR (CR/IR), those who obtained CR but not IR (CR/noIR), those not in CR but in IR (noCR/IR) and those who did not achieve either CR or IR (noCR/noIR). Time to CR and IR was evaluated excluding patients who were in CR and IR at the time of transplant, respectively.

Genetic abnormalities Although single evaluation of chromosome 13 deletion (del(13)) is no more considered an optimal prognostic marker, it still has value as it is frequently associated with t(4;14), del(17) or t(14;20). Thus patients presenting del(13) with/without other cytogenetic aberrations were considered as at high risk [24].

Statistical methods Primary endpoints were overall survival (OS) and event free survival (EFS) in the 4 patient cohorts defined by the achievement of CR and/or IR. OS was defined as time from transplant to death by any cause, and EFS as the time from transplant to progression/relapse/death as a result of any cause, whichever occurred first. Alive patients were censored as of October 1st, 2013. OS and EFS curves were estimated by the Kaplan-Meier method and compared using the log-rank test. OS and EFS were then analyzed by the univariate and multivariate Cox proportional hazards model, comparing by the Wald test the following risk factors: age at diagnosis (>55 vs. ≤55 years), gender (male vs. female), year of diagnosis (2008-2011 vs. 2004-2007 vs. 2000-2003), number of chemotherapy regimens (≥2 vs. 1), ISS (stage III vs. I-II), Durie and Salmon stage (IIIA-IIIB vs. IA-IB-IIA-IIB), donor gender (male vs. female), donor type (matched unrelated donor vs. sibling donor), cytogenetic profile (high risk vs. standard risk), EMD [21] in the clinical course before allografting, occurrence of acute and chronic GVHD (any vs. none) and disease response (CR/IR, CR/noIR, noCR/IR, noCR/noIR). Six- and twelve-month landmark analyses were performed to estimate survival by disease response. The occurrence of acute and chronic GVHD and post-transplant IR and CR were treated as time-dependent variables. Cumulative incidences of developing acute GVHD, chronic GVHD, overall relapse and extramedullary relapse were estimated by the Gray test to compare the cumulative incidence curves of the main event, in the presence of a competing event (defined as death without acute or chronic GVHD or relapse occurred before the development of acute or chronic GVHD for acute and chronic GVHD, as death without previous relapse for overall relapse, as death without previous extramedullary relapse or occurence of bone relapse for extramedullary relapse) [25]. Non-relapse mortality (NRM) was defined as death without previous relapse [25]. Patient characteristics were tested using the Fisher's exact test for categorical variables and the Mann-Whitney test for continuous ones. All reported p-values were two-sided, at the conventional 5% significance level. Data were analyzed as of January 2014 by IBM SPSS 21.0.0 (Chicago-IL, USA) and R 2.15.2 package cmprsk (The R Foundation for Statistical Computing, Wien-A).

RESULTS

Study population At diagnosis all patients presented with measurable disease and 12 out of 66 (18%) with EMD. Thirty-five out of 66 received the allograft as part of their first line treatment, whereas the remaining (31/66, 46%) were transplanted at relapse (Table 1). In 2/31 (6%) EMD presented at relapse before the allograft. Conditionings are summarized in Table 1. Post-grafting immuno-suppression consisted of calcineurine inhibitors (cyclosporine or tacrolimus) and mycophenolate mofetil in 60 (91%), and cyclosporine and methotrexate in the remaining. Patients did not receive maintenance therapies or donor lymphocyte infusion post allograft until relapse, with the exception of 6 recent patients who started lenalidomide at six months post transplant as per protocol. Due to the rather long study period, fluorescent in situ hybridization (FISH) was performed in only 20 (30%) patients: del(13) aberration was detectable in 6 patients, 1 patient presented del(13) associated with del(17) and t(4;14) and 1 patient resulted positive for t(4;14); the remaining 12 patients were negative for del(13).

All patients had suitable bone marrow aspirates for IR evaluation.

Non-relapse mortality and GVHD NRM of the overall population of 80 patients was 13.8% at 1 and 3 years, 15.3% at 5 years. In the 66 patients who survived at least 3 months and formed the study population, NRM was 6.1%, 9.1% and 10.8% at 1, 3 and 5 years respectively. After a median follow-up of 7.1 years (range 2.6-13.2), the incidence of acute and chronic GVHD was 44.6% and 52.4%. Patients transplanted at relapse developed more acute GVHD (p=0.03), whereas those transplanted upfront developed more chronic GVHD (p=0.034). Overall, main cause of death was disease relapse in both patients transplanted upfront and at relapse.

Disease response and relapse At the time of the allograft, 9 (14%) patients were in CR and 21 (32%) in IR, 5 of these were both in CR and IR. After the allograft, all 21 IR patients remained in IR and 25 additional patients entered IR for a total of 45/66 (68%), whereas 24/66 (36%) patients achieved CR, of whom only 7 were in CR pre-transplant. Median time to IR was 7 months (range 1-48, no. 23), whereas median time to CR was 8 months (range 1-60, no. 17). Among the 45 patients who achieved IR, 26 performed Magnetic Resonance Imaging (MRI) and 2 Computerized Tomography (CT) scans of the spine at the time of best response, 14 were in the CR/IR group and 14 in the noCR/IR group. Only 4 out of 28 MRI/TC scans showed myeloma infiltration, and all in the noCR/IR group. Seventeen patients in IR did not perform any MRI/CT scan. Twenty-one patients showed discrepant results with persistent serum and/or urine monoclonal component despite the absence of monoclonal marrow plasma cells. Overall, patients were divided into the following cohorts: 24 in CR and IR (CR/IR group); 21 in IR but not CR (noCR/IR group); 21 in neither CR nor IR (noCR/noIR group). No patient was in CR but not in IR

(CR/noIF group) (Table 2). Among patients in the CR/IR group, 5/24 only achieved CR before IR. Given the small cohort an analysis could not be carried out. Patients in the 3 cohorts were equally balanced for age, year of transplant, disease stage, median β2microglobulin, number of previous therapies, conditioning, donor gender and type. Conditioning regimen and acute GVHD were not correlated with disease response group (p=0.703 and p=0.282, respectively), whereas chronic GVHD (p=0.047) and previous therapy lines (p=0.015) were.

Overall, at follow up, cumulative incidence of disease relapse was 32%, 50% and 62% at 1, 3 and 5 years, respectively. At the same time-points, it was higher in the noCR/noIR group (67%, 81%, not applicable) as compared with the noCR/IR group (33%, 62%, 72%) and with the CR/IR group (0%, 13%, 30%, p<0.001). Among patients who achieved IR, median time to clinical relapse post-transplant was 9.7 months in the noCR/IR group and 30 months in the CR/IR one. The overall incidence of extra-medullary first relapse was 9%, 15% and 20% at 1, 3 and 5 years, respectively. At the same time-points, it was 5%, 14%, not applicable in the noCR/noIR; 24%, 33%, 44% in the noCR/IR; and 0%, 0%, 4% in the CR/IR group (p<0.001) (Figure 1). Sites of EMD are reported in Table 3. Fourteen (12 at diagnosis and 2 at pre-transplant relapse) out of 66 (21%) developed EMD before the allograft. However, only 3 of these 14 were among those who experienced EMD after the allograft.

Clinical outcomes Overall, after a median follow-up of 7.1 years (range 2.6-13.2), median OS and EFS were 5.5 and 1.4 years respectively. In patients in CR, median OS and EFS were not reached and 59 months as compared with 40 and 9 months in those not in CR (p<0.001). Median OS and EFS in patients who achieved IR were 96 and 41 months as compared with 36 and 6 months in those who did not (p<0.001). Landmark analysis showed that being in IR at six months post-transplant was not statistically associated with better OS and EFS (7.5 vs. 5.0 years. p=0.132 and 4.1 vs. 1.2 years p=0.065, respectively), whereas IR at 12 months post-transplant conferred an advantage in OS (10.3 vs. 2.4 p=0.018) but not in EFS (3.6 vs. 1 year, p=0.634).

By patient cohort, median OS and EFS were not reached and 59 months in the CR/IR cohort, 64 and 16 months in the noCR/IR cohort, and 36 and 6 months in the noCR/noIR cohort respectively ($p<0.001$, both for OS and EFS) (Figure 1). Among patients not in CR, there was a significant advantage in EFS and a trend for better OS for those who reached IR compared to the noCR/noIR group ($p=0.001$ and $p=0.063$, respectively).

With the limitations of the small sample size (only 20 patients evaluated), OS in high risk patients by FISH analysis was 39 months as compared with "not reached" in standard risk patients ($p=0.009$), whereas EFS was not statistically significant (19 months vs. 64 months, $p=0.097$).

All patients with EMD at first relapse (no.13) after the allograft eventually died of disease progression. OS in patients first relapsed with EMD was significantly shorter than in those relapsed without EMD (39 vs. 57 months, $p=0.034$). By contrast, there was no difference in OS and EFS between newly diagnosed patients with EMD and those without.

By univariate and multivariate analysis, belonging to the CR/IR cohort was the only significant predictor for prolonged OS and EFS ($p<0.001$) (Table 4, Table 5).

DISCUSSION

MRD analysis is currently used for evaluating treatment efficiency and patient risk stratification in several hematological malignancies [26]. In MM, not only is MRD of primary importance to assess tumor shrinkage, but it is now regarded as one of the strongest prognostic predictors, irrispective of any given treatment. MRD analysis by multicolor flow-cytometry has been introduced in many clinical trials on myeloma. The prognostic impact of achieving IR has been described after conventional chemotherapy, autografting and, more recently, after new drugs [9-13].

Despite some limitations due to its retrospective nature, our study underlines the clinical importance of achieving IR also after allografting (Figure 1). Post-transplant IR was associated with significantly better OS and EFS. Landmark analyses suggested that IR at 12 months post allografting had greater impact on OS than IR at 6 months. This might be explained by an ongoing and/or late occurrence of graft-vs-myeloma effect. However, in patients in IR, clinical outcomes were different in the light of CR status. OS and EFS were not reached and 59 months in the CR/IR group, and 64 and 16 months in the noCR/IR group, respectively ($p<0.007$ and $p<0.014$, Figure 1). To stress the role of IR, we also observed that patients in noCR/IR showed an intermediate clinical outcome compared with those in CR/IR and in noCR/noIR (Figure 1). IR and CR status was the only variable significantly associated with improved OS and EFS by multivariate analysis ($p=0.001$), whereas GVHD, the number of previous therapy lines, conditioning regimen, and year of transplant were not (Table 4, Table 5).Other authors reported similar outcomes between patients who were MRD negative but not in CR and those MRD positive [13]. Paiva et al. [11] reported 21% of patients in IR with persistent positive immunofixation after autografting. Moreover, progression free survival (PFS) was progressively shorter, 71, 65, and 37 months, in patients in IR/CR, in IR/noCR and in noIR/CR respectively ($p=0.001$). This study clearly showed that the achievement of remission by flow-cytometry had a higher prognostic value than remission by immunofixation. In our study, we cannot draw such a definitive conclusion on the role of IR, given the lack of patients in CR but not in IR.

The discrepancy between IR and not CR was observed in 32% of our transplant patients. This finding may be explained by a number of reasons. It may partly be argued that bone marrow aspirates do not systemically represent the marrow status and areas of marrow disease may persist. It may however be more plausible that residual extra-medullary plasma cells continue secreting monoclonal immunoglobulins in sanctuary sites where agents with anti-myeloma activity and/or a potential *graft-vs.-myeloma* may have little or slower effect. This hypothesis is supported by a higher incidence of extra-medullary relapse in the IR/noCR cohort: 44% in noCR/IR group vs. 4% in CR/IR group at 5 years ($p<0.001$). A high incidence of EMD following allografting after reduced-intensity conditioning was previously reported. In a series of 70 patients enrolled in a Spanish study, extra-medullary involvement was documented in 10 out of the 27 patients at first relapse (37%) [27]. Interestingly, the incidence of extra-medullary relapses was higher in patients who had developed chronic GVHD. Importantly, these patients had no evidence of disease recurrence in the marrow at the time of relapse. The Authors suggested that *graft-vs.-myeloma* effects may have been more efficient in the marrow or, alternatively, that monoclonal plasma cells involved in extra-medullary relapse were more resistant to donor T-cells. In another multi-center study, Minnema et al. reported an incidence of EMD of 20.4% in 54 relapsed MM patients from a total group of 172 treated with sequential autologous-allogeneic non-myeloablative transplantation [28]. Interestingly, no association with chronic GVHD and EMD at relapse was found. In our experience, chronic GVHD did not impact on extra-medullary relapse. Overall, the association between chronic GVHD and *graft-vs.-myeloma* effects, is still debated [29].

Finally, the recent observation of a possible increase of the occurrence of EMD, especially after multiple relapses, may partly be explained by the current natural history of myeloma where patients commonly live longer as compared to past decades [30]. A study on 1003 MM patients showed an increase in EMD incidence in the period 2000-2007 as compared with previous years raising concerns, despite a dramatic improvement in OS, about a correlation with the use of novel agents with potent anti-myeloma activity and/or a greater use of high-dose therapy [30]. However, the observation that the increase was evident both at diagnosis and at relapse suggests that other factors are contributory [31]. To reduce the risk of bias when comparing the patient cohorts of our study, we particularly focused on the presence of EMD at diagnosis and at first relapse post-transplant. In our series, the presence of EMD at diagnosis did not correlate with a higher risk of EMD development post transplant and only the noCR/IR status was significantly associated with extra-medullary relapse. Though EMD before allografting did not impact on survival, post transplant extra-medullary relapse was associated with poorer outcome in comparison with bone relapse (OS 39 vs. 57 months, p=0.034).

Although the potential role of positron emission tomography integrated with computed tomography (PET/CT) in the assessment of MM continues to be a matter of debate [32], it may be particularly informative to early diagnose EMD, together with other readily available laboratory assays such as serum free light chains assay [33]. Patients in IR/noCR could be ideal candidates for a clinical follow up that routinely includes PET/CT to possibly detect extra-medullary relapse before the occurrence of symptoms.

In conclusion, evaluation of MRD by flow-cytometry is a sensitive prognostic tool after allografting and should routinely be introduced in clinical practice. The achievement of IR is associated with better clinical outcomes. Moreover, the combination of IR and CR is helpful to identify a subset of patients (IR-noCR) at higher risk of developing extra-medullary relapse who may benefit from a more stringent follow up and consolidation treatment with new agents [34,35].

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FIGURE LEGEND

Figure 1. Clinical outcomes in three cohorts of patients defined by achievement of complete clinical remission (CR) and immunophenotipic remission (IR): patients in CR and IR (CR/IR) (green line); patients not in CR but in IR (blue line); patients not in CR and not in IR (pink line)