Title: Efficacy of Bortezomib to intensify the conditioning regimen and the Graft-versus-Host Disease prophylaxis for high risk myeloma patients undergoing transplantation

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Running head: Bortezomib in allo-RIC for high risk MM.

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Conflict of Interest:

This trial was supported by Janssen and Celgene. Dra. Teresa Caballero-Velázquez: has received honoraria derived from lectures from Janssen and Celgene. Jesús Martín: has receive accommodation and registration fee for Reunión Anual Pethema 2016 from Janssen. Dra. MV Mateos has received honoraria derived from

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Abstract:

This multicenter phase I trial was designed to evaluate the safety and efficacy of Bortezomib (Bz) as part of both the conditioning regimen and the graft-versus-host (GvHD) prophylaxis. Patients received fludarabine, melphalan and Bz (days -9 and -2). GVHD prophylaxis consisted of Bz (days +1, +4 and +7), Sirolimus (Siro) from day -5 and Tacrolimus from -3 (except the first 5 patients that did not receive Tk). 25 patients with poor prognostic multiple myeloma were included. 11 out of the 19 patients had high risk features. Out of the 21 patients evaluable at day +100, 14 were in CR (67%) and 7 (33%) in PR. Cumulative incidence (CI) of non-relapse mortality at 1 year was 24%. CI of grades 2-4 and 3-4 acute GvHD was 35% and 10%, respectively; CI of chronic GvHD was 35% and 55% at 1 and 2 years, respectively. Overall and event free survival at 2 years were 64% and 31%, respectively. Bz as part of the conditioning regimen and in the combination with Siro/tacrolimus for GvHD prophylaxis is safe and effective allowing an optimal disease control early after transplant and reducing the risk of GvHD.

INTRODUCTION

The use of novel drugs has improved the outcome of patients with multiple myeloma (MM)[1-9]. Nevertheless, considering that almost all patients with MM end up with refractory relapses, their management represents a critical area of basic and clinical research.

Although allogeneic stem cell transplantation (allo-SCT) may be the only curative approach for these patients, its results at the long term are hampered by a high morbidity and mortality[10-16]. With this background, the role of allo-SCT is being re-defined. Accordingly, different groups recommend its use only in relapsed patients displaying poor prognosis features and within clinical trials[17-20].

The development of reduced intensity conditionings (RIC) has allowed to decrease the toxicity of the allo-SCT, although the incidence of graft-versus-host disease

(GvHD) has not been reduced and, furthermore, in the RIC setting, a higher incidence of relapses has been reported[21,22].This is the reason why strategies aimed at optimizing the allo-SCT are needed, improving both the anti-tumor effect of the conditioning regimen as well as implementing new approaches that reduce the risk of GvHD without increasing the risk of relapse. In this regard, instead of considering allo-SCT versus novel drugs as competitive strategies, it would be reasonable to take advantage of the anti-myeloma effect of novel drugs[2-9] in the transplant setting and integrate them within the pre-, peri- or post-transplant period. In a previous study conducted by the Spanish myeloma group and the Spanish transplant group (GEM/GETH), we described for the first time a preliminary experience in which Bortezomib (Bz) was used within a conditioning regimen based on fludarabine and melphalan[23]. In this study, 15 out of 16 patients obtained stable disease or improved disease status at day +100, including 10 patients with pre-transplant active disease. Moreover, we and other authors have shown that Bz does exert a potent pro-apoptotic effect on activated T-cells preserving the viability of resting T-lymphocytes [24-28] as well as regulatory T cells (Tregs) [29], thus preventing GvHD. This concept has been translated into the clinical setting and the toxicity and efficacy of Bz as GvHD prophylaxis has been evaluated in several clinical trials[30-32].

In a preclinical model, we have previously shown a synergistic effect of the combination of Sirolimus (Siro) and Bz in the prevention of GvHD. Interestingly, the combination of both drugs inhibited the activation and proliferation of stimulated lymphocytes and the production of Th1 cytokines (IFN γ, IL-2 and TNF)[33]. On the other hand, this combination synergistically affects the viability of MM cell lines according to O'Sullivan e*t al*[34].

The present phase I trial was designed to evaluate the safety and efficacy of intravenous (iv) Bz as part of the conditioning regimen and in combination with Siro for GvHD prophylaxis in poor prognostic MM patients.

PATIENTS AND METHODS:

Study design:

Conditioning regimen was based on the combination of Bz 1.3 mg/m^2 iv on days -9 and -2, Fludarabine 30 mg/ m^2 iv on days -6 to -4 and Melphalan 140 mg/m^2 i.v on day -3. Progenitor stem cells, with a recommended target dose of > 5 x 10^6 CD34+ cells / kg,

were infused on day 0. As GvHD prophylaxis Bz 1.3 mg/m^2 was used on days +1, +4 and +7 after transplantation and Siro (range of serum levels: 6-12 ng/mL). After day +100, a slow taper was scheduled in the absence of GvHD.

Inclusion criteria:

Adult patients between 18 and 70 years of age with indication of transplant at the discretion of the attending physician were considered candidates. The first 10 patients had to be beyond first complete remission and having an HLA identical sibling was required. For the remaining patients. unrelated donors either matched or with a single mismatched at A. B. C or DRB1 were allowed. All patients signed informed consent to participate in the clinical trial. The original clinical trial was registered at www.clinicaltrialsregister.eu as EudraCT: 2010-018594-37. All the procedures were carried out. according to the ethical postulates of the Declaration of Helsinki. The trial was approved by the Autonomic Committee of Clinical Trials of Andalucía and by the local ethics committees of all participating centers.

Exclusion criteria:

Patients with any of the following criteria were excluded from the trial: HIV positive. hepatitis B surface antigen or active infection with hepatitis C virus; have had an acute myocardial infarction in the six months prior to entering the study or have functional class (NYHA) III or IV uncontrolled angina. uncontrolled arrhythmia or acute ischemia; previous history of malignant disease (except basal or squamous cell carcinoma in skin, carcinoma in situ in cervix) unless the patient was disease free for more than 5 years; uncontrolled high blood pressure or diabetes; previous history of cirrhosis, peripheral neuropathy ≥ grade 2, psychiatric disease. pericardial or diffuse pulmonary infiltrate and hypersensitivity to Bz, boric acid or mannitol.

Safety and efficacy analysis:

Safety was evaluated by assessing adverse events in all patients from the first procedure related to the study until 30 days after the end of treatment period, at least one year after transplant. The severity of adverse events was assessed according to the classification of the National Cancer Institute (NCI) common toxicity criteria (CTC), version 4.0. Safety was analyzed every 5 patients included into the trial.

The safety criteria were assessed in terms of graft, defined as > 500 granulocytes/µL and > 20,000 platelets/µL x 3 consecutive days, required for 9/10 patients; incidence of neuropathy grades 3-4 attributed to Bz > 20%; and incidence of gastrointestinal toxicity attributed to Bz > 15%.

The efficacy of the procedure was evaluated in terms of incidence of grades 2-4 acute GvHD (aGvHD), which had to be ≤ 3 for the first 5 patients or ≤ 6 out of 10 patients. Overall grading of aGvHD was assessed as defined by Glucksberg et al [35]. Regarding chronic GvHD (cGvHD), stages were defined according to NIH criteria [36].

If the efficacy criteria were met, but not the toxicity criteria, a reduction in Bz dose was planned. If efficacy criteria were not met in terms of incidence of GvHD, a modification in the GvHD prophylaxis by adding Tacrolimus (Tacro) at 0.02 mg/kg/day starting on day -3 (range of serum levels: 5-10 ng/mL) together with Bz and Siro was planned. A slow taper of Tacro was planned to start on day +50 post-transplant.

Disease status was evaluated according to the EBMT criteria[37].

Biological procedures and ex vivo monitoring

Blood cell counts, monoclonal component by electrophoresis and/or immunofixaion and chimerism assays in peripheral blood (PB) were performed on days +21/28. +100. +180. +270. +360. Different subpopulations of the immune system on PB and minimal residual disease (MRD) on bone marrow were evaluated by flow cytometry (FC) on days +100, +180, +270 and +365 after transplantation in patients who had received the triple combination; the pattern of cytokines in serum were calculated on days +7, +14, +21, +100, +180, +270 and +365 after transplantation. In addition. biological assays were also performed in 4 healthy controls and 7 patients undergoing transplantation with the same GvHD prophylaxis but without Bz (Siro and Tacro) on day +100 for immunophenotype analysis and on days +7, +14. +21 and +100 for the cytokines.

Immunophenotypic analysis

One hundred microliters of PB per tube was stained with the monoclonal antibodies to the surface antigens. After 30 minutes of incubation at room temperature in the dark samples were washed and acquired. Neutrophils, monocytes, eosinophils, basophils, Myeloid BDCA1 DCs, plasmacytoid DCs and monocyte-derived DCs were

identified with the following makers: CD16 (PB and FITC), HLADR (V450 and FITC), CD45-V500, BDCA1-PE, CD64-PE. CD11c-PerCP-Cy5.5, CD33- PerCP-Cy5.5., CD86-PE-Cy7, CD123-APC and CD14-APC-H7. The combination CD19+CD8-FITC/CD3+CD56-PE/CD4-Pe-Cy5/HLADR-APC was using to calculate all subpopulations of lymphocytes. CD45RA-FITC and CCR7-PE were used to distinguish the repertory of naive/effector/memory of CD4 and CD8 cells. The expression of CD16 and CD94 was studied on CD56bright and CD56low NK cells. For Treg assessment after incubation of surface antigens (CD25-FITC. CD127-PE and CD4-PerCP-Cy5.5), cells were washed in PBS and then fixed and permeabilized with FoxP3 Staining Buffer Set (eBiosciences) for FOXP3 staining. Activation assays were performed on 500 µl of PB added in 48-well plates; PB was stimulated or not with PMA (20µg/2ml) and ionomycin (0.91 µg/ml). Brefeldin A (10 µg/ mL) was added in both cases. After four hours. un-stimulated and stimulated cells were stained with surface antigens (anti-CD25-FITC and anti-CD3-PerCP-Cy5.5). Staining for cytoplasmatic IFN-gamma-PE and CD40L-APC was performed using Fix and Perm. All samples were acquired in a FACSCanto II flow cytometer (Becton Dickinson) using the Diva software (Becton Dickinson) and data analysis was performed using Infinicyt 7.1. software (Cytognos).

Cytokine assays

Serum levels of Th1/Th2 cytokines (IL-2. IL-4. IL-6. IL-10. tumor necrosis factor alfa (TNF)-α and interferon gamma (IFN-γ)) were determined by FC using the BD Human Th1/Th2 Cytokine CBA kit (BDB. Briefly, samples were analyzed using FACS array software II (Beckton Dickinson). The concentration of each cytokine was reported as pg/ml of PB.

MRD monitoring

Two milliliters of bone marrow were collected on EDTA for the detection of MRD by multiparametric FC with the following combination of antibodies: CD45-V450, CD138-V500, CD38-FITC, CD56-PE, CD27-PerCP-Cy5.5, CD19-PE-Cy7, CD117-APC and CD81-APC-H7. At least, a 1.000.000 of events was acquired. Blood contamination was evaluated based on the populations present in the bone marrow sample (erythroblasts, precursors and mast cells) and is considered positive when we detect at least one pathological population with a minimum of 30 events (limit of detection 0.003,

limit of quantification 0.005%). Plasma cells were identified by their expression pattern of CD138/CD38 and their light scattering (FSC and SSC). To determine whether it corresponds to pathological plasma cells, it must meet at least three of the following characteristics: CD45 and / or CD19 negative. under-expression of CD38, CD27 or CD81 or positive for CD56 or CD117. In case of doubt. its intracytoplasmic clonality was confirmed according to its cytoplasmic expression of light chain.

Statistical analysis

The following end-points were analyzed: response. aGvHD. cGvHD. non-relapse mortality (NRM). event free (EFS) and overall survival (OS).

Acute GvHD, cGVHD and NRM were analyzed and compared using the Gray test. The cumulative incidence was calculated with the cmprsk package for software R 2.14.0 (R Development Core Team (2011). http://www.R-project.org/). The competing events were defined as follows: in case of acute and cGvHD, the competitor event was death without the existence of the event of interest. For the NRM the competitor event was relapse. The OS, PFS and relapse free survival (RFS) were estimated by the Kaplan Meier method. using SPSS version 13.0 (SPSS Inc. Chicago. IL. USA).

The events were calculated from the time of transplantation as follows: NRM was defined as death from any cause without previous relapse or progression of the underlying disease. OS was calculated from transplant until death from any cause and patients who survived were censored at last follow-up. EFS was calculated from transplant to relapse or death. and those patients who did not reach at least partial response at any time after transplantation were also considered an event for EFS.

Patients who had received the triple combination were evaluated for both acute and cGvHD. Patients who engrafted were evaluable for aGvHD while patients who survived > 100 days were evaluable for cGvHD. For both acute and chronic GvHD the day of onset was considered as the time to the event. For cGvHD patients were censored at the time of relapse.

The biological studies were analyzed by nonparametric tests with the Mann Whitney U test or median test. The differences were considered statistically significant when $p < 0.05$.

RESULTS

Patient characteristics

The inclusion period was from March 2012 to June 2016 with a total of 25 patients planned. Median age was 53 (range: 40-69) and median number of prior lines of treatment was 3 (range: 1–7). Twenty-two patients had previously relapsed to an autologous stem cell transplantation, all of them had received treatment including proteosome inhibitors and immunomodulatory drugs (thalidomide, lenalidomide or pomalidomide). Treatments administered before and after transplantation are specified in supplementary table 1. Regarding cytogenetics, 11 out of the 19 patients with cytogenetic information available, had high risk features [del 17p and/or t(4,14)]. These characteristics are summarized in table 1 and supplementary table 2.

At the time of transplant 5 patients were in complete remission (CR), 17 in partial response (PR), 1 had stable disease (SD) and 2 patients had progressive disease (PD). Of the 5 patients transplanted in CR, 1 had a plasma cell leukemia and 2 patients had high-risk cytogenetic features (17q deletion and t(4;14))

As far as the transplant procedure is concerned 19 patients received allo-SCT from a matched related donor, 5 from a matched unrelated and 1 from a mismatched unrelated donor. Twenty four patients received progenitor stem cells from PB and 1 from bone marrow. These characteristics are shown in table 2.

Engraftment, toxicity and GvHD

All evaluable patients reached > 0.5 x 10^9/L granulocytes at a median of 15 days (range: 2-35) and 24 out of 25 patients reached > 20 x 10^9/L platelets at a median of 12 days (range: 9-174) after transplantation.

As far as the early post-transplant toxicities are concerned, 8 developed grades 1-3 mucositis, 12 gastrointestinal toxicity grades 1-3, 3 developed grades 1-2 thrombotic microangiopathy and 5 developed grades 1-2 neuropathy.

Regarding GvHD, the first 5 patients treated with the combination of Siro plus Bz develop aGvHD (3 grades 2, 1 grade 3 and 1 grade 4). According to the safety rules, for the remaining 20 patients, Tacro was also added to the combination of Siro + Bz. With this triple combination, 5 patients developed grades 2-4 aGvHD (3 patients had grade 2 aGvHD and 2 patients had grade 3), while 7 and 2 patients developed mild and moderate cGvHD, respectively. These data are shown in tables 2 and 3. The cumulative incidence of grades 2-4 and 3-4 aGvHD with the triple combination was 35% (95% CI: 15-55%) and 10% (95% IC: 1-27%), respectively, while the cumulative incidence of overall

cGvHD was 35% at 1 year (95% CI: 13.8-57.8%) and 55% (95% CI: 26.6-76.2%) at 2 years (figure 1).

Response and relapse rates and overall outcomes

Out of the 21 patients evaluable at day +100, 14 were in CR (67%) and 7 (33%) in PR. Cumulative incidence of NRM was 24% at 1 year post-transplant (CI-95%: 9.4-42.2). Relapse rate at 1 year was 21.4% (CI-95%: 4.7-45.9) (Figure 2). These data are shown in table 3. Causes of death include: septic shock (n=2), acute myocardial infarction without ST elevation (n=1), acute GvHD in a patient receiving Siro + Bz (n=1) and progression of MM (n=2).

Median of follow up for alive patients was 33 months (CI-95%, 19-47). OS and EFS at 2 years were 64% (CI-95%: 42-79) and 31% (CI-95%: 14-59), respectively. At last follow-up, 11 patients (44%) were alive and 5 of them had not relapse (Figure 3).

Immune recovery and MRD monitoring

On days +100, +180, +270 and +365 post-transplant different immune populations were identified. The most significant differences between patients with the triple combination of Tacro plus Siro plus Bz (n=16) as compared to healthy donors (n=4) were: a decreased percentage of plasmocytoid DCs, lymphopenia with inversion of the CD4/CD8 ratio and low counts of B cells up to 1 year after transplantation. Concerning naïve/memory/effector T cells distribution, a lower percentage of naïve and central memory T CD4+ cells were observed. However, percentage of Tregs (CD4+CD25+CD127-/+wFoxp3) were similar as compared to healthy controls. Also, no differences were observed regarding the percentages of NK cells and KIR, although the ratio of NK CD56 + cells "bright" and "weak" was different, with a higher percentage of CD56 "bright" cells among patients after 6 months of transplantation. A significantly lower expression of CD40L and higher levels of intracellular IFN stood out in patients until 9 months of transplant (Supplementary table S3). In addition, when we compared these populations on day +100 with those observed among patients undergoing transplantation with Siro plus Tacro without Bz as GvHD prophylaxis (7 patients), the most remarkable difference was a significantly lower expression of CD94 in NK cells among patients receiving Bz (Supplementary table S4).

The pattern of Th1/Th2 cytokines in serum on days +7, +14, +21 and +100 was also compared among patients receiving Siro and Tacro with (n=19) or without (n=7) Bz. Remarkably, a trend towards maintaining higher levels of pro-inflammatory cytokines was observed among patients who did not receive Bz and the contrary occurred for IL-10. The most significant differences were observed for IL2 (from day +7 up to +100 after transplant) and IL6 (on day +21) as well as for IL10 (on day +21) (figure 4). Regarding healthy donors, this pattern was different, with lower levels of IL6, IL10 and higher levels of IL4 than patients with triple combination (Supplementary table S5).

Regarding MRD monitoring, it was evaluated in 19 patients: 12 of them achieved MRD negative during the first year after transplantation and 2 additional patients achieved it beyond 1 year post-transplant. Eight of these 14 patients relapsed, four of them only with extramedullary disease and 1 more with extramedullary disease and MRD positivity. Finally, 7 patients remained MRD positive within controls established along the first year posttransplant; five of them relapsed and 2 achieved MRD negativity after 1 year of transplantation (figure 5).

Statistical analysis

The following end-points were analyzed: response. aGvHD. cGvHD. non-relapse mortality (NRM). event free (EFS) and overall survival (OS).

Acute GvHD, cGVHD and NRM were analyzed and compared using the Gray test. The cumulative incidence was calculated with the cmprsk package for software R 2.14.0 (R Development Core Team (2011). http://www.R-project.org/). The competing events were defined as follows: in case of acute and cGvHD, the competitor event was death without the existence of the event of interest. For the NRM the competitor event was relapse. The OS, PFS and relapse free survival (RFS) were estimated by the Kaplan Meier method. using SPSS version 13.0 (SPSS Inc. Chicago. IL. USA).

The events were calculated from the time of transplantation as follows: NRM was defined as death from any cause without previous relapse or progression of the underlying disease. OS was calculated from transplant until death from any cause and patients who survived were censored at last follow-up. EFS was calculated from transplant to relapse or death. and those patients who did not reach at least partial response at any time after transplantation were also considered an event for EFS.

Patients who had received the triple combination were evaluated for both acute and cGvHD. Patients who engrafted were evaluable for aGvHD while patients who

survived > 100 days were evaluable for cGvHD. For both acute and chronic GvHD the day of onset was considered as the time to the event. For cGvHD patients were censored at the time of relapse.

The biological studies were analyzed by nonparametric tests with the Mann Whitney U test or median test. The differences were considered statistically significant when $p < 0.05$.

DISCUSSION:

Even in the relapse setting, different combinations of novel drugs [1-9] have greatly improved the outcome of patients with MM, with PFS not reached at 24 months and overall and CR rates over 50%, among patients treated in 1st-3rd relapse. With these data in mind and considering the morbidity and mortality associated to allo-SCT, several cooperative groups recommend the use of allo-SCT only in relapsed patients displaying poor prognosis features and within clinical trials[17-20]. Nevertheless, survey data from the EBMT clearly shows that the activity of allo-SCT is not decreasing but, on the contrary, slightly increasing although in a more heavily pre-treated population[38], thus suggesting that, in spite of the efficacy of novel drugs, most MM patients finally relapse and eventually became candidates to be considered for allo-SCT, although in more advanced phases of the diseases. Accordingly, the development of novel approaches to improve the safety and efficacy of allo-SCT in MM, especially in this high risk group of patients, is an important medical need.

The only comparison available between allo-SCT and novel drugs in the relapse setting has recently been updated by Patriarca et al [39]. In this study, 169 patients previously relapsed after an autologous transplant were included. Seventy nine patients with a suitable donor were compared with 90 patients without a donor who were treated with salvage treatment including Bz and/or immunomodulatory agents. Seven-year PFS was 18% in the donor group and 0% in the no-donor group ($p < .0001$) while OS was 31% in the donor group and 9% in the no-donor group ($p < .0001$).

In the current study, we aimed to demonstrate that the combination with novel drugs might improve the efficacy of allo-SCT. More specifically, we decided to evaluate the use of Bz to both increase the efficacy of the conditioning regimen in terms of response rate and to decrease the risk of GvHD in combination with Siro and Tacro.

As far as the use of Bz within the conditioning regimen is concerned, in a previous study we already showed the feasibility of including Bz within a RIC23. Moreover, the use of Bz together with Siro for GvHD prophylaxis early after transplant might also contribute to enhance the antimyeloma effect of the procedure. In fact, in vitro studies indicate that Bz and Siro exert a potent synergistic antimyeloma effect [34]. Remarkably, in the current study, only 5 out of 25 patients underwent transplantation in remission. Moreover, out of these 5 patients, one had a plasma cell leukemia, 3 patients had required more than three lines of therapy to obtain complete remission and 2 patients had high-risk cytogenetic features (17q deletion and t(4;14)). Of note, 9 patients had received ≥ 5 lines of treatment and most of them displayed poor prognostic cytogenetic features. In spite of these characteristics, on day +100 evaluation, 14 were in CR (67%), and 7 (33%) in PR, thus confirming the efficacy of the procedure in terms of response rate early post-transplant. Cumulative incidence of NRM and relapse rate at 1 year was 24% and 21.4% with an OS and EFS at 2 years of 64% and 31%, respectively.

Considering the differences in terms of study design, end-points definitions or patients characteristics, it is difficult to compare the outcomes of patients included in different studies. Nevertheless, it is worth mentioning several studies, such as the retrospective analysis by Sobh et al, in a large series of 570 patients who underwent RIC allo-SCT after having relapsed to a previous autologous SCT. At the time of transplant, 70% of the patients were at least in PR. Median OS was 32, 31 and 20 months within patients receiving SCT from matched unrelated, mismatched unrelated donor or cord blood, respectively[40].

In the current study, most patients displayed poor risk cytogenetic features. In a previous study, Kröger et al described a 5-year PFS of 29% among 73 patients undergoing allo-SCT, with no significant differences between patients with 17p13 / t (4; 14) (n=16) and others (24% vs. 30%; p = 0.7)[41]. In addition, a recent prospective trial by Knop et al demonstrated that, among patients with high-risk cytogenetic features such as 13q or 17p, autologous transplantation followed by allogeneic significantly improved PFS[42].

As far as the risk of GvHD is concerned, we previously showed in a mouse model that the combination of Bz and Siro synergistically decreases the risk of aGvHD[33]. Remarkably, it has been described the efficacy of Bz in combination with Tacro and MTX as GvHD prophylaxis in prospective phase I/II trial conducted among 83 patients who received an allo-SCT from a mismatched unrelated donor. The risk of grades 2-4 and 3-4 aGvHD was 22% and 7%, respectively [31]. More recently, Koreth et al reported the

results of an open-label three-arm 1:1:1 phase II randomized controlled trial comparing Tacro/ methotrexate (MTX) versus Bz/ Tacro/ MTX versus Bz/ Siro/ Tacro in patients lacking HLA-matched related donors. The risk of grades II-IV aGvHD was 32.6%, 31.1% and 21%, respectively. In a subgroup analysis for the 8/8 HLA-matched recipients, Bz-based regimens had a borderline trend towards a lower risk of grades II-IV aGvHD as compared to Tacro/ MTX, with a cumulative incidence of 17% *vs.* 33%, respectively, (p=0.08)[43]. The stringent requirements for success could have limited their ability to detect lower, albeit potentially clinically meaningful benefit with Bz. It is also worth mentioning that the use of Bz within the conditioning, early before transplant, as performed in the current study, might also influence on the risk of GvHD considering the potential effect of Bz not only in T cells but also on antigen presenting cells (APC)[44]. Thus, blocking APC activation before transplantation might favor a pro-tolerogeneic immune response. In addition, Ahmad-Samer Al-Homsi et al described the results of a phase I trial in which patients undergoing allo-SCT received GvHD prophylaxis based on the combination of Bz and post-transplant cyclophosphamide. The incidence of grades 2-4 and 3-4 GvHD was 20% and 6.7%, respectively[32]. In fact, the use of post-transplant cyclophosphamide (Cy) has been one of the most disruptive concepts in the GvHD prophylaxis in the last years, allowing the widely use of haploidentical donors. Using Cy together with Tacro and mycophenolate mophetil, the group of Baltimore[45], described their experience on 39 patients diagnosed with MM. Cumulative incidence of grades 2-4 and 3-4 GvHD was 41% and 8% respectively, quite similar to the data reported in the current study. Nevertheless, cumulative incidence of relapse was 46% at 1 year. Another multicenter trial using post-transplant Cy in 30 MM patients undergoing haploidentical transplantation has been reported, with a cumulative incidence of grades 2-4 aGVHD of 29% and cGvHD of only 7% at 1 year. PFS and OS at 1.5 years were 33% y 63%, respectively[46]. Interestingly, in the current study we performed biological assays at different time-points after transplant. As compared to a series of patients receiving Siro / Tacro but not Bz, the most remarkable differences at day +100 were a higher percentage of neutrophils and lower levels of CD94 for NK cells and higher of CD158a and NKAT1 for NK cells among controls. The pattern of Th1/Th2 cytokines in serum on days +7, +14, +21 and +100 was also compared. Remarkably, patients who did not receive Bz maintained higher levels of pro-inflammatory cytokines and the contrary occurred for IL-10.

It is noteworthy to point out that although all patients engraftment neutrophils, two patient reached > 0.5 x 10^9/L granulocytes after 20 days after transplantation (upper range 35 days). Considering the acceptable toxicity profile of the procedure including a low NRM as well as the optimal disease control obtained early after transplant, and assuming that the strategies for GvHD prophylaxis described in the current as well as in other studies might help to minimize the risk of GvHD, relapse rate will remain as the main factor limiting the success of the procedure at the long term, especially taking into account that MM patients are nowadays undergoing transplantation in more advanced phases of the disease. Unfortunately, taking into account this and other trials, allo-SCT might be able to maintain disease control at the long term only in a minority of patients undergoing transplantation in advanced phases of the disease. Transplant in earlier phases of the disease might contribute to overcome this problem47 but, as previously mentioned, the current scenario40 will force us to face patients in advanced phases of the disease. Accordingly, other strategies such as including maintenance after allo-SCT, as shown outside the transplant setting, must be evaluated and are currently underway using immunomodulatory drugs. Remarkably, this approach has been shown to be quite effective when used as a rescue therapy in MM patients relapsing after allo-SCT, although in some of these studies its use might have increased the risk of GvHD48,49. Lower doses than those used in MM and a delayed administration of these drugs seem to decrease this risk and are being currently evaluated.

Another aspect to be considered in order to control the disease at the long term should be to perform an accurate monitoring of MRD in order to perform early interventions. In the current study 19 patients were evaluated, 12 of them achieved MRD negative during the first year after transplantation and 2 additional patients achieve it beyond 1 year post-transplant. 8 of these 14 patients relapsed, four only with extramedullary disease and 1 more with extramedullary disease and MRD positivity. As reported in the current manuscript and previously described, extramedullary relapses are common after allogeneic stem cell transplantation[22,50] and, accordingly, other strategies such as imaging assays using PET/CT or other similar approaches might also help to identify early relapses in an attempt to guide early interventions among patients at risk of relapse.

In conclusion, in the present trial the use of Bz to intensify both reduced intensity conditioning as well as GvHD prophylaxis is safe. An optimal disease control is obtained early after transplant. The risk of GvHD is low among patients receiving the triple

combination of Bz/Siro/Tacro. Relapse rate does remain as the main factor limiting the success of the procedure at the long term and other strategies such as including maintenance after allo-SCT and close monitoring including imaging assays should be evaluated.

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Contributions:

Study design: TCV, LLC, CMRF, JSM, JPS. Patient enrolment: LLC, JM, EPL, DC, M-VM, JPS. Data collection: CCC, LLC, EPL, JM, CMRF. Biological studies: TCV, NP, CGC. Biological analysis: TCV, NP, CGC. Data analysis. TCV, FMM, JM, JPS. Data interpretation: TCV, CCC, LLC, JSM, JPS. Wrote or contributed to the writing of the manuscript: all authors.

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FIGURE LEGENDS:

Figure 1: A cumulative incidence of grades 2-4 aGvHD (A), grades 3-4 aGvHD (B) and overall cGvHD (C). Acute GvHD and cGVHD were analyzed and compared using the Gray test. cumulative incidence was calculated with the cmprsk package for software R 2.14.0. The competitor event was death without the existence of the event of interest

Figure 2 cumulative incidence of NRM (A) and relapse (B). NRM were analyzed and compared using the Gray test. Cumulative incidence was calculated with the cmprsk package for software R 2.14.0. The competitor event was relapse.

Figure 3 overall (A) and event free survival (B). The OS and EFS were estimated by the Kaplan Meier method. using SPSS version 13.0. OS was calculated from transplant until death from any cause and patients who survived were censored at last follow-up. EFS was calculated from transplant to relapse or death. and those patients who did not reach at least partial response at any time after transplantation were also considered an event for EFS.

Figure 4 Cytokine pattern along the first year post-transplant. Seven patients receiving Siro plus Tacro without Bz were used as controls. The biological studies were analyzed by nonparametric tests with the Mann Whitney U test or median test

Figure 5 minimal residual disease monitoring.