FULL DONOR CHIMERISM AFTER ALLOGENEIC HEMATOPOIETIC STEM CELLS TRANSPLANT FOR MYELOFIBROSIS: THE ROLE OF THE CONDITIONING REGIMEN

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Word count: 2715

Key words

Myelofibrosis; allogeneic transplantation; busulfan; melphalan, thiotepa, fludarabine chimerism;

ABSTRACT

The aim of this retrospective study, was to assess the rate of full donor chimerism (F-DC) in patients with myelofibrosis, prepared for an allogeneic stem cell transplant, with one or two alkylating agents. We analyzed 120 patients with myelofibrosis, for whom chimerism data were available on day +30**.** There were two groups: 42 patients were conditioned with one alkylating agent (ONE-ALK), either thiotepa or busulfan or melphalan, in combination with fludarabine, whereas 78 patients were prepared with two alkylating agents, thiotepa-busulfan and fludarabine (TBF). Patients receiving TBF were older (57 vs 52 years), were less frequently splenectomized pre-HSCT (31% vs 59%), had more frequently intermediate-2/high DIPSS scores (90% vs 74%), were grafted more frequently from alternative donors (83% vs 33%) and received more frequently ruxolitinib pre-HSCT (26% vs 7%). The proportion of patients with F-DC on day +30, in the TBF vs the ONE-ALK group, was respectively 87% vs 45% ($p<0.00001$). The 5-year cumulative incidence of relapse was 9% in the TBF group, versus 43% for the ONE-ALK group ($p<0.0001$). The 5-year actuarial disease free survival was 63% for TBF and 38% for the ONE-ALK group ($p=0.004$).

In conclusion, early full donor chimerism is a prerequisite for long term control of disease in patients with myelofibrosis, undergoing an allogeneic HSCT. The combination of two alkylating agents in the conditioning regimen, provides a higher chance of achieving full donor chimerism on day+30, and thus a higher chance of long term disease free survival.

INTRODUCTION

Allogeneic hemopoietic stem cell transplantation is currently the only curative approach for patients with myelofibrosis **(1)**, and for this reason the number of allografts for this indications have been growing over the past years : in Europe the number of patients has increased from 305 in the year 2007 to 647 in the year 2016 **(2).** Engraftment and/or graft function can be problematic in patients with myelofibrosis (MF) undergoing an allogeneic hemopoietic stem cell transplant (HSCT). This is suggested by at least three orders of events: the high proportion of patients with poor graft function (PGF) **(3-5),** the incidence of patients with mixed chimerism **(6)**, and the slow rate of hematologic recovery . As to the latter observation, in our data base, the median platelet count on day +50 after an allogeneic HSCT is 127x10^9/L (range 7-295) for acute leukemia patients, and 68x10^9/L (range 7-190) for patients with myelofibrosis (p=0.001) (unpublished observations).

Several conditioning regimens have been proposed for patients with MF, and reported in retrospective or phase 2 prospective trials: commonly used regimens are fludarabine melphalan (FLU-MEL) **(7),** fludarabine busulfan (BU-FLU) **(8)** and thiotepa based regimens **(9).** Recently a prospective randomized study has compared the BU-FLU regimen to the combination of thiotepa and fludarabine (THIO-FLU) **(10)**. The BUFLU regimen was administered , as proposed by the German group**(8)**, with a total dose of intravenous busulfan of 8 mg/kg; the THIO-FLU combination included thiotepa at a dose of 12 mg/kg, and a comparable dose of FLU. Sixty patients were randomized, 30 in each group. The proportion of patients with full donor chimerism (F-DC) on day +100 was 24% for the BU-FLU regimen and 68% for the THIO-FLU regimen **(10)**. The overall cumulative incidence of relapse was 36% (BU-FLU) vs 24% (THIO-FLU) and the non relapse mortality 21% in both arms, whereas disease free survival was 43% (BU-FLU) vs 55% (THIO-FLU) **(10).** This study highlights the criticisms of allogeneic HSCT in MF patients: low rate of complete donor chimerism, relatively high rate of relapse, with an impact on disease free survival. The FLU-MEL regimen has been reported to induce a lower risk of relapse, but a higher mortality as compared to BU-FLU **(11)**; in keeping with this result, mortality was particularly high in unrelated donor grafts, prepared with the FLU-MEL regimen, in a prospective phase 2 study **(7).** A very recent report from MD Anderson confirms a high rate of mixed chimerism (43%) in patients with myelofibrosis, grafted with a BU-FLU regimen, associated with a high rate of relapse (41%) **(6).**

A combination of thiotepa , busulfan and fludarabine (TBF) has been described by Sanz and coworkers , as particularly effective in patients undergoing a cord blood transplant **(12).** We have shown encouraging results with the use of TBF in patients with myelofibrosis, also when grafted from donors other than HLA identical siblings **(13).** This has been confirmed in a recent study by Shouval and coworkers **(14)**: in this report the progression free survival at 1 year was significantly superior with TBF (81%) when compared to other preparative regimens (54% and 45%). **(14).** The hypothesis would be that the combination of two alkylating agents improves engraftment and thus reduces relapse.

We have therefore retrospectively studied donor chimerism in 120 patients with myelofibrosis, prepared for transplantation with one alkylating agent (busulfan, melphalan or thiotepa) (ONE-ALK), or two alkylating agents (busulfan and thiotepa) (TBF), and are here reporting the results of this study.

METHODS

Patients. This is a retrospective analysis of 120 consecutive patients with histologically proven myelofibrosis, allografted in two transplant Units (Genova San Martino and Roma Gemelli), between January 2000 and January 2019, and with chimerism data on day 30. The minimum follow up for surviving patients is one year. The study was approved by the IRB of the Institute of Hematology,Catholic University, Policlinico Gemelli, Rome, Italy on July 2020. Patients were retrospectively classified according to dynamic international prognostic scoring system (DIPSS) **(15).** Clinical data of patients are outlined in Table1, stratified by conditioning regimen: ONE ALK (n=42) and TBF (n=78). The TBF group was significantly older, had more advanced disease, less splenectomized patients , more donors other than HLA identical siblings (SIB) , and patients were transplanted more recently **(Table 1)**.

Chimerism studies and definition of full donor chimerism: chimerism was assessed by PCR analysis of short tandem repeats (STR). The proportion of donor recipient chimerism was calculated using the PowerPlex Fusion System (Promega, srl, Italy) on 24 STR loci. Full donor chimerism (F-DC) was defined as having >95% donor alleles.

Driver mutations. The JAK2 v617F mutation was found in 55 patients, CALR1 mutation was found in 4 patients and MPL mutation in 1, whereas 44 were triple negative. Patients

diagnosed before 2013 were retrospectively analyzed for both JAK2 and CALR mutations. Sixteen patients lacked molecular analysis pre-HSCT.

Conditioning regimens. Patients in the ONE-ALK group were prepared with fludarabine (FLU) combined with melphalan (MEL) (140 mg/m^2) (n=6), busulfan (BU) (8 mg/kg) (n=18) or thiotepa (THIO) (12mg/kg) (n=18) . The choice to use ONE-ALK was based on current procedures at the time of transplant, or else on prospective protocols : the FLU-MEL combination was used in 6 patients entered in the Myeloproliferative Disease Research Consortium 101 trial **(7),** and the BU-FLU or THIO-FLU combinations were used in 10 patients randomized in the Gruppo Italiano Trapianti di Midollo Osseo (GITMO) trial **(10).** TBF patients received thiotepa (5 mg /kg/dayx2) , fludarabine (50 mg/m^2x3) and 3 days of intravenous BU 3.2 mg/kg/day (TBF-BU3) as originally described **(12)**; in patients with comorbidities or over the age of 60, the busulfan dose was reduced to 2 days (6.4 mg/kg total dose) (TBF-BU2) or one day (3.2 mg/kg total dose)(TBF-BU1), in patients over 70 years of age, or patients with severe comorbidities; overall 25 patients received TBF-BU3, 50 TBF-BU2 and 3 patients TBF-BU1.

Supportive care. Management of bacterial, viral and fungal infections, followed standard operative procedures in the 2 transplant Centers.

Stem cell source. Bone marrow (BM) was the stem cell source in 23 and 58 patients in the two groups, mobilized peripheral blood (PB) in 19 and 20 respectively **(Table 1).**

GvHD prophylaxis. HLA identical siblings received cyclosporin (CyA) + short course metothrexate (MTX); patients grafted from unrelated donors, received CyA+MTX+ anti-thymocyte globulin (ATG) (Thymoglobulin , Sanofi Aventis, France) (3,75 mg/kg) on days -3 and -2 pre-transplant. Patients receiving a family haploidentical donor transplant (HAPLO), were given CyA from day 0, MMF from day +1, CY 50 mg/kg on days +3 and +5 **(13).**

Relapse. Relapse of myelofibrosis was diagnosed with the following markers: baseline drive mutations again detectable, declining bone marrow chimerism , a bone marrow biopsy showing increasing bone marrow fibrosis, systemic symptoms, spleen enlargement, abnormal peripheral blood counts . Patients with declining chimerism and or reappearance of driver mutations had immunosuppression discontinued, if still present, followed by a program of escalating dose donor lymphocyte infusions (DLI).

Statistical analysis

The NCSS 19 (NCSS Kaysville, Utah, USA) was used for statistical analysis. Comparison between groups was carried out using the chi-square test for categorical variables and the

non-parametric Mann-Whitney test for continuous variables. Multivariable analyses on the proportion of patients with full donor chimerism on day +30 were carried out by logistic regression analysis. Events for disease free survival, were death and relapse; the event for overall survival was death due to any cause; events for graft and relapse free survival, were acute GvHD grade III-IV, severe chronic GvHD, relapse and death. When calculating the cumulative incidence (CI) of non relapse related mortality (NRM) , the competing risk was relapse. When calculating the CI of relapse, the competing risk was non relapse mortality. The log rank test was used for univariable comparison of survival curves. The Fine and Gray test was used for univariable comparison of cumulative incidences. The Cox model was used for multivariable analysis on time dependent events.

RESULTS

Engraftment and GvHD. A neutrophil count of 0.5x10^9/l was achieved in all patients, who were then tested for chimerism on day +30. However secondary poor graft function developed in a number of patients, and was the primary cause of death in 3 patients: all three had mixed donor chimerism on day +30. Acute GvHD grade II-IV developed in 42% and 24% of patients given ONE-ALK or TBF (p=0.1), and moderate severe chronic GvHD developed in 26% and 21% of patients respectively (p=0.5).

Chimerism on day +30 after HSCT. On day +30 after HSCT, 11 patients (26%) and 5 patients (6,5%) had donor chimerism <50%, in the ONE-ALK vs the TBF group respectively; 12(29%) and 5(6,5%) had donor chimerism between 51% and 95%; 19 (45%) and 68 (87%) had donor chimerism greater than 95%, or full donor chimerism (p<0.0001) **(Fig.1).** On day +60 full donor chimerism was detected in 13% vs 93% (p<0.0001) and on day +90 in 21% vs 90% (p<0.0001).

The other predictor of full donor chimerism on day +30, was donor type: SIB donors (61%), alternative donors (69%) (p=0.04), possibly due to the fact that more alternative donors received TBF as a conditioning regimen. There were no other factors predicting full donor chimerism on day +30: DIPSS int1-in2 vs DIPSS high (74% vs 69%, p=0.5); TS low-intermediate (73%) high (60%)(p=0.1); recipient age <60 (71%), ≥60 years (77%) (p=0.4); recipient gender, male (72%), female (72%)(p=0.9); interval diagnosis transplant <2 years (78%) ≥ 2 years (68%) (p=0.2); donor age <30 years (75%), ≥30 years (68%) (p=0.5). In a

binary logistic regression analysis, the conditioning regimen was the only predictor of F-DC on day + 30 chimerism (p=0.00003).

Relapse. The cumulative incidence of relapse was 14% (95%CI 7-25%) versus 40% (95%CI 26-60%) for patients with full or mixed donor chimerism on day +30 (p=0.01 Fine and Grey) **(Fig.2).** Relapse was 9% for the TBF group and 42% for the ONE-ALK group, **(Fig.3)**; it was 14% vs 31% in patients with DIPPS int1-2 or DIPSS high (p=0.03). In multivariable Cox analysis the conditioning regimen was the strongest predictor of relapse (**Table 2),** with a risk ratio (RR) of 0.1 for TBF compared to ONE-ALK (p=0.0002). Complete chimerism on day +30 showed a strong interaction with the conditioning regimen and was not predictive, but, when tested without the conditioning regimen, in the same Cox model, it was highly predictive of relapse (RR 0.3, p=0.0007) . A High DIPSS also predicted relapse (p=0.001) **(Table 2).**

Non relapse mortality. The cumulative incidence of non relapse mortality (NRM) 24% for the ONE-ALK group (95% CI 13-45%) and 21%, at 5 years in the TBF group (95% CI 13-33%) (p=0.4). In univariable analysis, patients age had a borderline effect on NRM: it was 16% vs 35% for patients aged <60 or $\geq$ 60 years (p=0.08). There were no significant predictors of NRM in multivariable analysis **(Table 2),** except for a borderline effect of F-DC (P=0.06).

Disease free survival. Patients receiving the TBF conditioning had a 5 year DFS significantly superior to patients receiving ONE-ALK conditioning (63% vs 43%, p=0.004)(**Fig.4).** In multivariable Cox analysis predictors of DFS were the conditioning regimen, complete donor chimerism on day+30 and DIPSS score **(Table 2).** Patients age and donor type were not predictive of DFS.

Survival and GRFS. The overall 5-year survival for TBF and ONE-ALK patients was 71% and 43% (p=0.002): predictors in multivariable analysis were the same as for DFS. The 5 year GvHD free, relapse free survival was 51% for TBF and 24% for ONE-ALK (p=0.002).

DISCUSSION

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Reduced intensity conditioning regimens (RIC) are currently widely used in patients with myelofibrosis undergoing an allogeneic HSCT **(16,17)**, and are usually based on the combination of one alkylating agent -busulfan, melphalan, or thiotepa- with fludarabine **(7-**

9,16-18). The proportion of patients with early F-DC in these studies is less than 70%, and can be as low as 24% **(10).** In a recent report from Houston, the proportion of patients with F-DC within day+100, was 57% **(6),** and another study reported a rate of F-DC of 44% on day +30, in patients prepared with BU-FLU **(17).** In the present study we show that the combination of two alkylating agents (busulfan and thiotepa) with fludarabine (TBF) produces a high rate (87%) of F-DC on day +30 compared with the use of either agent and fludarabine, which we refer to as ONE-ALK (45% F-DC). There were no other predictors of F-DC , including DIPSS score, with the exception of alternative donor transplants, showing a higher rate of F-DC: this may be due to the fact that the majority of patients (82%) in this groups were prepared with TBF, compared to 31% TBF in HLA identical sibling transplants ($p<0.000001$). In keeping with our results, other studies suggest that combination of two alkylating agents produces a high rate of F-DC: one study used TBF and showed 81% F-DC **(14)**, whereas a second study reported 100% F-DC with busulfan melphalan fludarabine, compared to 44% with BU-FLU **(17).** In the present study we have used a fixed dose of thiotepa (10 mg/kg) but different doses of busulfan, according to patients age and comorbidities: 24 patients received 3 doses of BU (9.6mg/kg total dose) (TBF-BU3), 51 received 2 doses of BU (6.4 mg/kg total dose) (TBF-BU2). The proportion of patients achieving a F-DC within day +100 was 92% and 94% for TBF-BU3 and TBF-BU2 respectively; the cumulative incidence of relapse was 15% and 6% ($p=0.2$) and non relapse mortality was 17% and 31% ($p=0.2$), the latter figure possibly due to significantly older patients in the TBF-BU2 group (55 vs 49 years, $p=0.002$). Disease free survival and survival was comparable in patients receiving TBF-BU3 or TBF-BU2. In a multivariate analysis on DFS for patients receiving TBF-BU2 or TBF-BU3, the hazard risk for TBF-BU3 compared to TBF-BU2 is 0.78 (0.28-2.1), $p=0.6$.

Taken together these data strongly argue in favor of a combined effect of two alkylating agents in producing best engraftment of donor myeloid cells. Probably this can be achieved with different combinations of the three most widely used agents, busulfan, melphalan and thiotepa. We also suggest that 6.4 mg/kg of BU in combination with 10 mg/kg of thiotepa and fludarabine 150 mg/m^2, appears to be sufficient to ensure full engraftment, and therefore we are currently using this dose for all patients with myelofibrosis, independent of donor type. The question is whether this dose of Bu can be further reduced: our initial experience with 3.2 mg/kg of BU in three older patients, seems encouraging, with all three patients being disease free one year post HSCT, but this is very preliminary.

Stringent criteria were adopted to define relapse: patients with molecular evidence of driver mutations or patients with declining chimerism, were considered as relapsed. Full donor chimerism in our study, had a significant impact on the cumulative incidence of relapse, which was 14% versus 40% for patients with or without F-DC on day +30. F-DC chimerism showed a strong interaction with the conditioning regimen, in the multivariable Cox analysis on relapse, precisely because patients receiving TBF were the ones with high levels of donor chimerism. The other predictive variable of relapse in the Cox model, was the DIPSS score, which is not unexpected **(22)**.

Disease free survival is the final end point of any curative approach such as an allogeneic HSCT in patients, with a chronic, but otherwise uncurable disease: DFS was predicted in a multivariable analysis by the use of TBF and achievement of early F-DC. DFS was also predicted by DIPSS . Superimposable results were obtained when looking at overall survival or survival free of GvHD and relapse (GFRS).

One may argue that improved results with TBF could be due to a larger proportion of patients receiving ruxolitinib pre-HSCT in this group, as compared to ONE-ALK patients: however, when selecting only TBF patients, there was no difference in F-DC in patients receiving or not ruxolitinib pre-HSCT (90% vs 86%). Other main transplant outcomes were also comparable. Other differences between TBF and ONE-ALK were mostly in favour of the latter , such as less advanced disease , younger patients age and higher proportion of HLA identical sibling HSCT. In addition multivariable analysis confirmed the role of the TBF in promoting F-DC. Nevertheless, one may argue that this is a retrospective study, and that TBF actually succeeded the use of single alkylant agent conditioning: the only proof or disproof, of these findings can come from a prospective randomized study, as the GITMO study which compared BU-FLU versus THIO-FLU.

In conclusion: in patients with myelofibrosis, a double alkylating conditioning (TBF) appears to produce a higher rate of full donor chimerism early post-HSCT, as compared to single alkylating agent conditioning, and early F-DC is a prerequisite for disease control. As to non relapse mortality, a reduced dose of busulfan, may be considered for older patients, and this is still capable of inducing a high rate of F-DC, when combined with a second alkylating agent.

Aknowledgement. This study was supported by AIRC , Milano, grant to AB, and FARITMO, Genova.

Contribution of Authors: PC and AB designed the study, performed the analysis and wrote the manuscript. PC, LC SG performed the chimerism analyses. PC, SB, SG, TL, AD, AMR, CDG, FG, FS,LL, SS, GB, FG, MR, EM, ER, VD, AV, EA provided patients and patient care; AS provided statistical analyses;

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