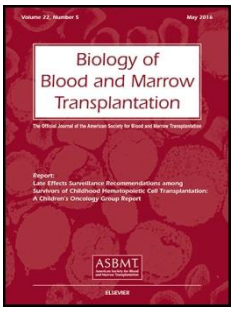
Accepted Manuscript



Title: Post–Transplant Cyclophosphamide and Tacrolimus–Mycophenolate Mofetil Combination Prevents Graft Versus Host Disease in Allogeneic Peripheral Blood Hematopoietic Cell Transplantation From HLA–Identical Donors

Author: Fabrizio Carnevale–Schianca, Daniela Caravelli, Susanna Gallo, Valentina Coha, Lorenzo D'Ambrosio, Elena Vassallo, Marco Fizzotti, Francesca Nesi, Luisa Gioeni, Massimo Berger, Alessandra Polo, Loretta Gammaitoni, Paolo Becco, Lidia Giraudo, Monica Mangioni, Dario Sangiolo, Giovanni Grignani, Delia Rota–Scalabrini, Antonino Sottile, Franca Fagioli, Massimo Aglietta

PII: S1083-8791(16)31214-9
DOI: http://dx.doi.org/doi: 10.1016/j.bbmt.2016.12.636
Reference: YBBMT 54527

To appear in: *Biology of Blood and Marrow Transplantation*

Received date: 28-9-2016
Accepted date: 21-12-2016

Please cite this article as: Fabrizio Carnevale–Schianca, Daniela Caravelli, Susanna Gallo, Valentina Coha, Lorenzo D'Ambrosio, Elena Vassallo, Marco Fizzotti, Francesca Nesi, Luisa Gioeni, Massimo Berger, Alessandra Polo, Loretta Gammaitoni, Paolo Becco, Lidia Giraudo, Monica Mangioni, Dario Sangiolo, Giovanni Grignani, Delia Rota–Scalabrini, Antonino Sottile, Franca Fagioli, Massimo Aglietta, Post–Transplant Cyclophosphamide and Tacrolimus–Mycophenolate Mofetil Combination Prevents Graft Versus Host Disease in Allogeneic Peripheral Blood Hematopoietic Cell Transplantation From HLA–Identical Donors, *Biology of Blood and Marrow Transplantation* (2017), http://dx.doi.org/doi: 10.1016/j.bbmt.2016.12.636.

Title: Post–transplant cyclophosphamide and tacrolimus–mycophenolate mofetil combination prevents graft versus host disease in allogeneic peripheral blood hematopoietic cell transplantation from HLA–identical donors.

Brief Title: Post–transplant Cy and FK 506/MMF as GVHD–prophylaxis in PBSCT.

Authors: Fabrizio Carnevale–Schianca[1], Daniela Caravelli[1], Susanna Gallo[1,6], Valentina Coha[1], Lorenzo D'Ambrosio[2,6], Elena Vassallo[3], Marco Fizzotti[2], Francesca Nesi[3], Luisa Gioeni[4], Massimo Berger[3], Alessandra Polo[5], Loretta Gammaitoni[2], Paolo Becco[1,6], Lidia Giraudo[2], Monica Mangioni[5], Dario Sangiolo[2,6], Giovanni Grignani[2], Delia Rota–Scalabrini[2], Antonino Sottile[5], Franca Fagioli[3]* & Massimo Aglietta[1,2,6]*

F. F & M.A: equally contributed to this work

Affiliation:

[1] Medical Oncology, Hematopoietic Stem Cells Unit, Turin Metropolitan Transplant Center, Candiolo Cancer Institute–FPO, IRCCS, Candiolo, Italy

[2] Medical Oncology, Candiolo Cancer Institute–FPO, IRCCS, Candiolo, Italy

[3] Pediatric Onco–Hematology, Stem Cell Transplantation, and Cellular Therapy Division, Turin Metropolitan Transplant Center, A.O.U. Citta' della Salute e della Scienza di Torino, Ospedale Infantile Regina Margherita, Torino, Italy

[4] Regulatory Affairs Candiolo Cancer Institute–FPO, IRCCS, Candiolo, Italy

[5] Collection and Processing Laboratory Candiolo Cancer Institute–FPO, IRCCS, Candiolo, Italy

[6] University of Torino, Department of Oncology, Turin, Italy

Correspondence: Fabrizio Carnevale Schianca MD

Hematopoietic Cells Transplant Unit

Candiolo Cancer Institute – FPO, IRCCS

Strada Provinciale 142 Km 3,95

10060 - Candiolo (TO), Italy

Phone 00390119933896/3951

Fax 00390119933290

fabrizio.carnevale@ircc.it

Keywords: allogeneic bone marrow transplantation; graft-versus-host disease; post-transplant cyclophosphamide

Disclosure and Conflicts of Interest: All authors declare no relevant competing conflicts of interests.

Article Highligths

- Post-transplant cyclophosphamide was safely conjugated with one of the most-utilized GVHD prophylaxis regimens.
- Non-relapse mortality was contained to 3% at two years.
- Extremely low acute- and chronic-GVHD rates (17% and 7%) allowed limited steroid and immunosuppressive drug use

- Most non-relapsing patients can live without chronic-GVHD therapy.
- The presented strategy appears to be widely reproducible across the majority of transplant centers.

Abstract

Allogeneic hematopoietic cell transplant (HCT) remains the only curative therapy for many hematological malignancies, but it is limited by high non-relapse mortality (NRM), primarily from unpredictable control of graft-versus-host disease (GVHD). Recently, post-transplant cyclophosphamide demonstrated improved GVHD control in allogeneic bone marrow HCT. Here we explore cyclophosphamide in allogeneic peripheral blood stem cell transplantation (alloPBSCT).

Patients with high-risk hematological malignancies received alloPBSCT from HLA-matched unrelated/related donors. GVHD prophylaxis included combination post-HCT cyclophosphamide 50mg/kg (days +3 and +4) and tacrolimus/mofetil mycophenolate (T/MMF) (day +5 forward). Primary objective was the cumulative incidence of acute- and chronic-GVHD.

Between 03/2011 and 05/2015, 35 consecutive patients received the proposed regimen. MMF was stopped in all patients at day +28; tacrolimus median discontinuation was day +113. Acute- and chronic-GVHD cumulative incidences were 17% and 7%, respectively, with no grade 4 GVHD events, two only patients requiring chronic GVHD immunosuppression control, and no deaths from GVHD. Two-year NRM, overall survival, event-free survival and chronic-GVHD event-free survival were 3%, 77%, 54%, and 49%, respectively. Graft-versus-tumor effect was maintained as five of 15 patients (33%) who received HCT with evidence of disease experienced further disease response.

A post-transplant cyclophosphamide+T/MMF combination strategy effectively prevented acute- and chronic-GVHD after alloPBSCT from HLA-identical donors, and achieved an unprecedented low NRM without losing efficacy in disease control or impaired development of graft versus-tumor effect. This trial is registered at clinicaltrials.gov as #NCT02300571.

INTRODUCTION

Allogeneic hematopoietic cell transplant (HCT) remains the only curative therapy for many hematological malignancies.[1-3] However, broad application of the procedure has been limited by the difficult control of graft-versus-host disease (GVHD)—the principal complication and cause of mortality in allogeneic-HCT.[1,2] The GVHD prophylaxis used most commonly in HCT is a calcineurin-inhibitor (CNI) combined with a short course of methotrexate (MTX), which in an unrelated donor setting is often supplemented by anti-thymocyte globulin. Even so, 30% to 80% of allogeneic-HCT patients will develop GVHD,[4-8] suggesting that development of strategies to control this potentially fatal complication are key to broadening its clinical applicability.

Cyclophosphamide given post-HCT is a novel and promising approach[9-11] that can be safely administered in high doses even after allogeneic-HCT without hematopoietic stem cell toxicity. Therefore, it may be possible to exploit it to target early-proliferating allo-reactive T-cells involved in GVHD onset.[10,11] Post-transplant cyclophosphamide (PTCy) has already been proved safe and active in both haplo-identical and unrelated bone marrow allografts.[12-16] On the contrary, PTCy has seldom been administered in the HLA-identical peripheral blood stem cell transplant (alloPBSCT) setting, despite the use of this stem cell source in more than 75% of HCTs from unrelated adult donors.[17-23] This study explored the performance of PTCy infusion, measured by transplant morbidity and outcome, when added to tacrolimus/MMF as GVHD prophylaxis regimen in alloPBSCT.

METHODS

All patients underwent HCT from PBSC and were matched for HLA–A, B, C, DRB1, and DQB1 alleles to either a related or unrelated donor. The following were deemed acceptable levels of recipient–donor mismatch: an a*llele–match* for HLA–A, B, C, DRB1, and DQB1; a s*ingle allele disparity* for HLA–A, B, C, or DRB1 or DQB1; t*wo allele disparities* for HLA–A, B, or C; a s*ingle allele disparity* for HLA–DRB1; and a s*ingle antigen plus single allele disparity* for HLA–A, B, or C. The criteria for clinical eligibility included age ≤70 years, first remission at high–risk of relapse, or second remission obtained after relapse or refractory hematological malignancy. The principal exclusion criteria were refractory central-nervous-system disease, active infection, pregnancy, HIV+ serology, or serious organ dysfunction (left ventricular ejection fraction <45% or pulmonary forced vital capacity <50% of predicted). All patients signed informed consent before study entry.

The study was approved by local IRB and Ethics Committee. The trial is registered at clinicatrials.gov (NCT02300571). Our primary objective was to determine the capability of the drug combination to control GVHD both in acute (aGVHD) and chronic GVHD (cGVHD) manifestations based on their cumulative incidences, assuming an expected rate of aGVHD around 80% and chronic GVHD around 35%[8]. Secondary objectives were measures of non-relapse mortality (NRM), infections, overall survival (OS), event-free survival (EFS), cGVHD EFS, and relapse rate. Acute GVHD was diagnosed based on standard criteria, for cGVHD we applied both traditional and NIH criteria (defined as requiring systemic immunosuppressive treatment).[24-26] Given the heterogeneity of patients, we also assessed disease-risk index by the refined criteria, which takes into account disease status, stage and cytogenetics.[27]

Conditioning regimen, post-graft immunosuppression, and supportive care

Conditioning regimens are reported in Table 1. Considering that the study objective was GVHD prophylaxis, the regimens adopted were disease-oriented. In seven of them cyclophosphamide was administered also before PBSC reinfusion on two consecutive days at a dose of 14,5 mg/kg (five regimens) and of 10mg/kg (two regimens). Immunosuppression began on days 3 and 4 after transplant with administration of intravenous cyclophosphamide (50 mg/kg/day). On day 5 and forward, tacrolimus (0.03 mg/kg in two daily doses; target through levels 5-10 ng/mL) and MMF (15 mg/kg in three daily doses) were given. Both agents were continued until day +28 when MMF was discontinued and day +84 when a tacrolimus taper was started. G-CSF (5 mcg/kg/day) was started on day 5 and continued until the absolute neutrophil count (ANC) exceeded 1.0×10^9/L for three consecutive days.

Patients received prophylaxis for bacterial, fungal, and viral infections, as well as for Pneumocystis jeroveci.[28] Standard cytomegalic virus (CMV) monitoring by polymerase-chain-reaction (PCR) started on day +10 and continued until day +365. Treatment with ganciclovir or valganciclovir began when the number of CMV-DNA copies rose above 100/mL (unrelated donors) or 500/mL (related donors) for two consecutive measurements or after a viral load change of >0.5 log IU/mL in peripheral blood plasma. Epstein-Barr virus (EBV) was monitored by PCR via biweekly plasma samples.[29]

Surveillance weekly blood cultures were drawn until patient discharge; in cases of fever, (>38.5°C) blood and urine cultures were collected and wide spectrum antibiotic coverage (*i.e.*, piperacillin/tazobactam 4.5 g/iv/q 8 hours, vancomycin 500 mg/iv/q 6 hours) was undertaken until pathogen identification or clinical control was achieved. Diagnostic and invasive procedures, were performed when clinically indicated. All specimens submitted for bacterial and fungal cultures were performed according to standard methods. Blood and platelet transfusions followed institutional protocols.[28]

Monitoring after transplant

Neutrophil engraftment was defined as the first of three consecutive days with an ANC of > 0.5×10^9/L after transplant, while platelet engraftment was defined as a platelet count of $20x10^9$/L with no transfusion during the preceding seven days. The degree of donor chimerism was assessed on day +30, +56, +90, +180, and +360 post–transplant on circulating myeloid and $CD3^+$ lymphocytes. Chimerism was determined using PCR on a panel of informative variable number tandem repeat (VNTR) regions, with full chimerism defined as more than 95% donor $CD3^+$ cells. Acute and chronic GVHD were graded as described elsewhere.[24-26]

Statistical Analysis

OS, EFS, and cGVHD-EFS were estimated using the Kaplan-Meier method with their respective 95% confidence intervals (CI).[30-32] Patient death from any cause constituted an OS event, while relapse or death from any cause was characterized as an EFS event. Most broadly defined were cGVHD-EFS events, which included any form of cGVHD (defined as per NIH criteria),[33] relapse, or death. OS, EFS, and cGVHD-EFS values were calculated from transplant date to date of event occurrence or upon censor at final follow-up for patients without an observed event. Discontinued immunosuppression time was determined from the date patients ended their taper from immunosuppressive drugs without subsequent resumption. NRM encompasses all deaths that occurred without evidence of relapse. Standard methods were used to estimate rates of acute and chronic GVHD, relapse or progression, and NRM. Death was treated as a competing risk for all other endpoints. Relapse was treated as a competing risk for NRM. The study was conceived as observational aiming to understand the timing and the role of cyclophosphamide as one of the tool to prevent GVHD, assuming an expected rate of aGVHD around 80% and of chronic GVHD around

35%.[8] Categorical variables were expressed as proportions and continuous variables were expressed as medians within their respective ranges. All statistics were computed using IBM-SPSS Statistics v.20 and GraphPad-Prism v.5.

RESULTS

Engraftment and immune-reconstitution

Between March 2011 and April 2015, we enrolled 35 consecutive patients (characteristic summary in Table 1) with high-risk hematological malignancies treated at our center. All the 10 related donors and 10 (40%) of the unrelated ones were 10/10 matched. Among the other 15 unrelated donors 8 (32%) and 7 (28%) were 9/10 and 8/10 matched, respectively. Sustained engraftment was documented in 34 of 35 (97%) patients with median times to neutrophil and platelet recovery of 15 (12-32) and 18 days (16-32), respectively. Only one patient (3%), who developed multi-resistant *Pseudomonas aeruginosa* septicemia, experienced primary graft failure.

Donor chimerism was >97% from day +28 and sustained in all non-relapsing patients. Absolute lymphocyte counts measured 400 (40-1980), 1020 (50-4900), and 1300/µl (400-5200) on day +28, + 56, and +84 after HCT (Table 2) with CD3+ cells being 310 (26-1670), 680 (28-3200), 890/µl (70-4000), respectively.

After transplant the median time of discharge was 22 (11-36) days. Three patients (9%) required readmission at day +29, +38 and +46, respectively, due to fever (two, 6%) or pneumonia (one, 3%). In all cases, complications were controlled and transfer to the outpatient clinic followed.

Infections and toxicity

Six of 35 (17%) patients experienced septicemia during the engraftment phase (days 0-26). *Staphylococcus* spp. was isolated in 3/35 (9%) and gram-negative bacilli (*Pseudomonas aeruginosa*, *Acinetobacter lwofii*, and *Klebsiella pneumoniae*) in 3/35 (9%) patients. Treatment with the appropriate antibiotic therapy resulted in complete control of all but one infection. Two of 35 (6%) patients colonized with *Klebsiella pneumoniae carbapenemase* (KPC)-producing bacteria before HCT suffered transient aplasia after transplant. Their fevers of unknown origin were successfully treated with antibiotics (meropenem 2g every 8 hrs, gentamycin 80 mg every 8 hrs, and tigecyline 50 mg every 12 hrs), which allowed both patients to be discharged after engraftment (day +18 and +23, respectively).

No patient developed a pulmonary fungal infection during transplant or follow-up.

CMV reactivation occurred in 21/35 (60%) patients at a median +38 days (range of 22-54 days). No case of primary CMV infection was reported and only one (3%) patient had late CMV reactivation (day +232). In all CMV cases, pre-emptive therapy was successful. No EBV-related disease was observed. Hemorrhagic cystitis with BK viriuria was witnessed in 3/35 (9%) patients on day +24, +41, and +46, respectively. However, complete resolution of the infection was achieved in all of them within four to six weeks.

Three of 35 patients (9%) were HBV-positive prior to HCT. Of these, two (67%) suffered viremia-reactivation after transplant. The first patient, who was on entecavir treatment for a mutated HBV form (YMDD) at the time of transplant, experienced a viremia flare-up on day +84. Association therapy (entecavir/tenofovir) was initiated and prompt control was achieved. The second patient, for whom lamivudine had been discontinued 12 months post HCT, was diagnosed on day +540 with HBV-mutated hepatitis (codon M250LM) that was successfully treated with tenofovir.

Grade 3 and 4 toxicities that occurred during the first 100 days after transplant are listed in Table 2. Grade 3 mucositis (20%, 7/35) and liver enzyme elevation (14%, 5/35) emerged most often. Mild sinusoidal-occlusion-syndrome (SOS) occurred in one patient (3%).[34]

Immunosuppression, GVHD and GVT

After discontinuation of MMF (day +28 for all patients) and tacrolimus (median +113 days, range 49-276), only two of the 21 patients (9%) alive without disease progression required immunosuppression restart. The overall cumulative incidence across all aGVHD grades was 17% (95% confidence interval [CI], 2-45%), of which 12% were grade II-III (CI, 1-48%), and none was grade IV. The median aGVHD onset was +75 days (range 22-98), (Figure 1A). No cases of late-onset aGVHD were reported. Three of 35 patients (9%) who required steroid therapy responded well, such that it was discontinued after a median 75 days (range 36-200). At two years, the cumulative incidence of NIH-defined cGHVD requiring systemic immunosuppression was 7% (CI 1-51%), (Figure 1B). We also analyzed the cumulative incidence of overall (limited + extensive) cGVHD defined by traditional criteria, and, at two-year, the incidence was 11% equally due to limited (1 patient) and extensive cGVHD (2 patients). No patient died from GVHD. Due to the low-event rate an analysis to assess the possible role of donor source on GVHD incidence was not conducted. After we enrolled the first 35 patients, the observed activity (aGVHD 17%, cGVHD 7%), was much greater than expected both in magnitude and overall duration ($p<0.0001$ and $p=0.0033$ respectively).

Among the 21 (60%) out of 35 patients alive without disease progression, five (24%), who were transplanted with evidence of disease (one acute myeloid leukemia, one acute lymphoblastic leukemia, one myelodysplastic syndrome, one multiple myeloma and one refractory follicular non Hodgkin lymphoma), achieved and maintained complete response after alloPBSCT .

Outcomes

The median follow-up period for the entire population was 20 months (range, 9-67 for patients without an event). The only (3%) patient who died of NRM accounted for the two-year NRM cumulative incidence of 3% (CI, 0-61%) (Figure 1C). Estimated one-year OS and EFS for all patients were 86% (CI, 69-94%) and 60% (CI, 42-74%), respectively; at two-years, they were 77% (CI, 59-88%) and 54% (CI, 37-69%), respectively (Figure 2A-B). The two-year cumulative incidence of relapse was 46% (CI, 28-62%) across all patients, and 25% (CI, 3-56%) for patients undergoing HCT in complete response (CR) (Figures 2 C). Among the 20/35 (57%) patients transplanted in complete response, only four (20%) relapsed: three of them with acute myeloid leukemia (one with FLT-3 positive disease on day +67, one with NPM1 positive form on day +162, and one with central nervous system extramedullary relapse on day +706). At relapse all patients were treated with MEC-regimen followed in the first patient by sorafenib and a second allogeneic HCT (intra-bone cord blood transplant), in the second patient by FLAG-liposome-encapsulated doxorubicin and a second allogeneic HCT (intra-bone cord blood transplant), and in the third patient by FLAG-Myocet (two cycles) followed by local conformational radiotherapy. Only one of them is still alive, the other two died from further relapse at day +245 and +1067, respectively. The fourth patient transplanted for myelodysplastic syndrome relapsed on day +128 and was treated with 5-Azacitidine. Overall, one- and two-year cGVHD-EFS were 54% (CI, 37-69%) and 49% (CI, 31-64%), respectively (Figure 2D). All employed patients returned to work after a median of nine months (168-462 days).

DISCUSSION

This manuscript described the impact of a modified strategy to prevent GVHD by using cyclophosphamide in early post-transplant days. We observed sharp reductions in acute and chronic GVHD to 17% and 7%, respectively. Consistently, NRM was reduced to a mere 3%.

When allogeneic HCT was introduced into clinical practice during the early 90s, it appeared to be a very effective therapy for many hematological malignancies that were otherwise incurable.[3] However, the procedure was characterized by extremely high toxicity that resulted in a 30-40% mortality risk.[35,36] Over the years, deeper knowledge of the HLA-system and transplant-immunology, better selection and matching of donors and patients, and advent of new immunosuppressive and antimicrobial drugs have led to a mortality risk reduction that is now approximately 15-20%.[1] Nonetheless, this rate still represents a burden that limits extensive application of HCT. Only by controlling GVHD will it be possible to reduce such toxicity to below 5%. As a result of the introduction of PTCy in allogeneic HLA-identical bone marrow transplant as well as in the haplo-identical setting, considerable progress in the prevention of GVHD has been made.[9,12-16] Only recently, initial data on the impact of this regimen after allo-PBSCT in HLA-identical donors have been reported.[17-19,37,38] The first clinical experience described the role of PTCy as sole GVHD prophylaxis in 11 patients; the II-IV aGVHD incidence of 45% and the NRM in up 36% of cases discouraged to further evaluate this approach[18] Subsequently the Seattle group published on 42 patients treated with PTCy to which cyclosporine was added as GVHD prophylaxis. This prophylaxys did not allow to consider HLA-mismatched unrelated donors –43% of our patients– and translated in a 70% incidence of grade II aGVHD but without any grade III-IV; the approach revealed very active in protection toward cGVHD and NRM being NIH defined cGVHD and NRM 16% and 14%, respectively.[17] Along with these studies Moiseev and colleagues reported on 86 patients affected by acute leukemias treated with PTCy and T/MMF. The main focus of

the study was a retrospective comparison with a historical control group of patients treated with antithymocyte globulin (ATG), calcineurin inhibitors and methotrexate. The incidence of grade II-IV aGVHD was 19% and of cGVHD of 16% and the NRM was as low as 16%. This study however did not described in details the post-transplant clinical course in terms of immunosuppression taper, discontinuation and number of patients requiring to restart it. [37]

Beside these three studies aiming to better define the ideal combination of PTCy and standard GVHD preventive regimens after allo-PBSCT, two more studies were reported where PTCy was followed by an experimental GVHD-prophylaxys with sirolimus; in the first one among the 26 patients treated aGVHD II-IV ranged around 45% and cGVHD was quoted at 31% with 37% and 11% of patients still on immunosuppression at one and two years, respectively, while NRM at 2 year was 13%.[19] In the second, where in unrelated patients MMF was added to cyclophosphamide and sirolimus, aGVHD II-IV was in the order of 30-35% and cGVHD 16% at one year. NRM was 14% at one year but the median follow up reported was of 225 days and this does not allow to draw any further conclusion.[38]

Given these premises, the results reported in our study add to the general picture some more pieces of information. First, PTCy after PBSC resulted in rapid engraftment as observed with conventional GVHD-prophylaxis.[20,21,39] Moreover, hematologic recovery made the procedure very tolerable, shortened the inpatient stay, and reduced the rate of hospital readmission. Second, the addition of T/MMF to PTCy was not detrimental to a rapid and sustained lymphocyte recovery,that helped to contain severe infection incidence rates to what seen in similar previous PTCy studies, as opposed to historical allogeneic allo-PBSCT data using standard GVHD regimens.[9,12,15,18,40]

A third important finding led to another set of conclusions. PTCy after PBSC resulted in high activity in acute and chronic GVHD prevention. In fact, PTCy synergy with T/MMF determined a lower incidence of aGVHD compared to both conventional allo-PBSCT (grade

II-IV, 45-80%),[1,2,8,41] and reduced-intensity regimen (42-64%),[42] as well as to PTCy after allogeneic bone marrow transplantation (43-51%).[9,12,13] Furthermore, despite PBSC use, this strategy maintained a robust protection against cGVHD that, with conventional prophylaxis, may be as high as 48%.[20] This high control rate allowed lower steroid use compared to conventional CNI-based strategies and earlier discontinuation of immunosuppressive therapy, staving off the need for its later re-introduction in most patients.[7,43,44]This datum distinguishes our study regimen from the previous ones of PTCy in either allo-BMT or allo-PBSC.[9,12,13,17-19] In BMT setting, at least 43-51% of patients required to re-start some form of immunosuppression after transplant; in the PBSC setting, the Seattle group described that regardless the high-grade of matching requested in unrelated transplants, at one year 30% of the 33 alive patients were still on immunosuppression and that among those diagnosed with cGVHD, 6/7 (86%) were still on immunosuppression at the time of the report.[9,17] Our finding, if confirmed in a larger patient cohort, is extremely appealing because a post-allogeneic HCT state that requires no further immunosuppression may be a platform to develop future post-transplant cellular therapies that safely and specifically act on minimal residual disease.

Two outcome indicators demonstrated the direct consequence of GVHD control. One is the NRM of 3% (after conventional allo-PBSCT and allo-BMT with PTCy it ranges between 21-30% and 15%, respectively).[9,12,20] Notably, a 3% treatment-related mortality compares closely to what observed in the autologous setting.[45] The second indicator is the cGVHD-EFS that nearly overlapped EFS, thus confirming the long-term tolerability of this regimen.

Finally, “double” post-transplant immunosuppression might raise concerns about relapse incidence as well as capability of generating an effective graft-versus-tumor (GVT) effect. In regard to the first point, even though also in our series relapse in patients transplanted not in CR remains a relevant issue, we reported an EFS and OS comparable to those described

following conventional allo-PBSC or allo-BMT with PTCy, suggesting no impact of our strategy on the general clinical.[9,17,19,20] The fast immunosuppression taper as well as the reduced need afterward of a new immunosuppressive treatment that we described, however may lay the ground for exploring in patients transplanted not in CR future studies aiming to increase disease-control with an early introduction of post-transplant cell therapies. In regard to the second point, GVT, although we did not give formal we immune-biological evidences for it, we reported that 5/15 (33%) patients in partial response achieved and maintained complete response after transplant as a consequence of the allo-based therapy. These data might support the intriguing concept that GVT is not sustained by early-proliferating donor T-cells (targeted by early-phase immunosuppressive drugs), but rather by a different T-cell population that needs time to develop and expand.[46]

We acknowledge our data mandate further confirmation because are limited by its observational nature, the relatively small sample-size (that is however very similar to other PTCy studies), heterogeneity of hematological malignancies treated and consequently, conditioning regimens utilized.[17,19] Notwithstanding, these weaknesses are partly mitigated by the fact that primary objective of the trial was GVHD control and a valid GVHD prophylaxis should be widely reproducible in the majority of transplant centers, should adapt to any disease and to specific conditioning regimen. For this reason, despite the above mentioned limitation, our results set the basis for the design of future clinical trials. This statement becomes more relevant in light of recent results achieved with ATG in related allo-PBSCT.[47] In this setting, a large phase III trial showed that ATG inclusion produced both a clear reduction of cGVHD incidence as well as an improvement in cGVHD-EFS. However, at two years 25% of patients were suffering from cGVHD. Acknowledging the non-randomized nature of our results, we did not observe any cGVHD late relapse both in related

and unrelated donors suggesting these two strategies should be compared in the near future as seems to suggest also another recent large retrospective study.[48]

In conclusion, the present study provides evidence that PTCy in association with T/MMF after allo-PBSCT, can substantially decrease both acute and chronic GVHD reducing NRM to less than 5%. If these results will be confirmed in a larger clinical trial, then the application of allogeneic-HCT might be broadened, and this strategy transformed into a safe immunological platform for development of future cellular therapies aimed at generating a more effective and long-lasting GVT effect.[49-51]

Acknowledgments

The authors thank the personnel of Turin Metropolitan Transplant Network, AVIS/I.R.C.C.S. Blood Service, the Pharmacy Department, and all medical, nursing, laboratory, and clinical staffs for their daily help and support in the conduction of the present study. We deeply thank Joan C. Leonard for her enlightened support in reviewing and editing the manuscript. We also wish to thank Alessandro Cignetti for helpful discussion. Above all, we are indebted to the patients and their caregivers for the courage and dedication shown during the study.

Authorship contributions: F.C–S. conceived of the idea and planned the clinical trial project. He also wrote the protocol, cared for patients, analyzed clinical data, and wrote the manuscript. D.C. contributed to study design, coordinated the trial and day–to–day patient clinical management, and participated in data analysis and manuscript writing. SG assisted with day–to–day clinical management, and participated in data analysis and manuscript writing. V.C, L.dA., M.F, P.B., G.G., and D.R.–S. each contributed to patient accrual, patient care and results analysis while, E.V., F.N., and M.B. all took part in donor selection, donor registry management, and results analysis. L.G. worked with the Ethics Committee and I.R.B. submission, study approval, and results analysis, whereas A.P., M.G., and A.S. were each involved in donor selection, PBSC collection and processing, and results analysis. L.G. and D.S. obtained peripheral blood lymphocytes, contributed to immune–reconstitution study, and results analysis. F.F. and M.A. contributed equally to this study; they had the idea for the study, wrote the protocol, obtained funding, analyzed results, and revised the paper.

All authors had access to the data, vouch for the completeness and accuracy of the data and analyses, and approved the final version of the manuscript. The corresponding author (FCS) had final responsibility for the decision to submit for publication.

Role of the funding source

This work was supported by "Associazione Italiana per la ricerca sul cancro" AIRC IG 2015 Id.17226 (GG); AIRC IG Id. 11515 (MA); MFAG N.15731 (DS); Ricerca Finalizzata-Ministero della Salute GR-2011-02349197 (DS); FPRC ONLUS-5x1000 Ministero della Salute 2012 (DS); University of Torino-Progetti di Ateneo 2011 grant Rethe-ORTO11RKTW (MA).

References

1. Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *N Engl J Med*. 2010;363(22):2091-2101.

2. Storb R, Gyurkocza B, Storer BE, et al. Graft-versus-host disease and graft-versus-tumor effects after allogeneic hematopoietic cell transplantation. *J Clin Oncol*. 2013;31(12):1530-1538.

3. Appelbaum FR. Hematopoietic-cell transplantation at 50. *N Engl J Med*. 2007;357(15):1472-1475.

4. Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. *N Engl J Med*. 1986;314(12):729-735.

5. Finke J, Bethge WA, Schmoor C, et al. Standard graft-versus-host disease prophylaxis with or without anti-T-cell globulin in haematopoietic cell transplantation from matched unrelated donors: a randomised, open-label, multicentre phase 3 trial. *Lancet Oncol*. 2009;10(9):855-864.

6. Nash RA, Antin JH, Karanes C, et al. Phase 3 study comparing methotrexate and tacrolimus with methotrexate and cyclosporine for prophylaxis of acute graft-versus-host disease after marrow transplantation from unrelated donors. *Blood*. 2000;96(6):2062-2068.

7. Martin PJ, Rizzo JD, Wingard JR, et al. First- and second-line systemic treatment of acute graft-versus-host disease: recommendations of the American Society of Blood and Marrow Transplantation. *Biol Blood Marrow Transplant*. 2012;18(8):1150-1163.

8. Flowers ME, Inamoto Y, Carpenter PA, et al. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. *Blood*. 2011;117(11):3214-3219.

9. Luznik L, Bolaños-Meade J, Zahurak M, et al. High-dose cyclophosphamide as single-agent, short-course prophylaxis of graft-versus-host disease. *Blood*. 2010;115(16):3224-3230.

10. Strauss G, Osen W, Debatin KM. Induction of apoptosis and modulation of activation and effector function in T cells by immunosuppressive drugs. *Clin Exp Immunol*. 2002;128(2):255-266.

11. Jones RJ, Barber JP, Vala MS, et al. Assessment of aldehyde dehydrogenase in viable cells. *Blood*. 1995;85(10):2742-2746.

12. Kanakry CG, Tsai HL, Bolaños-Meade J, et al. Single-agent GVHD prophylaxis with posttransplantation cyclophosphamide after myeloablative, HLA-matched BMT for AML, ALL, and MDS. *Blood*. 2014;124(25):3817-3827.

13. Kanakry CG, O'Donnell PV, Furlong T, et al. Multi-institutional study of post-transplantation cyclophosphamide as single-agent graft-versus-host disease prophylaxis after allogeneic bone marrow transplantation using myeloablative busulfan and fludarabine conditioning. *J Clin Oncol*. 2014;32(31):3497-3505.

14. Kasamon YL, Bolaños-Meade J, Prince GT, et al. Outcomes of Nonmyeloablative HLA-Haploidentical Blood or Marrow Transplantation With High-Dose Post-Transplantation Cyclophosphamide in Older Adults. *J Clin Oncol*. 2015;33(28):3152-3161.

15. Raiola A, Dominietto A, Varaldo R, et al. Unmanipulated haploidentical BMT following non-myeloablative conditioning and post-transplantation CY for advanced Hodgkin's lymphoma. *Bone Marrow Transplant*. 2014;49(2):190-194.

16. Jacoby E, Chen A, Loeb DM, et al. Single-Agent Post-Transplantation Cyclophosphamide as Graft-versus-Host Disease Prophylaxis after Human Leukocyte Antigen-Matched Related Bone Marrow Transplantation for Pediatric and Young Adult

Patients with Hematologic Malignancies. *Biol Blood Marrow Transplant*. 2016;22(1):112-118.

17. Mielcarek M, Furlong T, O'Donnell PV, et al. Posttransplantation cyclophosphamide for prevention of graft-versus-host disease after HLA-matched mobilized blood cell transplantation. *Blood*. 2016;127(11):1502-1508.

18. Holtick U, Chemnitz JM, Shimabukuro-Vornhagen A, et al. OCTET-CY: a phase II study to investigate the efficacy of post-transplant cyclophosphamide as sole graft-versus-host prophylaxis after allogeneic peripheral blood stem cell transplantation. *Eur J Haematol*. 2015.

19. Solomon SR, Sanacore M, Zhang X, et al. Calcineurin inhibitor--free graft-versus-host disease prophylaxis with post-transplantation cyclophosphamide and brief-course sirolimus following reduced-intensity peripheral blood stem cell transplantation. *Biol Blood Marrow Transplant*. 2014;20(11):1828-1834.

20. Anasetti C, Logan BR, Lee SJ, et al. Peripheral-blood stem cells versus bone marrow from unrelated donors. *N Engl J Med*. 2012;367(16):1487-1496.

21. Bensinger WI, Martin PJ, Storer B, et al. Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. *N Engl J Med*. 2001;344(3):175-181.

22. Mielcarek M, Storer B, Martin PJ, et al. Long-term outcomes after transplantation of HLA-identical related G-CSF-mobilized peripheral blood mononuclear cells versus bone marrow. *Blood*. 2012;119(11):2675-2678.

23. Alousi AM, Brammer JE, Saliba RM, et al. Phase II Trial of Graft-versus-Host Disease Prophylaxis with Post-Transplantation Cyclophosphamide after Reduced-Intensity Busulfan/Fludarabine Conditioning for Hematological Malignancies. *Biol Blood Marrow Transplant*. 2015.

24. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15(6):825-828.

25. Lee SJ, Vogelsang G, Flowers ME. Chronic graft-versus-host disease. *Biol Blood Marrow Transplant*. 2003;9(4):215-233.

26. Filipovich AH, Weisdorf D, Pavletic S, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant*. 2005;11(12):945-956.

27. Armand P, Kim HT, Logan BR, et al. Validation and refinement of the Disease Risk Index for allogeneic stem cell transplantation. *Blood*. 2014;123(23):3664-3671.

28. Carnevale-Schianca F, Cignetti A, Capaldi A, et al. Allogeneic nonmyeloablative hematopoietic cell transplantation in metastatic colon cancer: tumor-specific T cells directed to a tumor-associated antigen are generated in vivo during GVHD. *Blood*. 2006;107(9):3795-3803.

29. Boeckh M, Ljungman P. How we treat cytomegalovirus in hematopoietic cell transplant recipients. *Blood*. 2009;113(23):5711-5719.

30. TA G. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. In: W L, J C, BE S eds. Vol. 18: Stat Med.; 1999:695-706.

31. Kaplan, L. E, Meier, P. Nonparametric Estimation from Incomplete Observations. *J Am Statist Assoc*. 1958;53(282):457- 481.

32. Inamoto Y, Flowers ME, Sandmaier BM, et al. Failure-free survival after initial systemic treatment of chronic graft-versus-host disease. *Blood*. 2014;124(8):1363-1371.

33. Makar AP, Tropé CG, Tummers P, Denys H, Vandecasteele K. Advanced Ovarian Cancer: Primary or Interval Debulking? Five Categories of Patients in View of the Results of

Randomized Trials and Tumor Biology: Primary Debulking Surgery and Interval Debulking Surgery for Advanced Ovarian Cancer. *Oncologist*. 2016;21(6):745-754.

34. Coppell JA, Richardson PG, Soiffer R, et al. Hepatic veno-occlusive disease following stem cell transplantation: incidence, clinical course, and outcome. *Biol Blood Marrow Transplant*. 2010;16(2):157-168.

35. Thomas E, Storb R, Clift RA, et al. Bone-marrow transplantation (first of two parts). *N Engl J Med*. 1975;292(16):832-843.

36. Thomas ED, Storb R, Clift RA, et al. Bone-marrow transplantation (second of two parts). *N Engl J Med*. 1975;292(17):895-902.

37. Moiseev IS, Pirogova OV, Alyanski AL, et al. Graft-versus-Host Disease Prophylaxis in Unrelated Peripheral Blood Stem Cell Transplantation with Post-Transplantation Cyclophosphamide, Tacrolimus, and Mycophenolate Mofetil. *Biol Blood Marrow Transplant*. 2016;22(6):1037-1042.

38. Greco R, Lorentino F, Morelli M, et al. Posttransplantation cyclophosphamide and sirolimus for prevention of GVHD after HLA-matched PBSC transplantation. *Blood*. 2016;128(11):1528-1531.

39. Baron F, Baker JE, Storb R, et al. Kinetics of engraftment in patients with hematologic malignancies given allogeneic hematopoietic cell transplantation after nonmyeloablative conditioning. *Blood*. 2004;104(8):2254-2262.

40. Seggewiss R, Einsele H. Immune reconstitution after allogeneic transplantation and expanding options for immunomodulation: an update. *Blood*. 2010;115(19):3861-3868.

41. Jagasia M, Arora M, Flowers ME, et al. Risk factors for acute GVHD and survival after hematopoietic cell transplantation. *Blood*. 2012;119(1):296-307.

42. Mielcarek M, Martin PJ, Leisenring W, et al. Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. *Blood*. 2003;102(2):756-762.

43. Burroughs L, Mielcarek M, Leisenring W, et al. Extending postgrafting cyclosporine decreases the risk of severe graft-versus-host disease after nonmyeloablative hematopoietic cell transplantation. *Transplantation*. 2006;81(6):818-825.

44. Stewart BL, Storer B, Storek J, et al. Duration of immunosuppressive treatment for chronic graft-versus-host disease. *Blood*. 2004;104(12):3501-3506.

45. Giralt S, Garderet L, Durie B, et al. American Society of Blood and Marrow Transplantation, European Society of Blood and Marrow Transplantation, Blood and Marrow Transplant Clinical Trials Network, and International Myeloma Working Group Consensus Conference on Salvage Hematopoietic Cell Transplantation in Patients with Relapsed Multiple Myeloma. *Biol Blood Marrow Transplant*. 2015;21(12):2039-2051.

46. Rezvani AR, Storb RF. Separation of graft-vs.-tumor effects from graft-vs.-host disease in allogeneic hematopoietic cell transplantation. *J Autoimmun*. 2008;30(3):172-179.

47. Kröger N, Solano C, Wolschke C, et al. Antilymphocyte Globulin for Prevention of Chronic Graft-versus-Host Disease. *N Engl J Med*. 2016;374(1):43-53.

48. Moiseev IS, Pirogova OV, Alyanski AL, et al. Graft-versus-Host Disease Prophylaxis in Unrelated Peripheral Blood Stem Cell Transplantation with Post-Transplantation Cyclophosphamide, Tacrolimus, and Mycophenolate Mofetil. *Biol Blood Marrow Transplant*. 2016;22(6):1037-1042.

49. Rapoport AP. Donating used CARs. *Blood*. 2013;122(25):4007-4009.

50. Maus MV, Grupp SA, Porter DL, June CH. Antibody-modified T cells: CARs take the front seat for hematologic malignancies. *Blood*. 2014;123(17):2625-2635.

51. Sangiolo D, Mesiano G, Carnevale-Schianca F, Piacibello W, Aglietta M, Cignetti A. Cytokine induced killer cells as adoptive immunotherapy strategy to augment graft versus tumor after hematopoietic cell transplantation. *Expert Opin Biol Ther*. 2009;9(7):831-840.

Figure 1. Transplant–related complications. Panel A, cumulative incidence of acute graft–versus–host disease (aGVHD). Panel B, cumulative incidence of chronic graft–versus–host disease (cGVHD). Panel C, cumulative incidence of non–relapse mortality (NRM).

Figure 2. Kaplan–Meier survival curves. Panel A, overall survival. Panel B, event–free survival (EFS). Panel C, relapse incidence in all patients (solid line) and in patients who underwent bone marrow transplantation with complete remission (CR) of the underlying disease (dashed line). Panel D, chronic graft–versus–host disease–event–free survival (cGVHD-EFS).

Table 1. Patients' and Donors' Characteristics.

Patients' and Donors' Characteristics	Total (N=35)
Age at transplant , years Median Range	 49 y (23-69)
Sex Male Female	 24 (69%) 11 (31%)
Disease **AML** de novo AML relapsed AML **ALL** de novo ALL relapsed ALL **Multiple Myeloma** **Non Hodgkin Lymphoma** **MDS** **Hodgkin Lymphoma**	 **16 (46%)** 13 (37%) 3 (9%) **5 (14%)** 3 (8%) 2 (6%) **8 (23%)** **3 (8%)** **2 (6%)** **1 (3%)**
Disease status at BMT 1° CR > 1° CR Active disease	 15 (43%) 5 (14%) 15 (43%)
CIBMTR risk group, n **High** **Intermediate** **Low**	 20 (57%) 13 (37%) 2 (6%)
Source of stem cell peripheral blood stem cell	 35 (100%)
Sex mismatch No Yes Female into male	 22 (63%) 13 (37%) 9 (26%)
Donor age , y median range	 33 (20-68)
Source of graft sibling unrelated	 10 (29%) 25 (71%)
HLA match 10/10 9/10 8*/10	 20 (57%) 8 (23%) 7 (20%)
CMV serology CMV D-R- CMV D+R- CMV D-R+ CMV D+R+	 1 (3%) 0 (0%) 13 (37%) 21 (60%)

Conditioning regimen ^	
-Busulfan + Cyclophosphamide	13 (37%)
-Treosulfan + Cyclophosphamide	5 (14%)
-Melphalan+ Cyclophosphamide	4 (11.5%)
-Treosulfan + Cyclophosphamide + TBI 2Gy°	4 (11.5%)
-Melphalan + Cyclophosphamide + TBI 2Gy°	3 (8.5%)
-Fludarabine+ Thiotepa+ Cyclophosphamide	3 (8.5%)
-Thiotepa + Treosulfan	2 (6%)
-Treosulfan + Fludarabine + Cyclophosphamide	1 (3%)
Infused cell dose*	
CD34+ cell x 10^6/kg	
median	7.4 (range 2-15)
CD3+ cell x 10 ^8kg	
median	3.01 (range 1.240-9.788)
Total Nucleated Cells x 10^8/Kg	
median	12.1 (range 6.9-16.9)

AML Acute myeloid Leukemia, ALL Acute Lymphoblastic leukemia, MDS Myelodisplastic Syndrome BMT bone marrow transplantation, CR complete response, MDR minimal residual disease, disease risk corresponding to CIBMTR classification, * two patients had a antigen disparity at DQA1, CMV Cytomegalovirus, D donor, R recipient. ^Cyclophosphamide was given also before PBSCT at 14,5 mg/kg on two consecutive days, °Cyclophosphamide was given also before PBSCT at 10 mg/kg on two consecutive days ,TBI 2 Gy total body irradiation, MMF Mofetil Mycophenolate. *CD 34+ doses of cell was available for all patients, CD3+ doses only for 71% of patients.

Table 2. Post-transplant data.

Post-transplant data	
Engraftment median time Neutrophils engraftment > 0.5 x 10^9/L Platelets engraftment > 20 x 10 ^9/L	 15 days (range 12-26) 18 days (range 16-60)
Peripheral Blood Lymphocyte count ° **Day + 28** Median (U/µl) **Day + 56** Median (U/µl) **Day + 84** Median (U/µl) **Day + 180** Median (U/µl)	 400 (range 40-1.980) 1.020 (range 50-4.900) 1.300 (range 400-5.200) 1.900 (range 580-4.200)
Chimerism § **Day + 28** **Day + 56** **Day + 84**	 >97% of patients alive and not relapsed >97% of patients alive and not relapsed >97% of patients alive and not relapsed
CMV reactivation Incidence Median day of reactivation	 21/35 (60%) 38 (range 22-54)
Bloodstream infection during engraftment (day 0-26) Incidence	 6/35 (17%)
Toxicity (G3-G4)† **Liver enzymes elevation** **Hyperbilirubinemia** **Mucositis** **Hemorrhage ‡** **Sinusoidal obstruction disease (SOS)**	 5/35 (14%) 1/35 (3%) 7/35 (20%) 3/35 (9%) 1/35 (3%)

°Peripheral Blood Lymphocyte count was available on day + 28, + 56, +84 for all patients. § Chimerism on peripheral blood was available for all patients alive without disease relapse. †Toxicities were graded according to standard National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. ‡ Hemorrhagic cystitis.