A phase 2 trial of GVHD prophylaxis with PTCy, sirolimus, and MMF after peripheral blood haploidentical transplantation

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Key Points

- Sirolimus and PTCy/MMF GVHD prophylaxis effectively reduce the grade II-IV acute GVHD rates after haplo-HCT.
- Sirolimus and PTCy/MMF permit hematopoietic engraftment and result in low moderate/severe chronic GVHD rates and favorable survival.

The introduction of posttransplant cyclophosphamide (PTCy) made performing allogeneic hematopoietic cell transplantation (HCT) from HLA haplotype–incompatible donors possible. In a setting of PTCy and tacrolimus/mycophenolate mofetil (MMF) as a graft-versus-host disease (GVHD) prophylaxis, a peripheral blood (PB) graft source as compared with bone marrow reduces the relapse rate but increases acute GVHD (aGVHD) and chronic GVHD (cGVHD). This phase 2 trial assessed sirolimus and MMF efficacy following PTCy as a GVHD prophylaxis after PB haploidentical HCT (haplo-HCT). With 32 evaluable patients (≥18 years) enrolled, this study had 90% power to demonstrate a reduction in 100-day grade II-IV aGVHD to 20% from the historical benchmark of 40% after haplo-HCT using PTCy/tacrolimus/MMF. At a median follow-up of 16.1 months, the primary end point of the trial was met with a day-100 grade II-IV aGVHD cumulative incidence of 18.8% (95% confidence interval [CI], 7.5% to 34.0%). There were no graft-failure events and the 1-year probability of National Institutes of Health (NIH) moderate/severe cGVHD was 18.8% (95% CI, 7.4% to 34.0%), nonrelapse mortality was 18.8% (95% CI, 7.4% to 34.0%), relapse was 22.2% (95% CI, 9.6% to 38.2%), disease-free survival was 59.0% (95% CI, 44.1% to 79.0%), GVHD-free relapse-free survival was 49.6% (95% CI, 34.9% to 70.5%), and overall survival was 71.7% (95% CI, 57.7% to 89.2%) for the entire cohort. These data demonstrate that GVHD prophylaxis with sirolimus/MMF following PTCy effectively prevents grade II-IV aGVHD after PB haplo-HCT, warranting prospective comparison of sirolimus vs tacrolimus in combination with MMF following PTCy as GVHD prophylaxis after PB HCT. This trial was registered at www.clinicaltrials.gov as #NCT03018223.

Introduction

Posttransplant cyclophosphamide (PTCy), in combination with calcineurin inhibitor (CNI) and mycophenolate mofetil (MMF), controls graft-versus-host disease (GVHD) and makes the use of hematopoietic cell transplantation (HCT) from donors incompatible for 1 entire HLA haplotype safer.1-8 Today, haploidentical HCT (haplo-HCT) is increasingly offered as a curative treatment of patients with hematological malignancies who have no suitable HLA-identical sibling or matched unrelated donor options, whereas PTCy followed by tacrolimus/MMF is the most commonly used GVHD prophylaxis regimen.1-15 In a Blood and Marrow Transplant Clinical Trials Network (BMT CTN) phase 3 randomized trial, peripheral blood compared with bone marrow graft source resulted in a lower graft-failure rate but significantly higher risk of chronic GVHD after HCT from unrelated donors.16 Additionally, peripheral blood
and bone marrow grafts were compared after haplo-HCT with PTCy followed by tacrolimus and MMF in a large registry study by the Center for International Blood and Marrow Transplant Research (CIBMTR). Peripher al blood was associated with a significantly lower risk of relapse (28% vs 45%; *P < .001) compared with bone marrow, however, it increased the risks of both grade II-IV acute GVHD (47% vs 25%; *P < .001) and chronic GVHD (41% vs 20%; *P < .001) after transplant. In a retrospective study, sirolimus and MMF following PTCy after a treosulfan-based myeloablative regimen with peripheral blood haplo-HCT resulted in a low incidence of grade II-IV acute GVHD (of 15%) at day 100 after transplant. Sirolimus promotes the relative expansion and preserves the potency of suppressive regulatory T cells (Tregs) that play an important role in GVHD prevention after HCT, whereas CNIs suppress Treg activity. Over the past decade, several studies reported on the prevention of GVHD with sirolimus-based immune suppression after HCT from donors with various relationships and degrees of histocompatibility. We here hypothesized that sirolimus in combination with MMF after PTCy as a CNI-free immune suppression would prevent GVHD after peripheral blood haplo-HCT.

Methods

Study design and patients

This is a single-institution, prospective phase 2 trial to assess the efficacy of PTCy followed by sirolimus and MMF after peripheral blood haplo-HCT in patients with hematological malignancies. This trial was approved by the University of South Florida Institutional Review Board. The study was conducted in accordance with the Declaration of Helsinki.

Inclusion criteria of the trial were the following: first allogeneic HCT for hematologic malignancy with peripheral blood as the only graft source used for HCT, patient age ≥18 years, Karnofsky performance status of ≥80% for the myeloablative and ≥60% for the nonmyeloablative (NMA) regimens, cardiac left ventricular ejection fraction >45% assessed by multigated acquisition scan or echocardiogram and no myocardial infarction within 6 months of HCT evaluation, pulmonary forced expiratory volume and adjusted diffusing capacity for carbon monoxide ≥50% of predicted values, liver transaminases (aspartate aminotransferase/alanine aminotransferase) <2 times upper limit of normal values, and estimated creatinine clearance ≥50 mL/min. Exclusion criteria were progressive or advanced hematological malignancy, active infection not controlled with appropriate antimicrobial therapy, presence of donor-specific antibodies, and inability or unwillingness to provide signed informed consent or comply with the study requirements.

Eligible donors were HLA-haploidentical relatives with a shared donor DNA typing at HLA-A, -B, -C, and -DRB1 loci. Granulocyte–colony stimulating factor (G-CSF)-mobilized donor peripheral blood hematopoietic cells were targeted to a CD34+ cell dose of 5 × 10⁶/kg with a minimum accepted dose of 2 × 10⁶ cells per kg and a maximum dose of 10 × 10⁶ cells per kg.

Treatment

The trial allowed either a myeloablative or an NMA conditioning. The myeloablative conditioning consisted of daily IV fludarabine (Flu) 30 mg/m² and pharmacokinetically targeted busulfan (Bu) with an average area under the curve of 5300 daily for 4 days. NMA conditioning consisted of daily IV Flu 30 mg/m² for 4 days, cyclophosphamide (Cy) 14.5 mg/m² for 2 days, and single 200 cGy dose of total-body irradiation (TBI).

GVHD prophylaxis consisted of Cy, sirolimus, and MMF. Cy 50 mg/kg was administered IV at posttransplant days +3 and +4. Sirolimus was administered as a 9-mg oral loading dose on day +5, followed by a maintenance daily dose at a target level of 8 to 14 ng/mL until day +90 and was subsequently tapered off by day +180 in the absence of GVHD. MMF was administered at 15 mg/kg every 8-hour dose (maximum daily dose of 3000 mg) from day +5 to day +35 and was discontinued in the absence of GVHD.

We followed institutional standards for antimicrobial prophylaxis and monitoring of infections. Quantitative cytomegalovirus (CMV) polymerase chain reaction blood samples was monitored weekly starting on day +7 after HCT, and preemptive antiviral therapy was initiated if ≥500 copies per milliliter viral reactivation was detected. Anti-infectious prophylaxis started at day +1 after HCT and consisted of fluoroquinolone for bacterial infections, either echinocandin or triazole for fungal infections, and acyclovir for viral infections. No letermovir was used for CMV prophylaxis in this trial. Standard prophylaxis for Pneumocystis jirovecii infection was initiated at day +30 and continued for at least 1 year after HCT and until discontinuation of immunosuppression. G-CSF at 5 µg/kg dose was initiated on day +5 and continued daily until an absolute neutrophil count of >1000/mm³ for 3 consecutive days.

End points and definitions

The primary objective of the trial was to evaluate the efficacy of PTCy followed by sirolimus and MMF in preventing acute GVHD after peripheral blood haplo-HCT. The primary end point of the study was the cumulative incidence of grade II-IV acute GVHD at day 100 after transplant. With 32 evaluable patients enrolled, the study had 90% power with a error of 0.1 to demonstrate reduction in grade II-IV acute GVHD to 20% from the historical benchmark of 40% at day 100 after transplant. Secondary end points included neutrophil and platelet engraftment, chronic GVHD, nonrelapse mortality (NRM), relapse incidence, GVHD-free relapse-free survival (GRFS), disease-free (DFS), and overall survival (OS). Exploratory end points were the cytokine release syndrome (CRS), chimerism, infections, and immune reconstitution.

Acute GVHD organ staging and overall grade were assessed as previously reported. Chronic GVHD severity scoring was assessed using National Institutes of Health (NIH) Consensus guidelines. The cumulative incidences of acute and chronic GVHD were estimated, considering malignancy relapse and nonrelapse death as competing risk events. Neutrophil engraftment was defined as the first of 3 consecutive days with AN absolute neutrophil count >0.5 × 10⁹/µL, and platelet engraftment was defined as having a platelet count of ≥20 × 10⁹/µL and being transfusion-free in the preceding 7 days. NRM was defined as the time from HCT to death of any cause without evidence of relapse, and relapse was considered as a competing event. Relapse was defined as recurrence of hematologic malignancy after HCT, and nonrelapse death was considered as a competing event. GRFS was defined as previously reported. DFS was defined as the time to relapse or death from any cause. OS was defined as the time
from transplant to death from any cause. All surviving patients were
censored at the time of last follow-up.

The Hematopoietic Cell Transplantation-specific Comorbidity Index
(HCT-CI) was defined as reported by Sorror et al.30,31

CRS grading was assessed as reported by Lee et al.32 CRS
assessment was performed between post-HCT day 0 and day +14
according to published reports after peripheral blood haplo-HCT.33
The World Health Organization grading system was used for
mucositis assessment.34 Hepatic veno-occlusive disease (VOD)
was graded by the European Society for Blood and Marrow
Transplantation (EBMT) classification.35 Quantitative measurement
of unseparated bone marrow and lineage-specific peripheral blood
donor chimerism were performed at posttransplant days +30, +90,
+180, and +365 with polymerase chain reaction–amplified short
tandem repeats.36 Frequency and density of bacterial, fungal, and
viral infections were studied within post-HCT time intervals of days
0 to +45, days +46 to +90, days 91 to +180, and days +181 to
+365.37,38 Immune cell recovery parameters, including total absolute
lymphocyte (CD45+), CD3+ T-cell, CD8+ T-cell, CD4+ T-cell, Treg
(CD4+CD25+FoxP3+CD127−), natural killer (NK)-cell (CD16+CD56+),
and B-cell (CD19+) counts, were prospectively studied on
peripheral blood of patients prior to transplant and at days +30,
+90, +180, and +365 after HCT using the flow cytometry method.

Statistical analysis

Patient, disease, and transplant characteristics and immune-
reconstitution measures were summarized by standard descriptive
statistical methods. The cumulative incidence estimator was used
to calculate the incidences of acute GVHD and chronic GVHD,
neutrophil engraftment, platelet engraftment, NRM, relapse, and
first occurrence of infection to accommodate competing risks.39
The differences in rates of grade II-IV acute GVHD between PTCy/
HCT-CI was defined as reported by Sorror et al.30,31

| Table 1. Patient characteristics |
|---------------------------------|
| **Variable** | **Strata** | **n (%)** |
| Total patients | | 32 |
| Patient age, y | Median (range) | 50 (23-75) |
| Donor age, y | Median (range) | 34 (15-64) |
| Patient sex | | |
| Male | 20 (62.5) |
| Female | 12 (37.5) |
| Donor sex | | |
| Male | 22 (68.8) |
| Female | 10 (31.2) |
| Patient race/ethnicity | | |
| White | 13 (40.6) |
| African American | 7 (21.9) |
| Hispanic | 7 (21.9) |
| Asian | 3 (9.4) |
| American Indian | 2 (6.2) |
| HCT-CI | | |
| 0-2 | 20 (62.5) |
| ≥3 | 12 (37.5) |
| Karnofsky score | | |
| ≥90% | 23 (71.9) |
| <90% | 9 (28.1) |
| Disease type | | |
| AML | 16 (50.0) |
| CML | 4 (12.5) |
| MDS | 2 (6.2) |
| Other myeloid* | 2 (6.2) |
| ALL | 7 (21.9) |
| Hodgkin disease | 1 (3.1) |
| Conditioning regimen | | |
| Flu/Cy/TBI 200 cGy | 21 (65.6) |
| Flu/Cy/TBI 200 cGy | 11 (34.4) |
| CMV serostatus | | |
| Donor—Recipient— | 7 (21.9) |
| Donor—Recipient+ | 2 (6.2) |
| Donor+—Recipient+ | 10 (31.2) |
| Donor+—Recipient+ | 13 (40.6) |
| Donor relation to patient | | |
| Offspring | 16 (50.0) |
| Sibling | 13 (40.6) |
| Parent | 3 (9.4) |
| Donor HLA matching | | |
| 7/8 | 1 (3.1) |
| 6/8 | 6 (18.8) |
| 5/8 | 13 (40.6) |
| 4/8 | 12 (37.5) |
| Donorrecipient sex match | | |
| Male/Male | 13 (40.6) |
| Male/Female | 9 (28.1) |
| Female/Female | 3 (9.4) |
| Female/Male | 7 (21.9) |
| Year of HCT | | |
| 2017 | 14 (43.8) |
| 2018 | 18 (56.2) |
| TNC, ×10⁶ cells/kg | Median (range) | 10.19 (4.07-65.5) |
| CD3⁰, ×10⁶ cells/kg | Median (range) | 3.68 (1.38-5.06) |
| CD3⁴⁺, ×10⁶ cells/kg | Median (range) | 7.19 (4.99-10.40) |

| ALL | acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; TNC, total nucleated cell. |

*Other myeloid indicates myelofibrosis and blastic plasmacytoid dendritic cell neoplasms.

Results

Patient characteristics

A total of 32 patients with hematological malignancies were
enrolled in this trial between February 2017 and August 2018.
The median age of patients at HCT was 50 years (range, 23-75
years) and 59% of all patients were of a racial or ethnic minority
(Table 1). Nearly 40% of study patients had multiple comorbidities
(HCT-CI ≥3) and 28% had a Karnofsky score of <90% at
transplant. All except 1 patient with Hodgkin disease had either
acute leukemia or myeloid malignancy. The majority of patients
(66%) received myeloablative conditioning with Flu/Bu, whereas
34% received NMA Flu/Cy/TBI. Most patients (72%) were CMV
seropositive, and, in 22% of the cases, a female donor was used for
a male recipient. The median infused CD34⁺ dose was 7.2 × 10⁶
(range, 5.0 × 10⁶ to 10.4 × 10⁹) cells per kg; the total nucleated
cell dose was 10.2 × 10⁴ (4.1 × 10⁸ to 65.5 × 10⁹) cells per kg;

and the CD3⁺ T-cell dose was 3.7 × 10⁶ (1.4 × 10⁶ to 5.1 × 10⁸)
cells per kg.

Engraftment and early toxicity

The median time of neutrophil engraftment was 17 days (range,
12-30 days; Figure 1) and platelet engraftment was 26 days (range,
12-63 days) after transplantation. We observed no primary or secondary
graft failure events in this trial. As early as day +30 after HCT, median
unseparated bone marrow and peripheral blood lineage-specific CD3⁺
and CD3⁴⁺ donor chimerism were all 100% (range, 73.1% to
100%). Median chimerism remained 100% donor at all post-HCT
time points examined (days +90, +180, and +365).
The majority of study patients (78%) developed CRS, and the median CRS grade was grade 1 (range, 0-2). The median CRS onset was day 1 (range, day 0 to day 3) after HCT and the median duration of CRS was 4 days (range, 2-8 days). None of the study patients required vasopressors, intubation, or had organ toxicities as a result of CRS. In addition, there was no need for interleukin-6 receptor inhibitor, tocilizumab,33 for management of CRS. The median mucositis grade was grade 2 (range, grade 0 to grade 3). The distribution of study patients by mucositis grade was: 25% with no mucositis, 6.2% with grade 1, 43.8% with grade 2, and 25% with grade 3 mucositis. We observed 2 cases of VOD both presenting with late onset (>21 days) after myeloablative HCT. One VOD case was mild and resolved with supportive therapy, whereas the second was severe resulting in multiorgan failure and death despite therapy with defibrotide. None of the study patients developed thrombotic microangiopathy (TMA) after HCT.

**Acute and chronic GVHD**

The study primary end point was met with cumulative incidence of grade II-IV acute GVHD of 18.8% (95% confidence interval [CI], 7.5% to 34.0%) at day 100 after HCT (Figure 2A). In this study of GVHD prophylaxis with PTCy followed by sirolimus and MMF, the incidence of grade II-IV acute GVHD was significantly lower than the 40% historical benchmark6 with PTCy followed by tacrolimus and MMF ($P = .01$). The cumulative incidence of grade III-IV acute GVHD was 9.4% (95% CI, 2.3% to 22.5%) in this trial. In patients presenting with acute GVHD, skin was the most commonly involved organ in 56.2% of the cases, followed by low gastrointestinal (GI) in 37.5%, and skin combined with GI involvement in 6.2% of the cases. None of the study patients had hepatic involvement with acute GVHD. Steroid-refractory acute GVHD was observed in 2 patients with fatal, stage IV lower GI involvement. The median duration of systemic glucocorticoid use for treatment of grade II-IV acute GVHD was 88 days (range, 12-98 days).

The cumulative incidence of moderate to severe chronic GVHD was 18.8% (95% CI, 7.4% to 34.0%) at 1 year of HCT (Figure 2B). The rate of any grade chronic GVHD was 46.9% (95% CI, 28.6% to 63.2%): 31.2% of patients had mild, 15.6% moderate, and 3.1% severe chronic GVHD. Three patients required systemic glucocorticoids for chronic GVHD therapy, whereas the rest of the patients with chronic GVHD responded to reinitiation or increase in sirolimus.
The GRFS probability was 49.6% (95% CI, 34.9% to 70.5%) at 1 year after HCT (Figure 2C). The cumulative incidence of discontinuation of total systemic immunosuppression in all surviving patients from HCT to last follow-up was 55.4% (95% CI, 22.8% to 78.9%; Figure 2D), and the median time of discontinuation of systemic immunosuppression was 255 days (range, 155-518 days).

**Relapse and survival**

The median follow-up of all surviving patients was 16.1 months (range, 11.5-26.2 months) at the time of study analysis. The cumulative incidence of NRM was 18.8% (95% CI, 7.4% to 34.0%) at 1 year after HCT (Figure 3A). NRM events included bacterial infections in 3 patients, steroid-refractory acute GVHD in 2 patients, and very severe VOD in 1. The cumulative incidence of relapse was 22.2% (95% CI, 9.6% to 38.2%) at 1 year after HCT (Figure 3B). DFS at 1 year after haplo-HCT was 59.0% (95% CI, 44.1% to 79.0%; Figure 3C) for all patients and 1-year OS was 71.7% (95% CI, 57.7% to 89.2%; Figure 3D).

**Infection frequency and immune reconstitution**

Infection density analysis that accounts for multiple infection events in an individual patient demonstrated that the frequency of infections is the highest within the first 45 days, followed by between days 46-90 and 91-180 after transplantation, whereas infections were infrequent thereafter (Figure 4). The frequency of viral infections was the highest followed by bacterial infections, whereas fungal infections were uncommon. BK cystitis was the most common viral infection seen in 50% of all study patients. However, in the majority of the cases (75%), the hemorrhagic cystitis was limited to grades 1-2. CMV reactivation was observed in 25% of all study patients (35% of CMV-seropositive recipients), human herpesvirus 6 reactivation in 22%, and other viral infections in 38% of the cases. The most common bacterial infection was *Clostridioides difficile* colitis in 38% of the patients and 47% had other bacterial infections. Fungal infections were uncommon events and were observed in 9.4% of patients.

We studied the immune reconstitution after haplo-HCT with PTCy, sirolimus, and MMF by examining the median absolute cell counts of total lymphocytes, CD3⁺, CD8⁺, CD4⁺ T cells, Tregs, NK cells, and B cells pre-HCT and up to 1 year after transplant (Figure 5). We subsequently compared immune cell recovery parameters to their
pretransplant values. We observed recovery of Tregs and NK cells to their baseline pre-HCT value as early as day +90 after HCT. The CD8\(^+\) T cells and B cells recovered by day +90 and exceeded baseline at day +180. CD4\(^+\) T cells gradually improved but remained below baseline at 1 year. The cumulative incidence of absolute CD4\(^+\) T-cell count recovery of 200 cells per microliter was

Figure 5. Immune reconstitution after HCT. Reconstitution of absolute lymphocyte count (A), total CD3\(^+\) T cells (B), total CD8\(^+\) T cells (C), total CD4\(^+\) T cells (D), Tregs (E), total NK cells (F), and total B cells (G) after HCT. The boxes show the interquartile range of absolute cell count (per \(\mu\)L) for each immune cell type. The bold horizontal lines inside of the boxes and the corresponding numbers on the bottom indicate the median absolute cell count (per \(\mu\)L). The whiskers represent 1.5\(\times\) the height of the box (or minimum/maximum values if there is no value in that range). The circles indicate the outliers. \(*P < .05\), compared with the pre-HCT cell count.
In this phase 2 prospective study, we observed that PTCy, sirolimus, and MMF mitigate grade II-IV acute GVHD after peripheral blood haplo-HCT. In comparison with the historical benchmark with PTCy/tacrolimus/MMF, which is considered a standard of care in GVHD prevention after haplo-HCT, PTCy/sirolimus/MMF results in significantly lower rates of grade II-IV acute GVHD. We also found that PTCy/sirolimus/MMF permits consistent hematopoietic engraftment and results in low risk of moderate and severe chronic GVHD.

A CIBMTR analysis found a lower relapse rate with the use of peripheral blood over bone marrow after haplo-HCT with PTCy/tacrolimus/MMF GVHD prophylaxis, with both acute and chronic GVHD rates significantly higher with peripheral blood graft. As a result, DFS was better with peripheral blood and GRFS with bone marrow whereas survival was not different due to the different pattern of treatment failure after peripheral blood vs bone marrow HCT. Similarly, the risk of chronic GVHD was significantly higher with peripheral blood compared with bone marrow graft in a BMT CTN phase 3 randomized trial after commonly used CNI/methotrexate GVHD prophylaxis in patients receiving HCT from unrelated donors. Acute and chronic GVHD are associated with morbidity and impaired quality of life after HCT, and therefore more effective GVHD-prevention regimens are needed to mitigate the GVHD risks. Our findings of lower GVHD rates with PTCy/sirolimus/MMF after peripheral blood HCT are consistent with a previous single-center retrospective experience. Similar results in 158 patients were recently reported in a registry study by EBMT, however, this study included various donor types and both peripheral blood and bone marrow graft sources. In addition, a single-center phase 2 trial studying the combination of sirolimus and PTCy as a GVHD prophylaxis after HCT included only 8 of 8 HLA-matched related or unrelated donors and had no MMF in a regimen. Although the results of our study offer valuable insight, these findings are currently limited to our single-arm phase 2 trial experience after peripheral blood haplo-HCT and retrospective reports in a relatively small number of patients after HCT from various donor types and graft sources. Moreover, our study included various hematological malignancies and both myeloablative and reduced-intensity conditioning regimens. If lower GVHD rates of PTCy/sirolimus/MMF vs PTCy/tacrolimus/MMF were confirmed by a randomized prospective study, PTCy/sirolimus/MMF would potentially become the new standard of care for GVHD prevention after HCT. This point is particularly important because PTCy/tacrolimus/MMF is currently being tested in a national phase 3 BMT CTN trial (NCT03959241), after multicenter randomized phase 2 study results showed significantly lower rates of both acute and chronic GVHD with PTCy/tacrolimus/MMF compared with the current standard regimen of tacrolimus/methotrexate for HLA-identical sibling or 7 to 8 of 8 HLA-matched unrelated donor HCT.

Our study did not find that sirolimus offers an advantage over tacrolimus in hastening hematopoietic recovery after haplo-HCT when each is used in combination with PTCy/MMF. However, we observed no graft-failure events in this trial, in contrast to ~10% primary graft failure rates reported after PTCy in combination with tacrolimus/MMF. Notably, we found that median chimerism values were 100% donor as early as day +30 after HCT in this trial. The use of peripheral blood grafts in combination with the relatively high (>5 × 10⁶ cells per kg) CD34⁺ cell dose target were both likely to facilitate donor engraftment in our study, so the contribution of sirolimus to consistent donor engraftment remains uncertain in absence of a controlled trial. Despite the administration of higher donor cell doses in our study, we observed no increase in risk of severe CRS. The efficacy of sirolimus in offsetting the association between peripheral blood grafts and CRS after PTCy-based GVHD prophylaxis needs to be further confirmed in larger number of patients receiving haplo-HCT. PTCy/sirolimus/MMF was overall well tolerated with low risk of VOD and no documented TMA events in our analysis. Similar findings of low risk of VOD and no increased risk of TMA have been previously reported in patients receiving CNI-free GVHD prophylaxis with sirolimus in combination with PTCy, MMF, or both. However, we still recommend a caution on patient selection and post-HCT close monitoring of VOD signs and symptoms as various conditioning intensity and regimen types have been used in these reported studies.

In this trial, NRM was within 20%, and 70% of the patients were alive at 1 year after HCT, despite a high proportion of patients with HCT-CI ≥3, CMV seropositivity, and the use of myeloablative conditioning in most subjects, all factors with a well-recognized risk of NRM after haplo-HCT. The risk of relapse by 1 year of HCT was relatively low (24%), despite the large majority of patients having had acute leukemia or a high-risk myeloid malignancy. A potential relapse risk-reduction effect of sirolimus needs to be explored in controlled trials. The rates of GRFS at 1 year after haplo-HCT in our trial compare favorably with CIBMTR study results with the use of either peripheral blood or bone marrow as a graft source after haplo-HCT. This is likely a result of low GVHD rates with PTCy/sirolimus/MMF, and low relapse risk with use of peripheral blood grafts in our study.

Infections after PTCy/sirolimus/MMF were mostly observed within the first 45 days of haplo-HCT, which includes the initial timeline of neutropenia, whereas infections were relatively uncommon after day +90. These findings were likely related to robust immune reconstitution of CD8⁺ T cells, NK cells, and B cells that recovered to their baseline pre-HCT values by day +90 of transplant. Despite the CMV-seropositive status of most study patients, the relatively low rates of CMV reactivation in our cohort could be explained by fast recovery of immunity after HCT. Sirolimus-based, CNI-free GVHD prophylaxis was also associated with lower risk of infections and/or robust immune reconstitution in prior reports. The absolute number of Tregs remained relatively preserved at all time points after HCT in our trial, an observation that further highlights the important role of GVHD prevention using the CNI-free, PTCy/sirolimus/MMF regimen. These findings warrant prospective comparison of PTCy/sirolimus/MMF vs PTCy/tacrolimus/MMF for GVHD prophylaxis.

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**Authorship**

**Contribution:** N.B., J.A.P., and E.A. conceived the study; X.W. and R.T. analyzed and interpreted data; N.B. wrote the manuscript; and all authors interpreted and edited the manuscript and approved the final manuscript.
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