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## A novel reduced intensity conditioning regimen for unrelated umbilical cord blood transplantation in children with non-malignant diseases

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### Abstract

Reduced intensity conditioning (RIC) regimens have the potential to decrease transplant-related morbidity and mortality. However, engraftment failure has been prohibitively high after RIC unrelated umbilical cord blood transplantation (UCBT) in chemotherapy-naïve children with non-malignant diseases (NMD). Twenty-two children with a median age of 2.8 years, many with severe comorbidities and prior viral infections were enrolled in a novel RIC protocol consisting of hydroxyurea, alemtuzumab, fludarabine, melphalan and thiotepa followed by single UCBT. Patients were transplanted for inherited metabolic disorders (N=8), primary immunodeficiencies (N=9), hemoglobinopathies (N=4) and Diamond Blackfan anemia (N=1). Most UCB units were HLA-mismatched with median infused total nucleated cell dose of  $7.9 \times 10^7/\text{kg}$ . No serious organ toxicities were attributable to the regimen. The cumulative incidence of neutrophil engraftment was 86.4% (95% confidence interval [CI], 65%–100%) in a median of 20 days, with the majority sustaining >95% donor chimerism at 1 year. Cumulative incidence of acute graft-versus-host disease (GVHD) grades II–IV and III–IV by day 180 was 27.3% (95% CI, 8.7%–45.9%) and 13.6% (95% CI, 0%–27.6%), respectively. Cumulative incidence of extensive chronic GVHD was 9.1% (95% CI, 0%–20.8%). The primary causes of death were viral infections (N=3), acute

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The authors report no potential conflict of interest.

#### **AUTHORSHIP CONTRIBUTIONS:**

SP contributed to study design, managed the clinical trial, collected and interpreted the data, and wrote the manuscript; PS designed research, obtained funding, interpreted the data, and wrote the manuscript; JK contributed to study design, interpreted the data, critically reviewed and edited the manuscript; DN contributed to study design; AM performed statistical analysis; CLB, KK, JA performed immunological studies and helped analyze that data; AP, GH, TAD, PLM, KP, KF, JM collected data and provided clinical input.

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GVHD (N=1) and transfusion reaction (N=1). One-year overall and event-free survivals were 77.3% (95% CI, 53.7%–89.8%) and 68.2% (95% CI, 44.6%–83.4%) with 31 months median follow-up. This is the first RIC protocol demonstrating durable UCB engraftment in children with NMD. Future risk-based modifications of this regimen could decrease the incidence of viral infections. ([www.clinicaltrials.gov/NCT00744692](http://www.clinicaltrials.gov/NCT00744692))

## Keywords

Umbilical cord blood transplantation; Reduced intensity conditioning; Pediatric disorders; Non-malignant diseases; Thalassemia; Hemophagocytic lymphohistiocytosis (HLH)

## INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) can cure a variety of non-malignant diseases (NMD) in children, either by direct replacement of abnormal bone marrow precursors as in hemoglobinopathies and primary immunodeficiencies (PID) or indirectly, by providing cellular enzyme replacement to diseased areas such as liver and brain, in inherited metabolic disorders (IMD) (1–3). For patients lacking matched related donors, unrelated donor umbilical cord blood transplantation (UCBT) is increasingly being utilized because of easy availability and less restrictive human leukocyte antigen (HLA) matching requirements. Myeloablative conditioning (MAC) with UCBT has been used successfully for 2 decades to treat a variety of malignant and NMD (4–7), however, high transplant-related mortality (TRM), and the risks of long-term adverse effects, such as infertility and secondary malignancies have remained a barrier for children with hopefully decades of life expectancy (8–10).

Mixed donor chimerism leads to disease resolution in patients with hemoglobinopathies and PID, which can remain stable over time (11–14). These observations provide a rational basis to design reduced intensity conditioning (RIC) regimens aimed at decreasing non-hematopoietic toxicity without compromising the clinical benefits of persistent donor hematopoiesis. There is a spectrum of RIC regimens, ranging from minimally intensive/non-myeloablative to near-ablative with reduced toxicity (13–18). However, successful RIC trials were reported mainly after HSCT from living related or unrelated donors. The use of UCBT after RIC has been successful in heavily pre-treated adults transplanted for malignant conditions, but has been associated with high rates of primary graft failure or graft rejection and autologous reconstitution in chemotherapy-naïve children, exceeding 50% in patients undergoing single unit UCBT for hemoglobinopathies (19–22). In these cases, graft failure might result from the fact that lower numbers of infused progenitors may have been unable to outcompete recovering host hematopoiesis and lymphopoiesis. Therefore, we developed a novel immunoablative, nevertheless, RIC regimen, designed to establish durable donor cell engraftment, and report the results of this prospective, single arm, phase I pilot study in chemotherapy-naïve children with NMD undergoing single donor UCBT.

## METHODS

### Patients

From December 2008 to June 2012, 22 children with NMD lacking matched related donors scheduled to undergo a single UCBT were enrolled at Duke University, Durham, N.C. (N=19) and All Children's Hospital, St. Petersburg, FL (N=3). The study was approved by the IRB of both institutions. Written assent or informed consent was obtained from all parents/caretakers or, when 18 years of age, patients, in accordance with the Declaration of Helsinki. Eligible patients had to be between 2 months and 21 years of age with a diagnosis

of NMD (PID, IMD, hemoglobinopathies, or other transfusion dependent anemias). Patients with IMD exhibiting high performance status, severe aplastic anemia, and those who had undergone HSCT within the previous 6 months were excluded. ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) NCT00744692).

## Donors

Grafts were selected based on HLA Class I (A, B) intermediate-resolution and HLA Class II (DRB1) allelic level typing. The pre-cryopreservation total nucleated cell count (TNCC) had to be  $>3 \times 10^7/\text{kg}$  for 5/6 or 6/6 HLA-matched grafts and  $>5 \times 10^7/\text{kg}$  for 4/6 HLA-matched grafts. Potential units were screened by international cord blood banking standards and for normal enzyme activity in IMD patients. Pre-cryopreservation graft characteristics were obtained from the supplying cord blood banks while the respective hospital laboratories that thawed, washed and tested the units provided post-thaw data.

## Conditioning Regimen, Graft-versus-host disease (GVHD) prophylaxis, Transplantation and Supportive care

All patients were treated with alemtuzumab 1mg/kg/dose I.V. on three successive days -21 to -19, following a test dose of 0.2mg/kg on day -22; hydroxyurea 30mg/kg/day orally from day -22 until day -10; fludarabine (Flu) 30mg/m<sup>2</sup>/day I.V. from days -9 to -5; melphalan (Mel) 70mg/m<sup>2</sup>/day I.V. on days -4, -3; and thiotepa 200mg/m<sup>2</sup> I.V. on day -2. Frequent baths (Q4 hours  $\times$  6) were prescribed after thiotepa administration. The thawed and washed UCB unit was infused intravenously on day 0 as previously described (23,24).

Starting day -3, all patients received GVHD prophylaxis with tacrolimus 0.03mg/kg/day I.V. and mycophenolate 15mg/kg/dose I.V. every 8 hours. Tacrolimus trough levels were maintained between 8–15ng/mL. In patients without active GVHD, mycophenolate was discontinued after 45 days and tacrolimus was tapered after 9 months post-transplant.

Alemtuzumab was administered in the outpatient clinic for most patients following intensive premedication with acetaminophen, diphenhydramine, ibuprofen and corticosteroids. All patients were hospitalized in the Pediatric Blood and Marrow Transplant unit of Duke University Medical Center or All Children's Hospital prior to day -4 and through engraftment and stabilization post-transplant. Median length of stay for the initial hospitalization was 47 days (range 29–263). Fungal and antiviral prophylaxis was voriconazole, and acyclovir respectively. Pneumocystis jiroveci pneumonia, veno-occlusive disease (VOD) prophylaxis, nutrition, and transfusion support was as previously described(24). All patients received intravenous immunoglobulin (500mg/kg/dose) weekly until day +100, starting day-1. Filgrastim (Amgen, Thousand Oaks, CA) was started at 5 mcg/kg/day on day +1 and weaned post-engraftment. Cytomegalovirus (CMV) and adenovirus DNA were monitored weekly post- alemtuzumab. One patient received irradiated, G-CSF-mobilized, parental granulocyte transfusions until engraftment for history of Mycobacterium avium, Pseudomonas, and Candida infections. Four patients subsequently enrolled on the ADV HALT study (Randomized, Placebo-controlled, Phase 2 Study of preemptive CMX001 treatment for adenovirus disease prevention); two on a multicenter study of banked third-party virus-specific T-cells to treat CMV, Epstein-Barr virus (EBV), or adenovirus infections after HSCT (25), and two on expanded access study of human mesenchymal stem cell (Prochymal<sup>®</sup>) infusion for steroid-refractory acute GVHD. (Tables 3 and 5)

## Post-transplant assessments

Engraftment studies were performed by short tandem repeat methods in whole blood, CD15<sup>+</sup> and CD3<sup>+</sup> fractions on days 30, 60, 100, 180, 270, 1 year and annually thereafter. In

parallel, immunophenotyping by 4-color FACS was performed to quantitate lymphocyte and dendritic cell subsets (26–28). Immunoglobulin levels were tested at regular intervals post-transplant. Organ function and disease-specific evaluations were performed per institutional practices.

### Assessment of functional T-cell responses to microbial antigens

Multiplex cytokine measurements using a cytokine bead array (CBA) and lymphoproliferative assays as internal controls were set up to test in parallel for the presence and magnitude of virus-specific T-cell response. Mononuclear cell preps were made and  $10^5$  mononuclear cells were seeded/well of 96 well plates in complete medium composed of RPMI 1640, 10% PHS, 100x antibiotic/antimycotic, L-glutamine, HEPES and  $\beta$ -mercaptoethanol, (Life Technologies Corp, USA). Viral antigens (Meridian Life Sciences, Memphis, TN) derived from cytomegalovirus (CMV, R9A104), herpes simplex virus (HSV, R9A002), and varicella zoster virus (VZV, R02030) were diluted from frozen stocks and added for a total 200ul. Negative control wells received no antigen; while positive control wells received  $10^5$  ClinExVivo<sup>®</sup> beads (Life Technologies, Carlsbad, CA) as described. Duplicate wells were incubated for 5 days at 37°C, and then 75ul medium was removed and frozen for batched CBA analysis. Secreted cytokines (IFN $\gamma$ , TNF $\alpha$ , IL-10, GM-CSF, IL-2 and IL-4) were quantitated using the BioPlex<sup>®</sup> (Biorad, Hercules, CA) CBA system. The wells were pulsed with 25 $\mu$ l of medium containing 1 $\mu$ Ci of <sup>3</sup>H-Thymidine (Perkin Elmer, Waltham, MA, USA) and incubated for 6–8 hours at 37°C. Results were expressed numerically as the mean counts per minutes of duplicates as described (29,30).

### T cell receptor excision circle (TREC) measurements

Peripheral blood mononuclear cells were prospectively cryopreserved at defined intervals before and after UCBT. Thymopoiesis was assessed by amplification and quantification of delta-deletion TRECs as previously described (31). Primers for the TREC sequence were 5'-CCC TTT CAA CCA TGC TGA CAC-3' (forward) and 5'-GGG TGC AGG TGC CTA TGC-3' (reverse), which produced an 80-bp fragment detected with the probe 5'-FAM-TCT GGT TTT TGT AAA GGT GCC CAC TCC TG-BHQ-1-3'. TREC quantification was normalized for cell input by parallel quantification of the human  $\beta$ -globin gene. The primers for human  $\beta$ -globin were 5'-GAA GAG CCA AGG ACA GGT ACG-3' (forward) and 5'-CCT GGG AGT AGA TTG GCC AA-3' (reverse), which produced an 85-bp fragment detected by the probe 5'-FAM-CTG TCA TCA CTT AGACCT CAC CCT GTG-BHQ-1-3'.

### Statistical Methods

The study was designed as a prospective, single arm, safety and efficacy pilot study to evaluate the RIC regimen for single UCBT. The primary hypothesis/objective was that the RIC regimen would result in adequate engraftment defined as the presence of >25% donor chimerism at 6-months post-UCBT. Simon's (optimum) two-stage design was employed to test the null hypothesis that the donor engraftment rate is 0.30 (Ho: p 0.30) versus the alternative that it is 0.65 (Ha: p 0.65). *Primary graft failure* was defined as the failure to reach ANC >500/mm<sup>3</sup> by day 42 or absence of donor hematopoiesis 10% in whole blood cells by day 100. The day 100 and 180 stopping rules for graft failure were not crossed. Cumulative incidence estimates of neutrophil recovery, platelet engraftment, acute GVHD grades II–IV, and III–IV, and chronic GVHD were assessed using standard competing risk analyses with death as a competing risk.(32) Neutrophil recovery was defined as the first day of an absolute neutrophil count (ANC) >0.5  $\times 10^9$ /L for 3 consecutive days not secondary to granulocyte infusions; and platelet engraftment as first day of platelet counts >20  $\times 10^9$ /L or >50  $\times 10^9$ /L for 7 consecutive days without transfusions as previously described (24). Acute and chronic GVHD were graded per established criteria (33,34). The

cumulative incidence of infection (any, bacterial, viral or fungal) was estimated from the start of the RIC regimen using death as a competing risk. The Kaplan-Meier method was used to describe overall survival (OS) and event-free survival (EFS) (35). OS considered a patient death as the event and censored on the last date of contact while EFS considered death, graft failure or rejection as the events. Descriptive statistics of immune reconstitution were provided at 3, 6, 9, 12 and 24 months post-UCBT. Analyses were conducted using the SAS System version 9.3 (Cary, NC).

## RESULTS

### Patient characteristics

Patient demographics (N=22) are shown in Table 1. The median age was 2.8 years (range 0.3–8.0) and median weight was 13.7 kg (range 5.3–26.9) on the day of transplant. Most patients were males (59%) and Caucasians (68%), while, 23% were African-American and 9% were Asian. Patients were diagnosed with immunodeficiency disorders (n=9), IMD (n=8), hemoglobinopathies (n=4) and Diamond Blackfan anemia (n=1). Twenty one patients received their first HSCT, while one patient with thalassemia major was enrolled 15 months after rejecting a matched sibling bone marrow transplant (BMT) after MAC regimen.

### Graft characteristics

The median pre-cryopreservation TNCC and CD34<sup>+</sup> doses were  $11.1 \times 10^7/\text{kg}$  (range 5.9–31.3) and  $4.35 \times 10^5/\text{kg}$  (range 1.71–8.1), respectively. Median infused TNCC and CD34<sup>+</sup> doses were  $7.9 \times 10^7/\text{kg}$  (range 4.1–22) and  $3.3 \times 10^5/\text{kg}$  (range 0.9–7.7), respectively. Using conventional HLA matching criteria to select UCB grafts, donor-recipient matching was 4/6 in 7 (31.8%), 5/6 in 11 (50%), and 6/6 in 4 (18.2%) patients. Review of HLA typing at allelic level including HLA-C revealed matches of 3/8 in 1 (4.5%), 4/8 in 3 (13.6%), 5/8 in 5 (22.7%), 6/8 in 8 (36.4%), 7/8 in 3 (13.6%) and 8/8 in 2 (9.1%). Thus, approximately 40% of recipients were mismatched for 3 or more loci at the allelic level. Donors were matched for gender in 12 (55%) and ABO blood type in 10 (45.5%) recipients. Major and/or bidirectional ABO mismatch was noted in 7 patient/donor pairs (31.8%).

### Neutrophil and Platelet Engraftment

The cumulative incidence of neutrophil engraftment with donor cells by day 42 was 86.4% (95% confidence interval [CI], 65%–100%; Figure 1A). Nineteen patients engrafted at a median of 20 days (range 12–28). Three patients experienced graft failure: primary graft failure (n=1) who died on day 24 (UPIN 14) with 100% donor cells before engrafting, and autologous reconstitution (n=2). The latter two patients with autologous reconstitution (UPIN 15, UPIN 4) successfully underwent second transplants, 3.5 and 5 months later; with double UCBT after myeloablative Busulfan (Bu)/Cyclophosphamide (Cy)/ATG, and with matched unrelated donor BMT after thiotepa/ATG conditioning, respectively. Both are alive with donor engraftment. There have been no secondary graft failures. The cumulative incidence of platelet engraftment (50,000/uL) by day 180 was 72.7% (95% CI, 47.1%–98.3%) occurring in a median of 48 days (range 33–144; Figure 1B).

### Donor Chimerism

A primary objective of this study was to determine the proportion of patients engrafted with >25% donor cells 6 months post-transplant. Eighteen patients survived >6 months, 16 of whom achieved this endpoint. Specifically, whole blood donor chimerism >90% was demonstrated in 15/16 patients at 180 days, 14/15 patients at 1 year, and 9/10 patients followed at 2 years. Similar levels of donor cell chimerism were seen in CD15<sup>+</sup> (15/16 at

180 days, 15/15 at 1 year, 9/10 at 2 years); and CD3<sup>+</sup> fractions (13/16 at 180 days, 13/15 at 1 year, 9/10 at 2 years). (Figure 2 and Table 4)

### Immune reconstitution

In engrafting patients, the median absolute lymphocyte and CD4<sup>+</sup> T-cell counts at 3, 6 and 12 months were 1291 (382–3008) and 154 (0–678) (N=17); 1405 (556–4470) and 418 (1–1507)(N=16); and 2193 (333–5070) and 805 (6–2013)(N=15), respectively (Figure 3). These values are similar to other pediatric reports using ATG-based rejection prophylaxis after myeloablative regimens (36–39).

At 180 days, normal, age-adjusted NK, B, and CD4<sup>+</sup> counts were found in 14/16 (87.5%), 11/16 (68.8%) and 5/16 (31.3%) patients respectively. At 1 year, normal, age-adjusted NK, B, and CD4<sup>+</sup> counts were seen in 13/15 (86.7%), 12/15 (80%), and 12/15 (80%) patients, respectively.(40)

Nine of 15 engrafted and surviving patients with a minimum 1 year follow-up are off systemic immunosuppression, seven of whom have started immunizations. IgA levels were available for 14 of 15 patients followed at least 1 year; and were normal in 11 patients reflecting effective T-cell dependent isotype switching by B cells unaffected by possible IVIG supplementation; and low in 3 patients. Two of the patients with low IgA had received rituximab, and 1 had extensive chronic GVHD.

Seven of 10 patients followed >24 months maintain normal IgG levels without IVIG support.

### Thymic output

The appearance of recent thymic emigrants (RTE) is a powerful marker of thymopoiesis critical for acquiring a broad, naïve TCR repertoire. It was quantitated by qPCR for TREC and independently by flow cytometry monitoring CD45RA and CD62L co-expressing CD4<sup>+</sup> cells. There was a notable paucity of RTE and TREC<sup>+</sup> cells in most patients within the first 100 days (Figure 4) followed by a steep rise at day 180 reflecting the resumption of thymopoiesis. The exceptions were UPIN 22, who died on day +258 with GVHD complications associated with profound lymphopenia and recurrence of pre-transplant CMV and adenovirus infections; UPIN 16, who had grade III acute GVHD; and UPIN 17, who had extensive chronic GVHD and had failed a prior myeloablative transplant.

### Anti-viral immunity

Sensitive BioPlex assay monitored the Duke cohort (N=19) for virus-specific cytokines following in vitro challenge with herpes family of viruses: CMV, HSV and VZV. The young age of this study cohort explains the relative low incidence of pre-transplant exposure; CMV (n=5), HSV (n=1) and VZV (n=4). There were no VZV specific responses detected but only one seropositive patient was alive/studied beyond day 100. The one patient (UPIN 17) with positive pre-transplant HSV serology demonstrated specific cytokine response first at day 180 detectable by simultaneous IFN $\gamma$  and GM-CSF secretion (93pg/ml and 210pg/ml) reflecting probable subclinical viral reactivation earlier.

There were 5 patients with pre-UCBT CMV exposure. Notably, two PID patients were viremic already pre-alemtuzumab, one died before day 30 due to CMV pneumonia (UPIN 21), the other had autologous reconstitution (UPIN 4). Of the other 3 patients, UPIN 22 died of adenovirus and CMV with practically no circulating T-cells (<10/ul) even at 6 months while UPIN 9 (107pg/ml) and 17 (77pg/ml) both demonstrated detectable virus-specific IFN $\gamma$  secretion and proliferative responses by day 100. Neither experienced recurrent CMV

viremia. CMV-specific GM-CSF, IL-13, and TNF $\alpha$  secretion was also detected but was discordant between these 2 patients. In sum, anti CMV, -HSV responses were detected only in seropositive patients and, when detected, there was no associated disease.

### Graft-Versus-Host Disease

The cumulative incidence of grades II–IV and III–IV acute GVHD by day 180 was 27.3% (95% CI, 8.7%–45.9%) and 13.6% (95% CI, 0%–27.6%), respectively (Figure 1C). The cumulative incidence of any chronic GVHD at 1 year was 40.9% (95% CI, 19.3%–62.5%). However, the cumulative incidence of extensive chronic GVHD was only 9.1% (95% CI, 0%–20.8%) (Figure 1D). Of the 10 patients developing chronic GVHD, 8 had disease limited to skin and 2 had extensive (skin N= 1; skin, gut N= 1) disease. Autoimmune hemolytic anemia (AIHA) was noted in 3 patients (isolated AIHA, N=2; AIHA with chronic GVHD, N=1; cumulative incidence 9.1% [95% CI, 0%–20.8%]) and responded in all patients to steroid and rituximab therapy. Of these 12 patients (10 with classic chronic GVHD and 2 additional patients with isolated AIHA), 5 remain on chronic immunosuppression 14–35 months post-transplant. Two patients received mesenchymal stem cell treatment for steroid refractory GVHD. One (UPN 17) has had a partial response, whereas the other (UPN 22) died of acute GVHD despite MSC treatment. (Table 5)

### Infections

Cumulative incidence of infectious events at 1 year was high at 90.9% (95% CI, 67.5%–100%) for any infection; 81.8% (95% CI, 57.9%–100%) for viral; 13.6% (95% CI, 0%–27.7%) for fungal; and 86.4% (95% CI, 63.1%–100%) for bacterial infections. CMV, adenovirus and EBV represented the majority of viruses encountered.

**CMV**—Six patients were at risk for CMV disease due to seropositivity and/or viremia (N=2). Systemic therapy was required for four of them pre-transplant (2 before and 2 after initiation of conditioning), two of whom (UPN 21, 22), both with PID, developed CMV disease and died post-transplant.

**Adenovirus**—Three patients had adenovirus detected pre-transplant (2 in stool alone, 1 in stool, urine and blood), all of whom developed adenoviremia post-transplant, including 2 with adenoviral disease. Overall, adenoviremia developed in 8 of 22 patients (36%) post-transplant, all of whom received systemic anti-adenoviral therapy. Two patients with inadequate response to cidofovir received banked third party virus specific cytotoxic T lymphocytes. One of these patients cleared adenoviremia (UPN 3), whereas the other patient did not and died on day 24 (UPN 14). An investigational agent, CMX001 was used to treat 4 patients. One patient (UPN 8) had clearance of adenoviremia and is off CMX001 treatment for >2years. Treatment is ongoing for UPN 17 due to continued risk resulting from extensive chronic GVHD and ongoing severe lymphopenia. Two patients eventually died, one (UPN 11) with fulminant adenoviral disease on day 25, and another (UPN 22) with severe acute GVHD on day 258 with adenoviral and CMV infections. In all, three patients developed adenoviral disease that was associated with mortality. (Table 3)

**EBV**—No patient had EBV infection pre-transplant. Post-transplant, 2 patients developed EBV infection. EBV viremia with mesenteric lymphadenopathy was noted in one patient (UPN 1) on day +88. He responded to rituximab and is alive and well 4 years post-transplant. Transient increase in EBV copy numbers in another patient (UPN 16) responded to 2 doses of rituximab, and is alive >1 year post-transplant.



*AFB bacteremia* was noted in 2 patients, one with PID (UPN 10) on day +123 and one with Krabbe disease (UPN 12) on day +134. Both patients cleared their infections with multidrug treatment, and are well >2 years post-transplant and off therapy.

### Organ toxicity

This regimen was well tolerated by patients, including those with significant pre-transplant comorbidities (Table 1). All patients developed some mucositis, which required parenteral nutrition and analgesia, but VOD, idiopathic pneumonitis, pericardial effusion, hemorrhagic cystitis, or neurologic events attributable to the regimen were not seen in anyone. One patient who had developed severe hemorrhagic cystitis during prior BMT needing approximately 3 months of platelet support, prostaglandin and hyperbaric oxygen therapy, was enrolled on this study for a second transplant and tolerated the RIC regimen well without recurrence of cystitis. Several other patients had pre-transplant comorbidities such as severe bronchiectasis, chronic pulmonary disease, enteroviral meningitis, and obstructive sleep apnea and tolerated the regimen well (Table 1).

### Survival

Median follow up of surviving patients is 30.89 months (range 14.31–52.93). Overall and event-free survival at 1 year post-transplant is 77.3% (95% CI, 53.7%–89.8%) and 68.2% (95% CI, 44.6%–83.4%), respectively (Figure 5). Five patients died (Table 2), of whom three died before day +28 associated with viral infections. One immunodeficient patient died on day +258 from severe acute GVHD with concomitant adenoviral and CMV disease. Pre-transplant T-cell deficiency with adenovirus and CMV detection in stool and urine, respectively, increased risk for reactivation post-transplant. One patient, a mixed chimera on day +100 (total chimerism 63%, 91% lymphoid, 58% myeloid) died on day +170 of a hemolytic transfusion reaction after empirically receiving donor type blood.

## DISCUSSION

We report the outcomes of a prospective, pilot study evaluating a novel RIC regimen consisting of alemtuzumab, hydroxyurea, fludarabine, melphalan and thiotepa in a pediatric cohort of 22 patients with NMD undergoing single unrelated donor UCBT. The majority of patients (19/22) were chemotherapy-naïve and 9/22 were transplanted for PID, most of whom arrived to transplant with pre-existent viral infections (n=7) reflecting their underlying T-cell defects. The regimen was designed to decrease exposure to alkylating agents, while strengthening the immunosuppressive component (fludarabine and alemtuzumab) with the overall goal of facilitating donor engraftment while reducing non-hematopoietic organ toxicity. The cumulative incidence of neutrophil engraftment was 86%, comparable to myeloablative UCBT, and significantly higher than that reported after RIC UCBT in pediatric NMD (37.5–70.6%) (19,22,24,41–45). Consistent donor engraftment was noted in patients belonging to diverse diagnostic groups, including those at a higher risk of engraftment failure.

Most patients achieved high levels of durable donor chimerism over the period of observation (median >24 months). High level (>90%) whole blood donor chimerism was noted in 14/15 patients at 1 year, and 9/10 patients at 2 years post-transplant. Whole blood chimerism remains >80% and T-cell chimerism remains >65% in all engrafted surviving patients (n=15). The durability of donor engraftment will be monitored long-term. We are optimistic that donor chimerism will persist given the high (>50%) level of T-cell chimerism in patients thus far. This level of chimerism is similar to our previously reported cohort of 159 patients with IMD undergoing myeloablative single UCBT (24). All 5 patients with

inherited transfusion dependent anemias and both patients with hemophagocytic lymphohistiocytosis (HLH) maintain donor chimerism >90% at the time of this report.

Engraftment after UCBT has been a challenge in hemoglobinopathies even with MAC regimens (19–21,46,47) due to factors such as compensatory marrow hyperactivity, alloimmunization from frequent transfusions, and the presence of an immunocompetent, chemotherapy-naïve host (48). Retrospective registry analysis of mostly myeloablative UCBT in hemoglobinopathies showed primary graft failure as the predominant cause of failure, occurring in 27 of 51 patients with 2-year DFS of 45% despite an optimal cell dose (20). With RIC regimens, engraftment is even more challenging. High graft failure rate with a 2-year EFS of 50% was recently reported with Bu/Flu/alemtuzumab (21). Similarly, in a multicenter study of sickle cell disease patients undergoing UCBT after a RIC regimen of alemtuzumab/Flu/Mel, 5/8 patients had autologous recovery (1-year EFS of 37.5%) (19). In these patients, stringent criteria were used for HLA matching (5/6) and cell dose (median pre-cryopreservation TNCC  $6.4 \times 10^7$ /kg, range 3.1–7.6), and thus did not appear to impact the outcome, suggesting that perhaps the regimen needed to be intensified to ensure consistent engraftment. In the current study, the alemtuzumab/Flu/Mel backbone (18, 19), which was reported with increased primary graft failure after single UCBT, was augmented with thiotepa and hydroxyurea, promoting engraftment. Thiotepa is a potent immunosuppressive and myelosuppressive drug and has been successfully used with melphalan (49–51). It has promoted engraftment in murine transplant models (52, 53) as well as in clinical studies of T cell depleted HSCT and UCBT (54–56). Hydroxyurea can induce cycling of the stem cell pool potentially sensitizing them to melphalan and thiotepa (57). Alemtuzumab was administered distal to UCBT to maximize host immunoablation while reducing its impact on infused donor lymphocytes.

Of the two patients with autologous recovery, cord blood unit of one with cartilage hair hypoplasia lacked in vitro growth in colony forming unit (CFU) assay at the time of thaw; the other with adrenoleukodystrophy received a unit with 3/8 allelic HLA matching that showed transient full donor engraftment before being replaced by host cells. Poor graft potency and immune rejection respectively may have contributed to graft failure (58,59). Interestingly, both patients reconstituted host granulopoiesis within 4–6 weeks post-transplant, consistent with the notion that the preparative regimen is not fully myeloablative (60). Both patients underwent successful second transplants and are long-term survivors.

The two HLH patients are surviving with >90% donor chimerism for >2 years. TRM is a major barrier for success of conventional myeloablative regimens in HLH. In a CIBMTR retrospective HLH study (N=91), mostly with matched unrelated donor BMT and Bu/Cy/VP16+/-ATG, day 100 TRM was 35% (61). Alemtuzumab/fludarabine/melphalan has been demonstrated to decrease mortality in children with HLH receiving BMT with a 3-year survival of 92% compared to 43% for myeloablative Bu/Cy/ATG (14). However, several patients needed donor lymphocyte infusions to sustain mixed chimerism in the RIC group. Excellent engraftment and survival was also reported for BMT from Europe (62), but data with UCBT is limited (63).

This regimen was well tolerated in terms of organ toxicity as there was no cardiac, pulmonary, CNS, renal or bladder toxicity recorded. Pericardial effusion, hemorrhagic cystitis, VOD, and multiorgan failure have been associated with Bu/Cy regimen (64). Long-term follow-up will determine the impact of this regimen on late effects.

The major obstacle associated with this regimen is the high incidence of viral infections. Several factors are likely contributory. Clearly, patients with underlying T-cell immunodeficiencies and pre-transplant viral disease were extremely high risk for recurrent

infections. Nevertheless, the alemtuzumab dose was relatively high compared to some other pediatric studies (14,18,62) and post-UCBT exposure may have depleted most of the T-cells accompanying the grafts leading to delayed T-cell reconstitution until thymic recovery (65). Alemtuzumab dose de-escalation could be explored in the future to allow faster lymphocyte reconstitution; however, insufficient host immunoablation could jeopardize engraftment. Patients with pre-existing viral burdens should either have their transplants delayed where feasible, or use graft sources that would allow transfer of virus-specific memory. To further enable the use of cord blood donor grafts, patients could pre-emptively receive aggressive antiviral or adoptive immunotherapy post-transplant.

Taken together, this conditioning regimen is the first RIC regimen to our knowledge to result in consistent and durable donor engraftment in children undergoing UCBT for a variety of diseases, including patients with intact immunity and high risk for graft failure. It was well tolerated even in patients with significant pre-transplant co-morbidities. Engraftment, survival, acute GVHD, and extensive chronic GVHD were not inferior to those previously reported after traditional myeloablative chemotherapy. Immune reconstitution and thymopoiesis was initially slow, but robust after 6 months in most patients. This regimen warrants further study and optimization for patients with non-malignant conditions undergoing UCBT.

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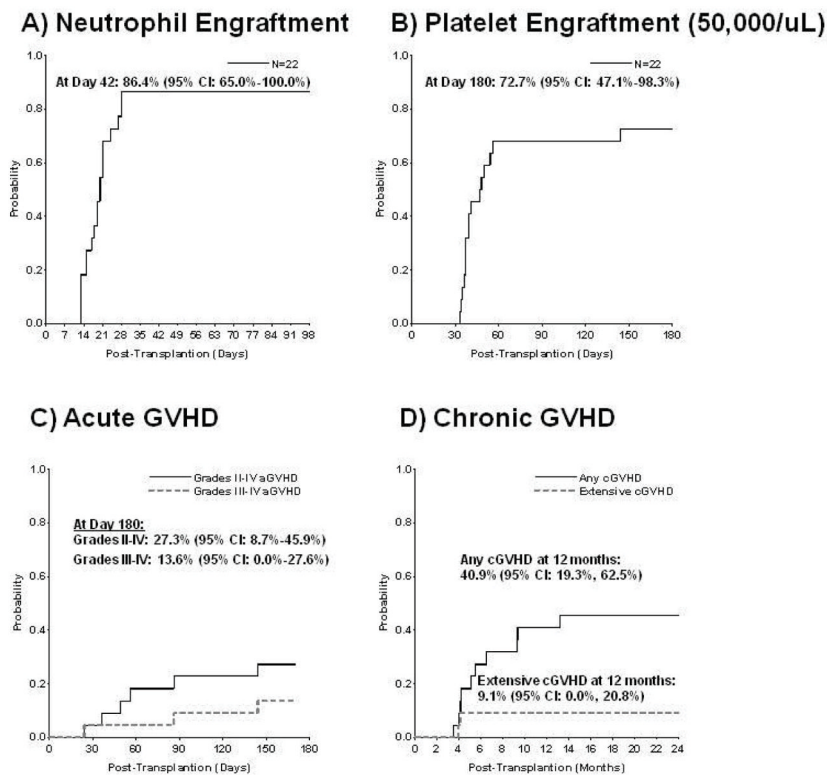
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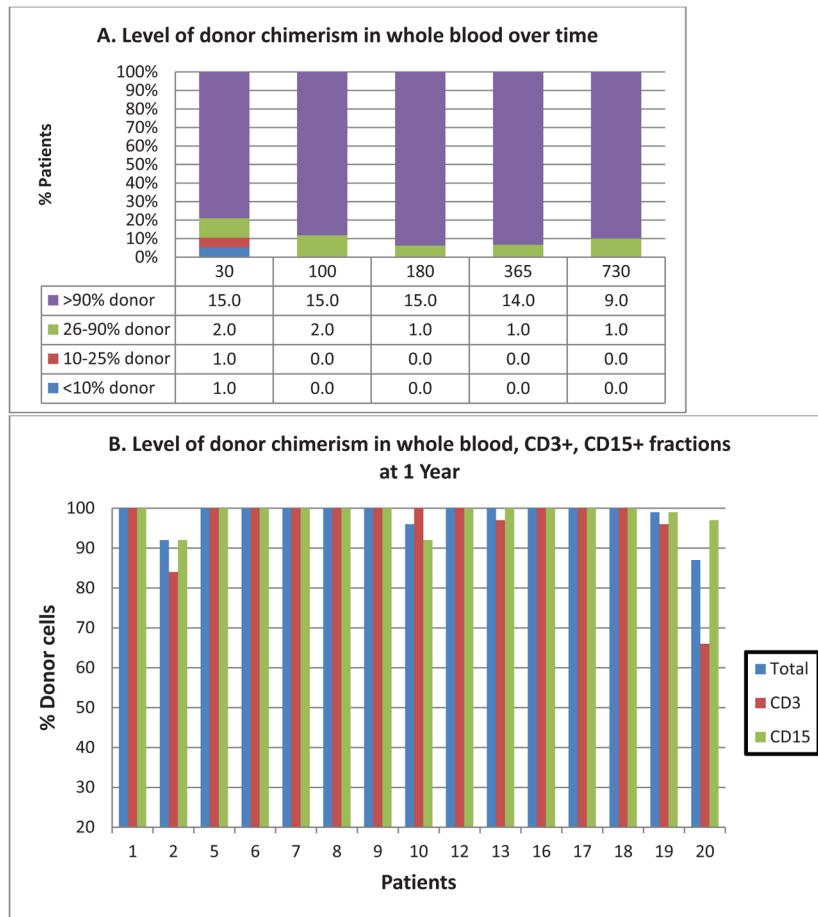
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**Figure 1. ENGRAFTMENT**

- (a) Cumulative incidence of neutrophil engraftment > 500 cells/mm<sup>3</sup> with donor cells by day 42
- (b) Cumulative incidence of platelet engraftment > 50,000/mm<sup>3</sup> by day 180 **GVHD**
- (c) Cumulative incidence of acute GVHD, grades II–IV (solid line) and III–IV (dotted line)
- (d) Cumulative incidence of chronic GVHD, overall (solid line) and extensive(dotted line)

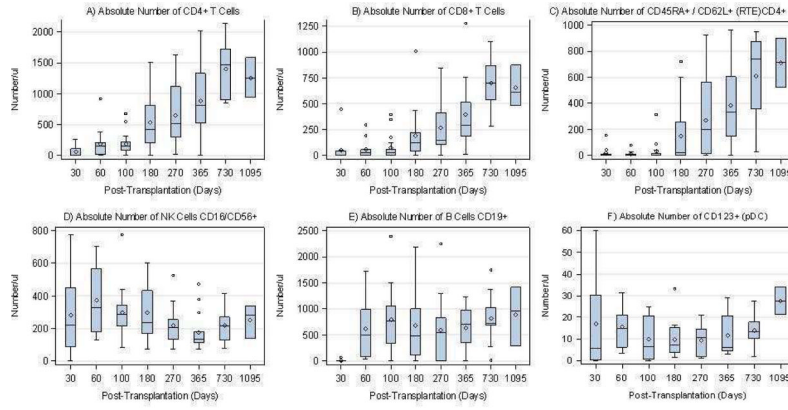




**Figure 2. DONOR CHIMERISM**

(a) Level of whole blood donor chimerism over time; X-axis = Days post-transplant; Y axis = % of evaluable patients

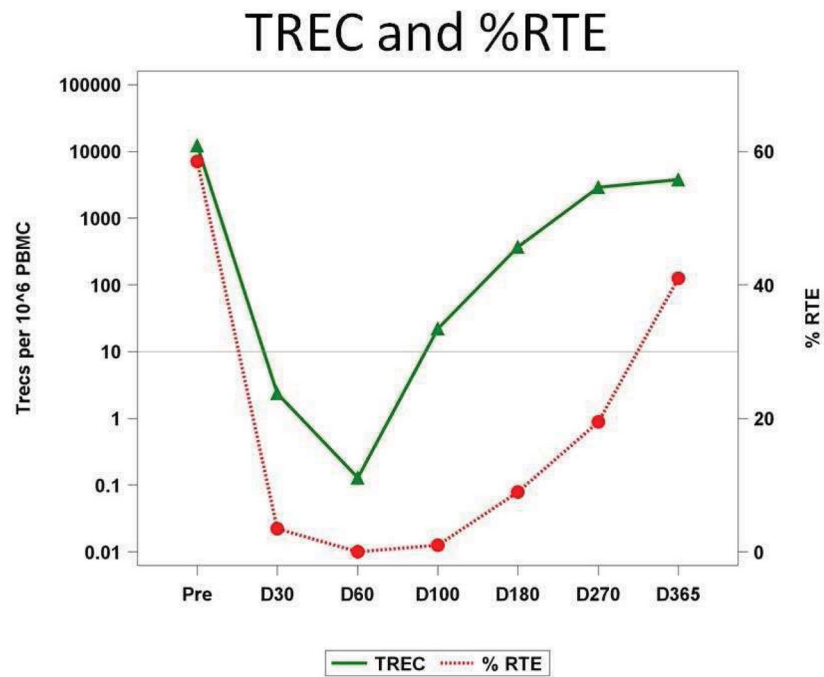
(b) Level of donor chimerism in whole blood, CD3+ & CD15+ fractions at 1 year (N=15)



**Figure 3. IMMUNE RECONSTITUTION**

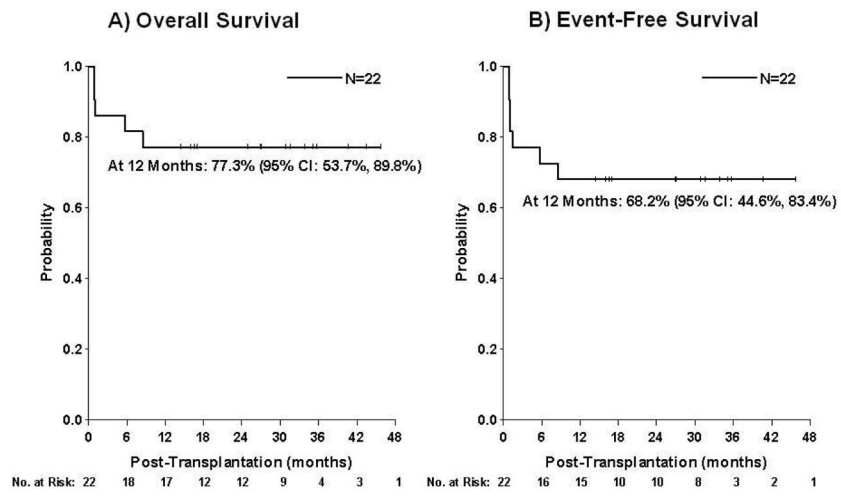
Absolute numbers of lymphocyte subsets: CD4+, CD8+, CD16,56+ (NK), CD19+, CD45RA+/62L+/CD4+ (RTE), CD123+(pDC)

Box plots are shown serially over time for above immune parameters. The diamond in the box represents the mean, while the horizontal line in the box represents the median. The lower box represents the 25<sup>th</sup> percentile and the upper box represents the 75<sup>th</sup> percentile. Upper and lower whiskers represent +/- 1.5 times the interquartile range



**Figure 4. Thymopoiesis (TREC and RTE% reconstitution)**

TREC = T-cell receptor excision circles; RTE = recent thymic emigrants (CD45RA+/CD62L+/CD4+); Median TREC (filled triangle) and %RTE (filled circle) are connected serially over time



**Figure 5. SURVIVAL**  
Kaplan-Meier estimate of (a) Overall survival (b) Event-free survival

Table 1

## Baseline patient and graft characteristics

UPIN	Diagnosis	Age (yrs)	Sex	Minority (Y/N)	Pre-transplant Co-morbidities	Wt (kg) on day 0	HLA Match 6 allele A, B, DRB1	HLA Match 8 allele Hi Res A, B, C, DRB1	CMV Pt	TNCC ( $\times 10^7/\text{kg}$ reinfused)	CD34 <sup>+</sup> ( $\times 10^5/\text{kg}$ reinfused)
1	Hunter	2.03	M	Y	Cardiomyopathy, obstructive sleep apnea	25.3	4/6	6/8	Neg	7.91	3.70
2	Sanfilippo B Syndrome	3.35	M	N		15.1	6/6	8/8	Neg	5.18	3.78
3	Sanfilippo B Syndrome	3.32	F	N		15.8	5/6	6/8	Neg	4.14	0.87
4	Cartilage Hair Hypoplasia	1.30	M	N	CMV viremia, transfusion dependent anemia, chronic neutropenia	8.2	6/6	7/8	Pos	13.90	4.31
5	MLD	6.76	M	N		20.4	5/6	6/8	Neg	4.58	2.43
6	Zap 70 deficiency	3.32	F	Y	Resp: MAI, Pseudomonas, Parainfluenza 1, Candida, bronchiectasis; Enteroviral meningitis, hydrocephalus, VP shunt	11.2	5/6	5/8	Neg	6.60	2.6
7	HLH	0.45	F	N		7.9	5/6	5/8	Neg	15.3	3.67
8	HLH	0.58	F	Y	Adenoviremia, adenovirus in stool and urine	6.20	4/6	4/8	Neg	11.80	2.48
9	$\beta$ thalassemia major	1.56	M	Y		9.5	5/6	6/8	Pos	8.17	5.10
10	CID	1.14	F	N	ITP, HHV-6 pneumonitis, Enterovirus encephalitis, colitis	10.1	5/6	6/8	Neg	7.80	2.73
11	Krabbe	3.09	F	Y	Blindness	12.2	4/6	5/8	Neg	14.30	4.58
12	Krabbe	3.25	F	N	Ataxia	19.1	4/6	4/8	Neg	7.36	1.55
13	DBA	2.56	M	N	Iron overload	15.9	5/6	7/8	Neg	6.22	1.50
14	Hurler	1.71	F	Y	Obstructive sleep apnea. Stool adenovirus +, h/o Parainfluenza 3 infection	11.8	4/6	4/8	Neg	10.8	4.86
15	ALD	8.02	M	Y		26.9	4/6	3/8	Neg	6.99	3.7
16	$\beta$ Thalassemia major	3.65	M	Y		14.2	4/6	5/8	Neg	7.07	1.63
17	$\beta$ thalassemia major	5.44	F	Y	Prior myeloablative transplant; h/o severe hemorrhagic cystitis	18	5/6	6/8	Pos	11	3.5
18	Omnem syndrome	0.45	M	Y		5.26	5/6	6/8	Pos	22	7.72
19	$\beta$ Thalassemia major	2.58	M	Y		13.2	6/6	6/8	Neg	5.04	4.58
20	CD40L deficiency	3.38	M	N		14.1	5/6	7/8	Neg	9.46	3
21	Omnem syndrome	0.31	M	N	Erythroderma, FTT, CMV	6	6/6	8/8	Pos	15.8	2.4
22	PNP deficiency	4.27	M	N	FTT, food intolerance, chronic enteritis, CMV urine, adenovirus in stool	16.6	5/6	5/8	Pos	5.58	1.3

ALD = adrenoleukodystrophy; CID = combined immunodeficiency; CMV = cytomegalovirus; DBA = Diamond Blackfan anemia; F = female; FTT = failure to thrive; HHV-6 = human herpes virus 6; Hi res = high resolution; HLH = Hemophagocytic lymphohistiocytosis; h/o = history of; ITP = immune thrombocytopenic purpura; kg = kilograms; M = male; MAI = Mycobacterium avium; MLD = metachromatic leukodystrophy; Neg = negative; PNP = purine nucleoside phosphorylase; Pos = positive; Pt = patient; UPIN = patient number; VP = ventriculoperitoneal; Wt = weight; Y/N = Yes/No; yrs = years; ZAP 70 = zeta chain associated protein kinase 70;

**Inherited metabolic disorders (IMD)** = Hunter, Hurler, Krabbe, Sanfilippo B, Metachromatic leukodystrophy (MLD), adrenoleukodystrophy (ALD);

**Primary immunodeficiency diseases (PID)** = Cartilage Hair Hypoplasia, Zap 70 deficiency, HLH, CID, Omenn syndrome, CD40Ligand deficiency, PNP deficiency)

**Table 2**

## Causes of death

UPIN	Day post-transplant	Diagnosis	Cause of death
3	170	Sanfilippo B	Hemolytic transfusion reaction
11	25	Krabbe	Disseminated adenovirus
14	24	Hurler	Adenoviremia, Parainfluenza 3
21	27	Omenn syndrome	CMV pneumonia
22	258	PNP deficiency	Acute GVHD, adenoviremia, CMV

CMV = cytomegalovirus; GVHD = graft-versus-host disease; PNP = purine nucleoside phosphorylase; UPIN = patient number

Table 3

Treatment and outcomes of EBV, CMV, Adenovirus infections

UPN	Infection	Treatment	Response	F/U (mo)	Outcome	Day died	Cause of death
1	EBV	Rituximab	Resolved	52.9			
2				45.5			
3	Adenoviremia	Cidofovir CTL	Resolved		Death	170	Hemolytic transfusion reaction
4	CMV viremia	Foscarnet, GCV	Resolved	AR			
5				40.4			
6				35.4			
7				34.8			
8	Adenoviremia	CMX001	Resolved	33.6			
9	CMV viremia	Foscarnet, GCV	Resolved	31.3			
10				30.9			
11	Adenoviremia	Cidofovir CMX001	Death	GF	Death	25	Disseminated adenoviral infection
12				26.6			
13	Adenovirus	Cidofovir	Resolved	26.4			
14	Adenovirus	Cidofovir CTL	Death		Death	24	Adenoviremia, Parainfluenza 3
15				AR			
16	EBV	Rituximab	Resolved	16.4			
17	Adenoviremia	CMX001	Ongoing treatment due to chronic GVHD	15.9			
18				15.5			
19				15.3			
20	Adenoviremia	Cidofovir		14.3			
21	CMV viremia	Foscarnet, GCV	Death		Death	27	CMV pneumonia
22	Adenoviremia CMV	Cidofovir CMX001	Death		Death	258	Acute GVHD, adenoviremia, CMV

F/U = follow-up; CTL = Trivirus (adenovirus, CMV, EBV) specific cytotoxic T lymphocytes



**Table 4**

Donor Chimerism(%)

UPN	Day 30			Day 100			Day 180			1 year			2 year		
	T	L	M	T	L	M	T	L	M	T	L	M	T	L	M
1	>98	95	>98	>98	96	98	>98	98	>98	>98	>98	>98	>98	>98	>98
2	>98	>98	>98	95	78	90	92	72	89	92	84	92	88	86	86
3	10	88	10	63	91	58	<b>Died day 170</b>								
4	0	0	0	<b>Autologous recovery day 30</b>											
5	>98	>98	>98	>98	86	>98	>98	86	>98	>98	>98	>98	>98	>98	>98
6	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
7	>98	>98	>98	>98	90	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
8	>98	>98	>98	>98	83	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
9	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
10	>98	n/a	n/a	94	86	92	>98	>98	96	>98	>98	92	91	>98	92
11	Engrafted day 13, died day 25														
12	>98	>98	>98	>98	>98	>98	>98	98	>98	>98	>98	>98	>98	>98	>98
13	>98	>98	>98	>98	>98	>98	>98	>98	>98	97	>98	>98	>98	>98	>98
14	Graft failure, died day 24														
15	91*	8*	99*	<b>Autologous recovery day 41</b>											
16	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
17	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
18	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
19	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98	>98
20	87	71	41	87	55	61	88	58	96	87	66	97			
21	Engrafted day 13, died day 27														
22	83	>98	68	>98	>98	>98	98	>98	97	<b>Died day 258</b>					

\* = 0% on day 41, thus considered autologous recovery

T = whole blood; L = CD3+ fraction; M = CD15+ fraction; donor chimerism %

**Table 5**

Details of acute and chronic GVHD

UPN	Match Conventional A, B, C, DRB1	Match 8 allele Hi Res A, B, C, DRB1	AGVHD Max Skin stage	AGVHD Max Gut stage	AGVHD Max Liver stage	Acute GvHD Consensus	Chronic GvHD	eGVHD Site	AIHA	Rituximab	MSC	Notes on outcome
1	4/6	6/8	2	0	0	1	No		Yes	Yes (AIHA, EBV)		Resolved Off IS
2	6/6	8/8	1	0	0	1	No					Resolved Off IS
3	5/6	6/8	1	0	0	1	No					Resolved Died
4	6/6	7/8	NE	NE	NE	NE	NE					
5	5/6	6/8	0	0	0	0	No					Off IS
6	5/6	5/8	3	0	0	2	Extensive	Skin	Yes	Yes (AIHA)		Ongoing
7	5/6	5/8	0	0	0	0	Limited	Skin				Resolved Off IS
8	4/6	4/8	0	0	0	0	Limited	Skin				Resolved Off IS
9	5/6	6/8	1	0	0	1	Limited	Skin				Resolved Off IS
10	5/6	6/8	0	1	0	2	Limited	Skin				Resolved Weaning IS
11	4/6	5/8	0	0	0	0	NE					Died
12	4/6	4/8	0	0	0	0	Limited	Skin				Resolved Off IS
13	5/6	7/8	2	0	0	1	Limited	Skin				Resolved Off IS
14	4/6	4/8	NE	NE	NE	NE	NE					Died
15	4/6	3/8	NE	NE	NE	NE	NE					
16	4/6	5/8	1	3	0	3	No			Yes (EBV)		Resolved Weaning IS
17	5/6	6/8	3	3	0	3	Extensive	Skin, gut			Yes	Ongoing
18	5/6	6/8	1	0	0	1	Limited	Skin				Resolved Off IS
19	6/6	6/8	3	0	0	2	No		Yes	Yes (AIHA)		Resolved Weaning IS
20	5/6	7/8	0	0	0	0	Limited	Skin				Resolved Weaning IS
21	6/6	8/8	0	0	0	0	NE					Died
22	5/6	5/8	3	4	0	3	No				Yes	Died

MSC = Mesenchymal stem cells; NE = not evaluable; AIHA = Autoimmune hemolytic anemia; IS = immune suppression