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Recipient Pretransplant Inosine Monophosphate Dehydrogenase Activity in Nonmyeloablative Hematopoietic Cell Transplantation

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Mycophenolic acid, the active metabolite of mycophenolate mofetil (MMF), inhibits inosine monophosphate dehydrogenase (IMPDH) activity. IMPDH is the rate-limiting enzyme involved in de novo synthesis of guanosine nucleotides and catalyzes the oxidation of inosine 5′-monophosphate to xanthosine 5′-monophosphate (XMP). We developed a highly sensitive liquid chromatography–mass spectrometry method to quantitate XMP concentrations in peripheral blood mononuclear cells (PMNCs) isolated from the recipient pretransplant and used this method to determine IMPDH activity in 86 nonmyeloablative allogeneic hematopoietic cell transplantation (HCT) patients. The incubation procedure and analytical method yielded acceptable within-sample and within-individual variability. Considerable between-individual variability was observed (12.2-fold). Low recipient pretransplant IMPDH activity was associated with increased day +28 donor T cell chimerism, more acute graft-versus-host disease (GVHD), lower neutrophil nadirs, and more cytomegalovirus reactivation but not with chronic GVHD, relapse, nonrelapse mortality, or overall mortality. We conclude that quantitation of the recipient's pretransplant IMPDH activity in PMNC lysate could provide a useful biomarker to evaluate a recipient's sensitivity to MMF. Further trials should be conducted to confirm our findings and to optimize postgrafting immunosuppression in nonmyeloablative HCT recipients.

INTRODUCTION

Postgrafting immunosuppression for allogeneic hematopoietic cell transplantation (HCT) recipients often consists of the combination of a calcineurin inhibitor and mycophenolate mofetil (MMF) [1,2]. The development of lower dose, nonmyeloablative conditioning increased the availability of this potentially curative procedure to patients who could not tolerate the toxicity of high-dose conditioning regimens due to age or comorbidity [3]. Nonmyeloablative HCT relies on achieving a delicate balance between recipient and donor cells, with the goal of ensuring sufficient immunosuppression of the recipient to maximize graft-versus-tumor effect but minimize toxicity.

After nonmyeloablative HCT, the recipient experiences at least a short-term mixed chimerism state in which the recipient and donor hematological cells coexist in the blood of the recipient. The level and rate of change in donor T cell chimerism have been correlated with several clinical outcomes such as graft rejection, graft-versus-host disease (GVHD), disease relapse/progression (ie, graft-versus-tumor effect), and progression-free survival [4,5]. The observed associations between donor T cell chimerism and subsequent clinical responses could, in part, reflect differences in each recipient's sensitivity to MMF. Early studies in nonmyeloablative HCT recipients administered MMF every 12 hours, regardless of donor type. Although recipients of related donor grafts had acceptable engraftment rates with MMF every 12 hours, patients receiving unrelated donor grafts had persistent problems with graft rejection. Engraftment

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Table 1
Participant Characteristics

	Donor Type		
	Related	Unrelated	All Participants
Total number	22	64	86
Sex, female/male (% female)	9/13 (41)	23/41 (36)	32/54 (37)
HCT-CI			
0	1 (5)	8 (13)	9 (10)
1-2	3 (14)	14 (22)	17 (20)
3-4	10 (45)	23 (36)	33 (38)
≥5	8 (36)	19 (30)	27 (31)
Median recipient age, yr (range)	55 (20-69)	62 (27-75)	62 (20-75)
CMV-seropositive recipients	10 (45)	35 (56)	45 (53)
Kahl disease risk [27]			
Low	4 (18)	22 (34)	26 (30)
Standard	14 (64)	26 (41)	40 (47)
High	4 (18)	16 (25)	20 (23)
Female donor to male recipient	8 (36)	17 (27)	25 (29)
Median donor age, yr (range)	55 (23-73)	31 (20-58)	35 (20-73)
HLA-mismatched graft	1 (5)	2 (3)	3 (4)
Conditioning regimen			
2 Gy TBI + FLU ± auto	9 (41)	25 (39)	34 (40)
2 Gy TBI + FLU + rituximab* ± auto	11 (50)	20 (31)	31 (36)
3 Gy TBI + FLU ± rituximab*	2 (9)	12 (19)	14 (16)
4-4.5 Gy TBI + FLU	0	7 (11)	7 (8)
Postgrafting immunosuppression			
MMF every 8 h	1 (5)	64 (100)	65 (76)
MMF every 12 h	21 (95)	0	21 (25)
Cyclosporine + MMF ± sirolimus†	12 (55)	49 (77)	62 (72)
Tacrolimus + MMF ± sirolimus‡	10 (45)	15 (23)	24 (28)

HCT-CI indicates HCT comorbidity index; FLU, fludarabine monophosphate; auto, autologous transplant.
Values are number of cases, with percents in parentheses, unless otherwise indicated.
* Rituximab given on days −3, +10, +24, and +38 relative to transplant.
† Ten participants received cyclosporine + sirolimus, 1 with a matched donor and 9 with unrelated donors.
‡ Five participants received tacrolimus + sirolimus, all with unrelated donors.

rates in these patients improved when the daily MMF dose was increased by shortening the administration interval to every 8 hours [1,6]. Because reliable engraftment was achieved, efforts have been ongoing to separate the graft-versus-tumor effect from GVHD. Examples of such efforts include examining the association of day +28 T cell chimerism or neutrophil nadirs within the first 3 weeks post-HCT with relapse rates (ie, graft-versus-tumor effect) and GVHD [4,5,7].

The active metabolite of MMF, mycophenolic acid (MPA), is a selective, reversible, and noncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH). IMPDH is the rate-limiting enzyme involved in de novo synthesis of guanosine nucleotides; IMPDH catalyzes the oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) by a nicotinamide adenine dinucleotide $(NAD)^+$-dependent pathway [8]. In renal transplantation patients, high recipient IMPDH activity is associated with rejection [9]. To date, no studies have evaluated the association of clinical outcomes in HCT participants with recipient pretransplant IMPDH activity, which is determined before allograft infusion and MMF administration. Characterizing the relationship between recipient pretransplant IMPDH activity and clinical outcomes is critical to understanding the potential benefit of alternative postgrafting immunosuppression or MMF dosing strategies to improve outcomes in HCT recipients.

Even with nonmyeloablative HCT, however, the conditioning regimen administered before the donor graft infusion suppresses the bone marrow and thus decreases the number of peripheral blood mononuclear cells (PMNCs) available to determine IMPDH activity. Various nonradioactive methods using chromatographic separations have been used to quantify XMP, the catalytic product of the enzyme, to indirectly evaluate IMPDH activity. Only recently were mass spectrometry–based detection methods, which provide more specificity and sensitivity, reported for XMP quantitation [8]. Here we report a liquid chromatography–mass spectrometry (LC-MS) method to measure recipient pretransplant IMPDH activity in PMNCs (ie, ex vivo) based on the quantification of XMP formation normalized by cell count. We evaluated and validated this method in PMNC lysates from healthy volunteers and nonmyeloablative HCT recipients. We also evaluated factors associated with recipient pretransplant IMPDH activity and the association of recipient pretransplant IMPDH activity with clinical outcomes in nonmyeloablative HCT recipients.

METHODS

Participant Characteristics

From November 2008 to February 2012, 105 patients participated in a prospective ancillary biomarker study in nonmyeloablative HCT recipients who received either a related or unrelated donor graft. Study participation did not influence the HCT procedure, including the conditioning regimen or postgrafting immunosuppression. Participants receiving fludarabine monophosphate (fludarabine) and total body irradiation (TBI) conditioning with postgrafting immuosuppression of a calcineurin inhibitor (cyclosporine or tacrolimus) with MMF were eligible to participate. This protocol was approved by the Institutional Review Board at the Fred Hutchinson Cancer Research Center (Protocol 1980, Clinicaltrials.gov identifier NCT00764829). Written informed consent was obtained from all participants before study procedures. Participant characteristics are summarized in Table 1.

The conditioning regimen (summarized in Supplemental Figure 1) comprised fludarabine (30 mg/m^2/day i.v.) from day −4 to day −2 (cumulative dose 90 mg/m^2) followed by a single fraction of 2 to 4.5 Gy TBI on day 0 [1]. In general, the postgrafting calcineurin inhibitor was either cyclosporine or tacrolimus given through day +177, although some participants also received sirolimus as part of postgrafting immunosuppression. MMF, at a dose of 15 mg/kg, was given at 2 different dose frequencies, either 3 times a day (every 8 hours) to unrelated graft recipients or twice a day (every 12 hours) to related graft recipients. Adjusted ideal body weight [10] was used to determine MMF dosing, and all doses were rounded to the nearest

250 mg. MMF doses were not adjusted based on recipient pretransplant IMPDH activity. Participants were asked to take MMF at the same time daily. MMF treatment started on day 0 and, in general, continued until day +27 (related donor) or day +40 (unrelated donor) at which time the MMF dose was reduced by 10% per week in the absence of GVHD.

Most donor grafts were matched for HLA A, B, C, and DRB1 at high resolution DNA typing and DQB1 by intermediate-resolution techniques, with the following exceptions: 1 related and 2 unrelated donor grafts with an antigen mismatch and 9 unrelated donor grafts with an allelic mismatch. The median follow-up among participants at the time of last contact was 1.9 years (range, .6 to 3.8 years).

Sample Collection

Recipient pretransplant IMPDH activity is the IMPDH activity in recipients' PMNCs obtained on day −4 before graft infusion and before MMF administration. Three peripheral blood samples (5 mL in vacutainers containing EDTA) were obtained to determine each recipient's IMPDH activity and also to characterize the within-individual variability in IMPDH activity. No MMF was administered on this day. These IMPDH samples were obtained concurrently with pharmacokinetic samples for 2-fluoro-ara-A (Supplemental Figure 1) after administration of the first fludarabine dose. In 4 participants, these samples were collected at 5 minutes after the end of the infusion, 90 minutes after the start of the infusion, and 6.5 hours after the start of the infusion [11]. Sample collection occurred in the ambulatory clinic, which necessitated a sampling schedule that would provide acceptably low withdrawal rates. The initial participant withdrawal rates were too high, with participants stating that remaining at the clinic for the 6.5-hour sample was a barrier to their continued participation. Thus, to lower participant withdrawal rates, the sampling schedule was revised to collect samples at the end of fludarabine infusion, 5 minutes after the end of infusion, and 90 minutes after the start of the infusion [11]. Most participants (77/86 [90%]) had 3 samples collected, demonstrating the success of this revised sampling schedule. The number of samples collected in the remaining participants was 4 samples in 1 participant, 2 samples in 8 participants, and 1 sample in 1 participant.

Chemicals and Reagents

Acetonitrile, ammonium acetate, methanol, sodium hydroxide, sodium phosphate monobasic, ammonium hydroxide, and potassium chloride were all purchased from Thermo Fisher (Waltham, MA). IMP, XMP, adenosine monophosphate (AMP), 8-bromo-adenosine 5′-monophosphate (BMP, internal standard), and NAD were obtained from Sigma (St. Louis, MO). Dulbecco's PBS was purchased from Invitrogen (Grand Island, NY). Ficoll Hypaque solution (density 1.077 g/mL) was obtained from GE Healthcare (Uppsala, Sweden). All chemicals were of reagent grade or better.

Isolation of Human PMNCs

Blood samples were stored at 4°C until processing. PMNCs were isolated within 6 hours of collection and were isolated by first diluting blood in PBS at a 1:1 v/v ratio and subsequently layering atop Ficoll. The height ratio of Ficoll to diluted whole blood sample was 3:4. This suspension was then centrifuged at 298 *g* for 30 minutes at 22°C. PMNCs were collected from the interface and diluted to a volume of 10 mL with PBS as a wash and centrifuged at 405 *g* for 15 minutes at 22°C. To facilitate cell counting and limit the variability in cell concentration, all but 1.1 mL of the supernatant was removed. The PMNC pellet was resuspended in the remaining 1.1 mL of PBS, and 1.0 mL of the PBS-cell slurry was transferred to a 2-mL tube. WBC counts in this sample were quantitated using an ABX Micro 60 (requires <10 μL; Horiba Diagnostics, Irving, CA). After cell quantitation, the sample was centrifuged at 325 *g* for 10 minutes at room temperature. From the cell pellet, 920 μL of the supernatant was removed; distilled water was added to adjust the cell concentration to $.5 \times 10^6$ cells/mL lysate. The cells were subsequently stored at −80°C until incubation. After thawing, insoluble fragments of disrupted cells were removed by centrifugation at 16,000 *g* for 2 minutes. The supernatant was kept at 4°C (or on wet ice) until it was used for the IMPDH activity assay.

Quantification of IMPDH1 and IMPDH2 Messenger RNA in PMNCs

Total RNA was extracted from cells from 15 participants using the MagMax-96 Total RNA Isolation Kit obtained from Life Technologies (Carlsbad, CA). The RNA yield was not determined from PMNCs because of low cell numbers. To concentrate the amount of total RNA isolated, RNA was precipitated using 5 M ammonium acetate followed by the addition of 5 mg/mL glycogen and 2 times the volume of ethanol. The tubes were then placed in a −20°C freezer for at least 25 minutes and then centrifuged at 15,000 *g* for 15 minutes. The supernatant was carefully removed, and the pellet was air-dried and resuspended in elution buffer. The generation of cDNA was performed using SuperScript III First-Strand Synthesis System (Life Technologies). Gene-specific TaqMan Gene Expression Assays (Life Technologies) were used to quantitate relative expression of the two *IMPDH* gene isoforms, *IMPDH1* (Hs00992210_m1) and *IMPDH2* (Hs00168418_m1), relative to the internal endogenous control β-glucuronidase (4333767F) on a StepOnePlus Real-Time PCR System (Life Technologies). Samples were analyzed in triplicate using .8 μL cDNA in a 10-μL reaction containing a TaqMan Gene Expression Assay primer/probe set and TaqMan Gene Expression Master Mix (Life Technologies). The methods for data analysis were performed as described previously [12].

PMNC Incubation

IMPDH activity in PMNCs was determined from the conversion of IMP to XMP according to a procedure adapted from Glander et al. [13] and Daxecker et al. [14]. For each incubation, a fresh reaction mixture was prepared from stock solutions: .8 mL IMP (6.0 mmol/L, stored at −20°C), .8 mL NAD (4.5 mmol/L, made fresh each day), 1.6 mL NaH_2PO_4 (120 mmol/L stored at 4°C), and 1.6 mL KCl (300 mmol/L, stored at 4°C); the pH was adjusted to 7.4 using 1 M NaOH, and the total volume was brought to 5.2 mL with deionized water. From this 5.2 mL of reaction mixture, 130 μL was used for each incubate. The enzymatic reaction was started via the addition of 130 μL of reaction mixture to 50 μL prewarmed (5 minutes) PMNC lysate (standard concentration of $.5 \times 10^6$ cells/mL). After 2.5 hours at 37°C, the enzymatic reaction was terminated by the addition of 1250 μL methanol. Internal standard (20 μL of 130 pmol/μL BMP in deionized water) was then added to each incubate, and then the mixture was centrifuged at 16,000 *g* for 10 minutes at 37°C. The supernatant was then transferred to 12 × 75 disposable culture tubes and evaporated to dryness under a stream of air at 37°C. The residue was then dissolved in 75 μL of deionized water and 5 μL was injected on the LC-MS.

A quality control (QC) lysate was prepared by pooling PMNCs from 5 healthy volunteers. This lysate was run in triplicate with every incubation, with the QCs for the incubation procedure, which were no substrate (IMP), no NAD, and no PMNC lysate.

LC-MS Quantitation of XMP in PMNCs

The HPLC separation was performed on an HPLC/MS series 1100 system equipped with a thermostatically controlled autosampler (Agilent Technologies, Santa Clara, CA). Agilent ChemStation (version B.01.03) was used for instrument control. Separation was achieved using a Thermo Scientific Hypercarb column (2.0 mm × 100 mm × 5 μm, part no. 35005-102130; Thermo Scientific, Bellefonte, PA). The mobile phase consisted of acetonitrile and .1 M ammonium acetate adjusted to pH 8.5 with ammonium hydroxide. A gradient system was used starting at 5% acetonitrile for .5 minutes, increasing to 30% at 4 minutes, held at 30% until 5 minutes, and then returning to 5% at 5.1 minutes. The total run time was 10 minutes. The injector was maintained at 4°C. The injection volume was 5 μL. The column thermostat was set to 30.0°C, and the solvent flow was maintained at .3 mL/min. Within an incubate, the typical retention times were 4.3 minutes for XMP, 5.6 minutes for AMP, 5.1 minutes for IMP, 7.0 minutes for BMP, and 7.5 minutes for NAD (Figure 1). Of note, the NAD was present in much higher concentrations than the analytes of interest and did not interfere with IMP, XMP, or AMP (Figure 1). Figure 2 shows the chromatograms from our QC lysate, with the various QC incubations of no substrate (no IMP, Figure 2A), no NAD (Figure 2B), no PMNC lysate (Figure 2C), and an incubation with all components (Figure 2D).

An Agilent G1946D mass selective detector (MSD) (Agilent Technologies) atmospheric pressure ionization-electrospray in positive ion mode was used. The temperature of the drying gas (nitrogen) was maintained at 350°C at a flow of 11 L/min. The nebulizing pressure was 35 psi, the capillary voltage was 2400 V, and the fragmentor voltage was 100 V. The MSD was run in the selective ion monitoring mode. Monitored ions included *m/z* 365 for the $(M+H^+)$ ion of XMP, *m/z* 348 for the $(M+H^+)$ ion of AMP, and *m/z* 426 for the $(M+H^+)$ ion of BMP, the internal standard. The MSD conditions for quantification were as described above; the fragmentor and capillary voltage were optimized under analytical conditions with Chemstation FIA software (Agilent Technologies).

An 8-point calibration curve was prepared by spiking reaction mixture and deionized water with both XMP and AMP. The dynamic range of the calibration curve for XMP was 0 to 2318 pmol and for AMP, 0 to 4748 pmol. The calibration curves were processed identically to the incubation samples. The respective relationship between the peak heights of XMP and BMP and between AMP and BMP and their respective concentrations were analyzed by second-order polynomial regression. The correlation coefficient (R^2) was used to evaluate the linearity of the calibration curves and in all experiments was >.9950. The limits of quantitation (signal-to-noise ratio > 60 and coefficient of variation [CV] < 2%) were 58 pmol for XMP and 102.5 pmol for AMP.

Method Validation

Long-term stability of analytes was determined by analyzing in triplicate QC lysates that were stored at −80°C for 27 months. The percent difference

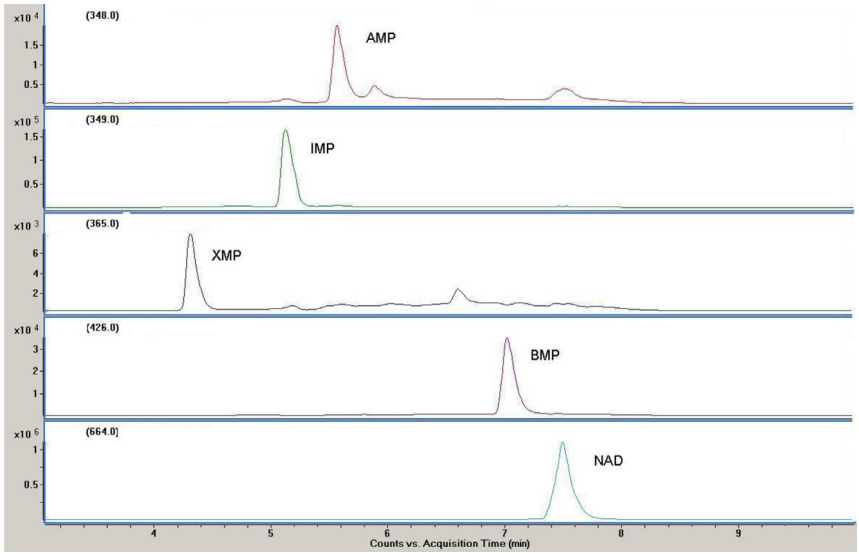

Figure 1. Chromatogram showing separation of analytes (.0066 mM AMP, .66 mM IMP, .0032 mM XMP, .0138 mM BMP, and .5 mM NAD) in a QC incubate (ie, after incubation with a QC lysate).

was −6.5% for XMP and −13.6% for AMP. To evaluate freeze–thaw stability, QC lysates were subjected to 4 cycles of freezing at −80°C and thawing at room temperature. We found a decrease of 33.6% for XMP and a decrease of 30.8% for AMP. Therefore, all samples analyzed were processed without any freeze–thaw cycles.

The intraday and interday precision were assessed based on the CV. The intraday precision was calculated using 12 replicates of the QC lysate on 6 different days. The intraday precision was 3.86% for XMP and 3.18% for AMP. The interday precision was calculated over 40 separate incubations of 3 replicates of the QC lysates per incubation. The interday precision for the incubation was 6.20% for XMP and 5.85% for AMP. The IMPDH activity was determined with the following equation:

$$\text{IMPDH activity} = \frac{\text{produced XMP} \times 10^6}{\text{incubation time} \times \text{measured cell count}}$$

XMP is expressed in pmol/L, incubation time in hours, measured cell count in cells, and IMPDH activity in pmol/10^6 cells/h. XMP formation was also normalized by AMP formation using the following equation:

$$\text{IMPDH activity} = \frac{\text{produced XMP/incubation time}}{\text{produced AMP}}$$

In this equation, XMP is expressed in pmol/L, incubation time in hours, and AMP in pmol/L.

For those samples with more than 1 replicate, the CV for each sample was evaluated by dividing a PMNC lysate into 2 or 3 aliquots and conducting separate incubations and LC-MS quantitation. Thus, the within-sample CV (ie, replicate variability within 1 sample) characterizes the variability of the incubation procedure and LC-MS assay. For this within-sample CV, the median of the CV was 1.94% (range, .07% to 19.2%) when normalized by cell count (ie, pmol/10^6 cells/h) and 1.45% (range, .08% to 44%) when normalized by AMP (ie, pmol XMP/h/pmol AMP). Within-sample CV for activity normalized by AMP could not be determined for 2 samples because of unmeasurable AMP concentration. For samples with more than 1 replicate, the lowest IMPDH activity divided by the highest IMPDH activity (ie, minimum/maximum) ranged from 72% to 100%, with a median of 96%. The average (± standard deviation) of IMPDH activity was 840 ± 337 pmol/10^6 cells/h for XMP/cells and .34 ± .14 pmol XMP/h/pmol AMP. Because the precision was similar between these 2 normalization methods, we chose to normalize all reported data by cell count because it is more intuitive for clinicians and facilitates communication of pretransplant recipient IMPDH as a predictive biomarker.

Clinical Outcomes

Clinical outcomes of interest were toxicity to MMF (ie, neutropenia, cytomegalovirus [CMV] reactivation), efficacy of MMF (ie, day +28 donor T cell chimerism, acute and chronic GVHD), and overall HCT outcomes (ie, relapse, nonrelapse mortality, and overall survival). Neutropenia post-HCT was assessed only through day +28, because multiple potential confounding variables (eg, viral infection or reactivation, corticosteroid therapy) could affect the neutrophil count after day +28. Neutropenia was evaluated by examination of complete blood counts with differential and assessment of absolute neutrophil count (ANC). CMV reactivation was also evaluated, because it represents a significant consequence of immunosuppressed status; CMV serological status was assessed in each participant and donor before HCT. All participants underwent weekly testing to detect the CMV pp65 antigen for the first 3 months after HCT.

On days +28, +56, and +84 after HCT, or as clinically indicated, all participants' peripheral blood samples were assessed for the percentage of donor $CD3^+$ T cells present. Flow cytometry was used to sort $CD3^+$ cells, and chimerism was measured using PCR of polymorphic microsatellite regions [15]. If donor $CD3^+$ cells were less than or equal to 5% at any of the assessed time points after HCT, then the participant was noted to have graft rejection.

Acute and chronic GVHD were graded according to established criteria [16-18]. It is recognized that gastrointestinal GVHD and gastrointestinal MMF toxicity have some similarities; however, studies have identified differences between these conditions that can be used in clinical diagnosis [19-26]. The gastroenterologists and pathologists worked closely to stay current with the literature and to properly diagnosis gastrointestinal GVHD (Supplemental Table 1). Hematological diseases were classified as low, standard, or high risk of relapse per the Kahl criteria to evaluate relapse rate in a consistent manner [27]. We defined disease relapse or disease progression as disease recurrence after complete remission or progression of persistent disease.

Statistical Analysis

The association of various covariates with recipient pretransplant IMPDH activity was evaluated via ANOVA and regression-based approaches. Participant covariates assessed included sex, age at the time of HCT, diagnosis (categorized as high, intermediate, or low risk), donor graft (categorized as related or unrelated), HCT comorbidity index, and recipient pretransplant IMPDH messenger RNA (mRNA).

Recipient pretransplant IMPDH activity was treated as a fixed covariate. Cumulative incidence curves for acute GVHD were estimated using previously described methods [28]. Cox regression analysis was used to model the impact of recipient pretransplant IMPDH activity on time-to-event endpoints. Death and relapse were treated as competing risks for analysis of acute and chronic GVHD. Relapse was treated as a competing risk for the analysis of nonrelapse mortality. Logistic regression was used to evaluate the relationship between IMPDH activity and the post-transplant neutrophil nadir. The effects of recipient pretransplant IMPDH activity on hazard ratios (HRs) and odds ratios (ORs) were expressed as the effect per doubling of IMPDH activity. All reported *P* values are 2-sided, and those estimated from regression models are derived from the Wald test. No adjustments were made for multiple comparisons. Statistical analysis was performed using SAS version 9.3 (SAS Institute, Cary, NC). All statistical tests were 2-tailed with a statistical significance level of .05.

RESULTS

Participant Characteristics

Recipient pretransplant IMPDH activity was available from 86 of 105 participants. Of those 19 participants without

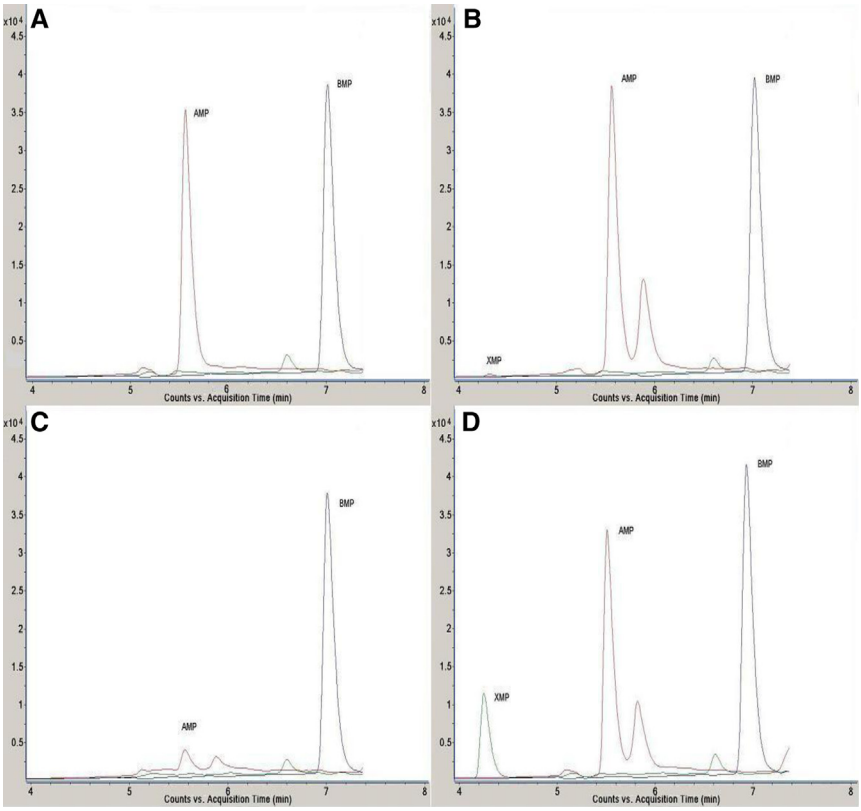

Figure 2. Chromatograms of XMP, AMP, and BMP for IMPDH activity measurement in PMNCs. (A-C) QC incubates included in each incubation procedure. (A) No substrate (ie, no IMP), (B) no NAD, and (C) no PMNC. (D) Results of an actual incubation.

IMPDH activity, samples from 15 were used for method development, and samples were not collected in the remaining 4 because of scheduling difficulties or patient withdrawal. The characteristics of the 86 participants with available recipient pretransplant IMPDH activity data are summarized in Table 1. The conditioning regimen and post-grafting immunosuppression were determined by the participant's HCT treatment protocol.

Recipient Pretransplant IMPDH Activity

We first documented acceptable variability of the analytical method to quantitate XMP and the incubation procedure to consistently provide accurate XMP formation (see Method Validation). We also evaluated the within-individual variability from samples drawn over a 1.5- to 6-hour time period. The within-individual variability was calculated by dividing the lowest IMPDH activity sample by the highest sample. Most participants (90%) had 3 samples collected, with 4 samples collected in 1 participant, 2 samples in 8 participants, and 1 sample in 1 participant (see Sample Collection). For the within-individual variability, these samples were within 46% to 98% of each other; the activities were within 90% of each other in 41 participants (48%) and 80% of each other in 71 participants (83%). For each participant, the recipient pretransplant IMPDH activity was calculated by averaging the XMP formation rate normalized

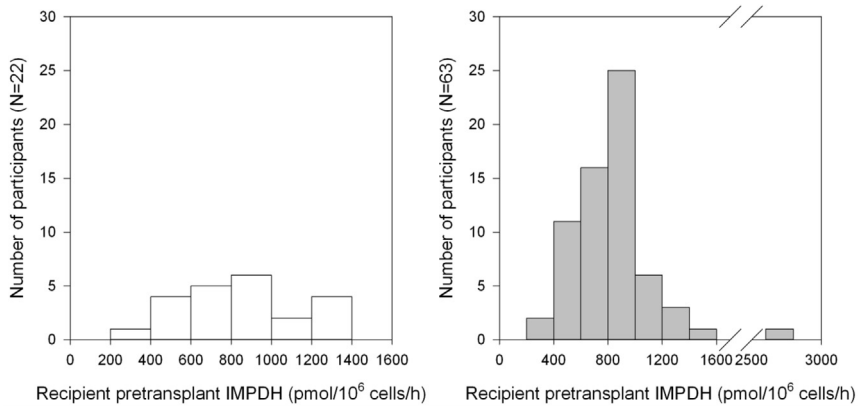

Figure 3. Between-individual variability of recipient pretransplant IMPDH activity in PMNC obtained before administration of a related (white) or an unrelated (gray) donor graft.

by cell count (ie, pmol XMP/10^6 cells/h) from all samples obtained within 1 day. Among all 86 participants, considerable between-individual variability (12.2-fold) was observed (Figure 3).

Covariate Analysis

The association of age, sex, disease (categorized as high, intermediate, or low risk), donor graft (related or unrelated), HCT comorbidity index, and recipient pretransplant mRNA with IMPDH activity were assessed. To achieve normal distribution, the extreme outlier (IMPDH activity >2500 pmol/10^6 cells/h) was excluded. Only age showed a statistically significant correlation ($P < .04$) with $R^2 = .05$. Although this reached statistical significance, it is clinically irrelevant because of the low R^2 value. IMPDH1/2 mRNA was only determined in 15 participants because of resource constraints resulting from the IMPDH activity method development. For the 15 samples assayed, there was no significant correlation between IMPDH1/2 mRNA level and activity.

Clinical Outcomes

Association of IMPDH activity with various clinical outcomes is shown in Table 2, Figure 4, Supplemental Figure 2, and Supplemental Figure 3. Table 2 also describes the number of participants with each clinical outcome of interest. Graft rejection occurred in 2 participants: 1 received a related donor graft and 1 had an unrelated donor. Both received MMF with cyclosporine, and their recipient pretransplant IMPDH activities were 571 and 925 pmol/10^6 cells/h, respectively.

Of the 86 participants, 78 (17 related, 61 unrelated) had an ANC nadir below 500/μL between days −8 and +28. Of those 78, 19 experienced their ANC nadir between days −8 and +7 and 59 between days +8 and +28. Of the 86 participants, 23 were CMV positive with a CMV-positive donor, 23 were CMV positive with a CMV-negative donor, 13 were CMV negative with a CMV-positive donor, and 27 were CMV negative with a CMV-negative donor. Among the 46 participants who were CMV seropositive before HCT, 30 (65%) experienced CMV reactivation. Specifically, 5 of 10 seropositive participants with related donors (50%) and 25 of 36 with unrelated donors (69%) experienced CMV reactivation. In CMV-negative recipients with a CMV-positive donor, CMV antigenemia was detected in 2 of 4 related (50%) and 1 of 9 unrelated (11%) donor graft recipients.

The median for day +28 donor T cell chimerism was 88% (range, 35% to 100%). Sixty-seven participants had acute GVHD with the median onset on day +37. Of these, 60 participants (16 related, 44 unrelated) had grades II to IV acute GVHD and 11 (4 related, 7 unrelated) had grades III to IV acute GVHD. Supplemental Table 1 includes detailed acute GVHD information, specifically the organ affected and its grade. Forty-seven participants had chronic GVHD. Twenty participants relapsed; 6 had related donors and 14 had unrelated donors. Thirteen of these participants died of relapse, 4 of whom had a related donor and 9 of whom had an unrelated donor. Seventeen participants died of nonrelapse mortality. Of these, 3 had a related donor and 14 had an unrelated donor. At last follow-up, 56 participants were still alive.

We sought to evaluate whether clinical outcomes were associated with recipient pretransplant IMPDH activity. Increasing IMPDH activity was associated with a decreased likelihood of day +28 T cell donor chimerism ≥95%, with an OR of .17 per doubling of IMPDH activity (95% confidence interval [CI], .1 to .6; $P = .003$). Thus, the odds of experiencing day +28 T cell chimerism ≥95% were decreased by 83% with each doubling of IMPDH activity. Increasing IMPDH activity was associated with a lower rate of grades II to IV acute GVHD (HR = .50 per doubling of IMPDH activity; 95% CI, .3 to .9; $P = .03$) and grades III to IV acute GHVD (HR = .30 per doubling of IMPDH activity; 95% CI, .1 to .9; $P = .03$). Increasing IMPDH activity was associated with a decreased risk of neutrophil nadir <165/mm^3 ($P = .01$), with an OR of .26 (95% CI, .1 to .7) per doubling of IMPDH activity. Thus, the odds of experiencing a neutrophil nadir <165/mm^3 were decreased 74% with each doubling of IMPDH activity. Similarly, higher IMPDH activity was associated with a lower rate of CMV reactivation (HR = .48 per doubling of IMPDH activity; 95% CI, .3 to .9; $P = .02$). IMPDH activity was not, however, statistically associated with chronic GVHD, relapse, nonrelapse mortality, or overall mortality (Table 2).

Table 2
Effect of Recipient Pretransplant IMPDH Activity on Clinical Outcomes

Clinical Outcome	Number of Events	OR/HR* (95% CI)	P
Day +28 T cell chimerism ≥95%	33	.17 (.1-.6)	.003
Grades II to IV acute GVHD	60	.50 (.3-.9)	.03
Grades III to IV acute GVHD	11	.30 (.1-.9)	.03
Chronic GVHD	47	1.03 (.6-1.8)	.91
Relapse	20	1.53 (.6-3.7)	.35
ANC nadir < 165/mm^3	44	.26 (.1-.7)	.01
CMV reactivation	33	.48 (.3-.9)	.02
Nonrelapse mortality	17	.58 (.2-1.5)	.27
Overall mortality	30	1.04 (.5-2.2)	.92

* Day +28 T cell chimerism and neutropenia analyzed as binary endpoint (OR), and all others as time-to-event endpoint (HR). OR and HR are effects per doubling of recipient pretransplant IMPDH activity. All analyses adjusted for Kahl risk category (low, standard, high), donor–recipient gender (female to male, other), and donor (related, unrelated).

DISCUSSION

In this analysis we evaluated recipient pretransplant IMPDH activity in 86 consecutive participants who were given nonmyeloablative conditioning before receiving allogeneic grafts to treat hematological malignancies. To our knowledge, this is the first analysis of recipient pretransplant IMPDH in HCT participants. There was considerable between-individual variability in the IMPDH activity (Figure 3). Low recipient pretransplant IMPDH activity was associated with increased day +28 T cell chimerism, more acute GVHD, lower neutrophil nadirs, and more CMV reactivation but not with chronic GVHD, relapse, nonrelapse mortality, or overall mortality (Table 2 and Figure 4).

The role of MMF as postgrafting immunosuppression in HCT recipients has gradually increased over the past 15 years [29]. There is considerable interindividual variability in MPA plasma exposure with weight-based dosing of either intravenous or oral MMF in HCT recipients [10,30]. Pharmacodynamic data suggest a relationship between clinical outcomes and MPA plasma exposure (as reviewed in McDermott et al. [31]), and some HCT centers personalize MMF doses to a target MPA exposure [32]. We recently observed that low total MPA plasma exposure was associated with increased grades III to IV acute GVHD and increased nonrelapse mortality in nonmyeloabative HCT recipients with an unrelated donor graft. In patients receiving a related donor graft after nonmyeloablative conditioning, however, total MPA plasma exposure was not associated with clinical outcomes, and additional biomarkers, such as recipient

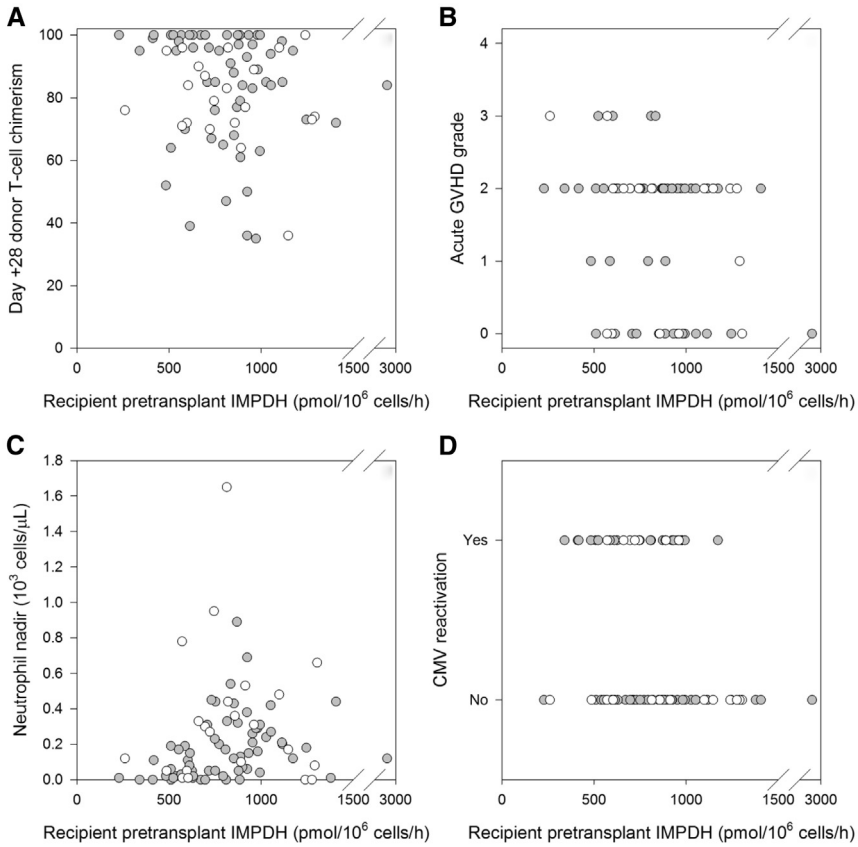

Figure 4. Clinical outcomes and recipient pretransplant IMPDH before administration of a related (white) or unrelated (gray) donor graft: (A) day +28 donor T cell chimerism, (B) acute GVHD grade, (C) neutrophil nadir, and (D) CMV reactivation.

pretransplant IMPDH activity, are of interest [31]. Unfortunately, because of a concurrent clinical trial comparing TBI versus fludarabine/TBI in related donor graft recipients [33], there is a paucity of nonmyeloablative HCT participants receiving an related donor graft in this reported population (Table 1).

PMNCs are the predominant cell population used to measure IMPDH activity [8,13,14,34], although Vethe and Bergan [35] evaluated IMPDH activity in $CD4^+$ cells. To our knowledge, IMPDH activity has only been assessed in these 2 cell populations, but mRNA expression of both IMPDH1 and IMPDH2 has been more extensively studied. MPA inhibits both IMPDH1 and IMPDH2, which are ubiquitously expressed and have similar catalytic activity in vitro but do differ in their tissue expression [36-40]. Compared with hepatic mRNA expression, IMPDH1 expression is higher in the pancreas, colon, and peripheral blood leukocytes, and IMPDH2 expression is higher in the pancreas, kidney, and skeletal muscle [36]. Both IMPDH1 and IMPDH2 are expressed in the small intestine and colon. Notably, grades II to IV gastrointestinal GVHD was infrequent (Supplemental Table 1).

Developing a rapid and robust analytical method that can be used in clinical practice is a necessity before evaluating IMPDH activity as a biomarker. The assays for IMPDH activity can be divided into either radiometric or nonradiometric quantitation methods [13,34]. The radiometric methods require low sample volume and also maintain intracellular MPA concentration. Substantive difficulties with storage of control and/or participant samples for longer than 8 hours, however, make these methods suboptimal. Nonradioactive liquid chromatography procedures such as ours are based on the quantitation of produced XMP in cell extracts supplemented with the substrate IMP and cosubstrate NAD^+ in excess and subsequently incubated. Laverdière et al. [8] described a LC-coupled tandem MS method for the quantification of XMP and AMP (for normalization) in PMNC lysates, including those from 19 HCT recipients. Our XMP assay had similar reproducibility to that of Laverdière (CV $<$ 7.5%) [8]. Our chromatographic conditions using the Hypercarb column provided excellent baseline separation of XMP from IMP and other endogenous adenosine-phosphate species (Figures 1 and 2). The carbon solid phase of the Hypercarb column eliminates the need for the ion pairing reagents, which can often be difficult to remove from the HPLC system. We chose not to use AMP to normalize results because precision was similar between normalization by cell count or by AMP concentration. We chose to normalize all reported data by cell count because it is more intuitive for clinicians and facilitates communication of pretransplant recipient IMPDH as a predictive biomarker.

Recipient pretransplant IMPDH activity has several advantages as a biomarker. First, an adequate number of PMNCs can be obtained because samples are drawn before administration of nonmyeloablative (ie, fludarabine/TBI) conditioning. The second advantage for recipient pretransplant IMPDH as a biomarker is that it would not have the same rapid turnaround time requirements for MPA plasma concentrations for MMF dose personalization to a target MPA exposure. High recipient IMPDH activity is associated with

rejection in renal transplant participants [9]. In this cohort, only 2 rejections occurred, and thus this relationship could not be evaluated.

Low recipient pretransplant IMPDH activity was associated with increased day +28 T cell chimerism, more acute GVHD, lower neutrophil nadirs, and more CMV reactivation but not with chronic GVHD, relapse, nonrelapse mortality, or overall mortality (Table 2 and Figure 4). An alternative way to present these findings is that higher recipient pretransplant IMPDH activity was associated with lower day +28 T cell chimerism, less acute GVHD, higher neutrophil nadirs, and lower CMV reactivation. If a recipient is not sensitive to MPA—potentially as demonstrated by a high pretransplant IMPDH activity—fewer immunologically competent cells will die [41], and therefore more of the recipient's immunologically competent cells are present to allow for tolerance between the recipient and donor cells. Thus, the trends between IMPDH activity and day +28 donor T cell chimerism, neutrophil nadir, or CMV reactivation are not unexpected.

At first, the finding that higher recipient pretransplant IMPDH activity is associated with less acute GVHD seems less intuitive. There are at least 2 mechanistic hypotheses to explain this finding. Previously, we have shown that high donor T cell chimerism on day +28 was associated with an increased probability of acute GVHD in 120 nonmyeloablative HCT recipients [5,7]. Based on these previous results and the association of increasing pretransplant IMPDH activity with lower day +28 donor T cell chimerism (Table 2), the association of a lower risk of acute GVHD with increasing pretransplant IMPDH activity is not surprising. Furthermore, these results agree with recent data regarding the influence of peritransplant neutrophil and lymphocyte counts upon the risk of acute GVHD or relapse in 459 nonmyeloablative HCT recipients [7]. Storb et al. found that low neutrophil nadirs within the first 3 weeks post-HCT had significant associations with increased risk of acute GVHD and 5-year nonrelapse mortality but not with relapse. In the study presented here, recipients with higher pretransplant IMPDH activity have higher neutrophil nadirs and therefore a lower risk of acute GVHD. Thus, the association between IMPDH activity and acute GVHD has at least 2 mechanistic rationales.

In addition, we hypothesize that recipients with higher pretransplant IMPDH activity have fewer immunologically competent cells die, allowing more of the recipient's immunologically competent cells to be present to build tolerance between the recipient and donor cells. Evaluating IMPDH activity early after the graft infusion may provide some insight regarding this hypothesis. We sought to evaluate IMPDH activity on day +2, but participants had too few cells to isolate PMNC successfully. Future studies could seek to evaluate IMPDH activity and T cell chimerism immediately after adequate cell numbers are present, possibly within 24 hours of engraftment. In addition, future work should address the effect of MPA-based postgrafting immunosuppression on various T cell subsets. Within human and animal models, there is accumulating evidence that regulatory T cells are involved in the development of GVHD [42,43]. In vitro data in human PMNCs indicate that MPA might favorably influence the balance between regulatory T cells and the newly identified subset of IL-17–secreting helper T cells [44]. Separate in vitro studies suggest that MPA hinders the antigen presenting and lymph node homing capacities of human blood myeloid dendritic cells, which may promote allograft tolerance by interfering with the initiation of acquired immunity [45]. The translation of these in vitro findings to nonmyeloablative HCT recipients receiving MPA-based postgrafting immunosuppression could offer novel insight to the pathophysiology of GVHD. Because of the complexity and multiscale nature of the human immune response, the translation of these in vitro findings could benefit from the use of mathematical modeling and simulation to gain a mechanistic understanding.

Such a mechanistic understanding can be gained from the recently developed fully integrated immune response model, created with a systems biology approach using mathematical models developed by integrating models of the humoral side of the immune response, the cellular side of immune response, and for cytokine kinetics [46]. This model accurately simulated the immune response to a tuberculosis infection, a bloodborne pathogen, immune-mediated tumor elimination, and tumor removal with regulatory T cells. Future work should include the application of immune models to allogeneic HCT. As a first step, we characterized recipient pretransplant IMPDH along with additional biomarkers to begin collecting adequate data to build such immune models. A limitation of our work was not evaluating donor IMPDH activity, which would be important after donor engraftment occurs. Because most of our donors were unrelated, however, PMNCs from the donor were not available pretransplant.

In conclusion, we developed a method with adequate sensitivity to quantitate recipient pretransplant IMPDH activity. We found that recipient pretransplant IMPDH activity was associated with an increased risk of neutropenia, CMV reactivation, and acute GVHD in nonmyeloablative HCT recipients. Because of the variable outcomes, future prospective trials should address the clinical benefit of alternative postgrafting immunosuppression or higher initial oral MMF doses to patients with high recipient pretransplant IMPDH activity to improve clinical outcomes in nonmyeloablative HCT recipients.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2014.05.032.

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