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ARTICLE

Pharmacometabonomic association of cyclophosphamide 4-hydroxylation in hematopoietic cell transplant recipients

Jeannine S. McCune[1] | **Ryotaro Nakamura**[1] | **Denis O'Meally**[2] | **Timothy W. Randolph**[3] | **Brenda M. Sandmaier**[3,4] | **Aleksandra Karolak**[1,5] | **David Hockenbery**[3,4] | **Sandi L. Navarro**[6]

[1]Department of Hematologic Malignancies Translational Sciences, City of Hope, and Department of Hematopoietic Cell Transplantation, City of Hope Medical Center, Duarte, California, USA

[2]Center for Gene Therapy, Hematologic Malignancies Research Institute, City of Hope, Duarte, California, USA

[3]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA

[4]Department of Medicine, University of Washington, Seattle, Washington, USA

[5]Division of Mathematical Oncology, Department of Computational and Quantitative Medicine, City of Hope, Duarte, California, USA

[6]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA

Correspondence
Jeannine S. McCune, Department of Hematologic Malignancies Translational Sciences, City of Hope, and Department of Hematopoietic Cell Transplantation, City of Hope Medical Center, 1500 E Duarte Road Shapiro Building Room 1025 Duarte CA 91010, USA.
Email: jmccune@uw.edu

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Abstract

The widely used alkylating agent cyclophosphamide (CY) has substantive inter-patient variability in the area under the curve (AUC) of it and its metabolites. Numerous factors may influence the drug-metabolizing enzymes that metabolize CY to 4-hydroxycyclophosphamide (4HCY), the principal precursor to CY's cytotoxic metabolite. We sought to identify endogenous metabolomics compounds (EMCs) associated with 4HCY formation clearance (ratio of 4HCY/CY AUC) using global metabolomics. Patients who undergo hematopoietic cell transplantation receiving post-transplant CY (PT-CY) were enrolled, cohort 1 ($n = 26$) and cohort 2 ($n = 25$) donating longitudinal blood samples before they started HCT (pre-HCT), before infusion of the donor allograft (pre-graft), before the first dose of PT-CY (pre-CY), and 24 h after the first dose of PT-CY (24-h post-CY), which is also immediately before the second dose of CY. A total of 512 and 498 EMCs were quantitated in two cohorts, respectively. Both univariate linear regression with false discovery rate (FDR), and pathway enrichment analyses using a global association test were performed. At the pre-CY time point, no EMCs were associated at FDR less than 0.1. At pre-HCT, cohort 1 had one EMC (levoglucosan) survive the FDR threshold. At pre-graft, cohort 1 and cohort 2 had 20 and 13 EMCs, respectively, exhibiting unadjusted p values less than 0.05, with the only EMCs having an FDR less than 0.1 being two unknown EMCs. At 24-h post-CY, there were three EMCs, two ketones, and threitol, at FDR less than 0.1 in cohort 2. These results demonstrate the potential of pharmacometabonomics, but future studies in larger samples are needed to optimize CY.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

We report the first pharmacometabonomic study of the association of plasma EMCs with cyclophosphamide pharmacokinetics, specifically the ratio of 4HCY/CY AUC.

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WHAT QUESTION DID THIS STUDY ADDRESS?

This study addresses the question regarding if EMCs in the plasma before PT-CY administration are associated with the ratio of 4HCY/CY AUC.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

This study adds to our knowledge that longitudinal collection of plasma EMC samples is feasible in HCT patients receiving PT-CY. In addition, the plasma EMC changes over the ~21-day time period that starts pre-HCT to 24-hr after the first PT-CY dose.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

This study demonstrates the possibility of pharmacometabolomic research to evaluate the pharmacokinetics of a drug – in this case, cyclophosphamide – with a complex pharmacokinetic disposition.

INTRODUCTION

The development of post-transplant cyclophosphamide (PT-CY) as an immunosuppressive regime was a benchmark in the field of allogeneic hematopoietic cell transplant (HCT) and is transforming HCT practice worldwide.[1] PT-CY has traditionally been administered as 50 mg/kg/day for 2 days, based on partially mechanistic and partially empirical data.[2,3] Preclinical studies suggest a dose-dependent effect of PT-CY upon graft-versus-host disease (GVHD), with the lowest GVHD rates occurring with moderate doses of PT-CY in small animal models.[2,3] Novel mechanistic insight was also gained from this preclinical work, which suggested a two-part mechanism of GVHD attenuation: direct effects on (1) alloreactive T cells; and (2) suppressive cell populations that control GVHD.[2,3] Specifically, PT-CY reduces proliferating conventional CD4+ (Tcon) T cells and preferentially expands CD4+CD25+FoxP3+ regulatory (Treg) T cells.[2] The precise mechanism(s) regarding how CY and its active endogenous metabolomic compounds (EMCs) are transported into and subsequently affect the T cell types of interest, here, Treg and Tcon, are unknown.

CY is a prodrug with a complex metabolic pathway (Figure S1). Dosing CY based on body surface area (BSA) or bodyweight leads to considerable interpatient variability in systemic exposure to CY and its metabolites, including 4-hydroxycyclophosphamide (4HCY), the precursor to CY's primary cytotoxic metabolite phosphoramide mustard.[4,5] We hypothesize that the area under the curve (AUC) of plasma 4HCY is a surrogate marker for the intracellular effects of CY on Tcon and Treg. However, quantifying plasma concentrations of 4HCY is time- and resource-intensive because this CY metabolite has an in vitro half-life of less than 3 min.[6] An understanding of 4HCY pharmacokinetics (PKs) is critical because 4HCY forms phosphoramide mustard, which covalently cross-links DNA. Further, because phosphoramide mustard does not cross cell membranes appreciably, the transport of its precursor 4HCY into the cell is a key step for CY's cytotoxic activity.[4] Thus, it is necessary to gain an improved understanding of the biological factors influencing the formation of 4HCY from CY—which is the 4HCY formation clearance defined as the 4HCY $AUC_{0-48\ h}$ divided by the CY $AUC_{0-48\ h}$ (referred to as the ratio of 4HCY/CY AUC hereafter). Identifying such pharmacometabolomic relationships in the ratio of 4HCY/CY AUC could be less resource-intensive than PK studies.

In addition, gaining such a mechanistic understanding of factors affecting the plasma ratio of 4HCY/CY AUC may lead to biomarkers of the effectiveness and toxicity of PT-CY. Alternative methods to predict 4HCY concentrations have been evaluated. Unfortunately, the hepatic activity of CY-metabolizing enzymes (Figure S1) are only weakly or moderately correlated with the expression of cytochrome P450 in peripheral blood mononuclear cells[7] or with plasma glutathione *S*-transferase activity (GST; $R^2 = 0.567$), respectively.[8] Thus, peripheral blood sampling is not an adequate surrogate for CY metabolic capacity in the liver. Additionally, the within-patient variability of the ratio of the 4HCY/CY AUC varies (i.e., this ratio was similar between two CY doses in approximately half of the patient population; Figure S29). This suggests that environmental factors influence the ratio of the 4HCY/CY AUC. Thus, it is not surprising that the pharmacogenetic studies of CY have produced conflicting results.[10,11] No consistent genotype-phenotype association has been found with the PKs, efficacy, or toxicity of CY.[10,11]

Against this background, we evaluated EMCs in HCT participants receiving PT-CY to further our mechanistic understanding of the ratio of 4HCY/CY AUC. Metabolomics, which is the study of small-molecule EMCs' profiles in biological samples, is a promising new technology in personalized medicine.[12] Concentrations of the EMCs represent sensitive downstream markers of genomic changes and responses of cells and tissues

to external stimuli.[13] Consequently, the development of robust metabolomic platforms will facilitate understanding of the in vitro and in vivo actions of drugs.[13] Thus, we hypothesized that differences in plasma EMCs are associated with the ratio of 4HCY/CY AUC in patients receiving CY-based GVHD prevention after allogeneic HCT.

METHODS

Study populations

Two prospectively collected datasets were analyzed: cohort 1 ($n = 25$) and cohort 2 ($n = 26$). These participants underwent the same study procedures, but their samples were analyzed in two separate runs. Thus, the datasets were analyzed only with samples from the same metabolomic run. The datasets were collected from participants who received HCT and post-graft immunosuppression with PT-CY from June 2018 to July 2020 under the aegis of two protocols approved by the City of Hope Institutional Review Board. All participants were diagnosed with hematologic disorders and had adequate renal (i.e., serum creatinine <1.5 mg/dl, and creatinine clearance or measured glomerular filtration rate >60 ml/min/1.73 m^2) and liver (i.e., total bilirubin <1.5 mg/dl and alanine aminotransferase <300 units/L) function at baseline. Demographic data were taken from the participants' medical charts (age, sex, height, total [i.e., actual] body weight, BSA, and clinical information [disease and conditioning regimen]). All participants provided written informed consent prior to study procedures. The conditioning regimen and post-graft immunosuppression were not affected by participation in these studies. Antiemetics, antibiotics, and antifungals were given per Institutional Standard Practice Guidelines.

PT-CY administration and pharmacokinetic sample collection

The PT-CY dose was 50 mg/kg i.v. (2-h] infusion every 24 h for two doses, administered on HCT days +3 and +4. PK samples were collected after each PT-CY dose; the PK samples were collected at 2 (end of infusion), 4, 8, 20, and 24 h from the start of the infusion.[14] The samples were processed within 30 min and subsequently placed in a −80°C freezer within 1 h.

Concentrations of CY, 4HCY, carboxyethylphosphoramide mustard (CEPM), deschloroethylcyclophosphamide (DCCY), and 4-ketocyclophosphamide (KetoCY) were measured as previously reported,[14] using liquid-chromatography tandem-mass-spectrometry (LC-MS/MS). The dynamic range of the assays of CY and its metabolites were as follows: CY, 0.96–19 2 μM; 4HCY, 0.125–25 μM; CEPM 0.043–17 μM; DCCY 0.063–25 μM; and KetoCY 0.022–9.1 μM. All assays had an interday precision of less than 10%. The AUCs were estimated using the trapezoidal numerical estimation function in MATLAB (version 2019a).

Global pharmacometabonomics sample collection

Figure 1 is a schema of the pharmacometabonomics sample collection relative to the HCT regimen and the PK sampling for CY and 4HCY AUC. Longitudinal blood samples (3 ml/sample) were scheduled to be collected in EDTA tubes at four time points during PT-CY dosing: up to 2 weeks prior to the first conditioning dose (pre-HCT or pre-transplant sample), immediately before administration of the allograft (pre-graft sample), immediately before administration of the first PT-CY dose (pre-CY sample), and 24 h after the first CY dose, which is also immediately before the second PT-CY dose (24-h post-CY sample).

With this dataset, our goal was to identify metabolomic pathways associated with the ratio of 4HCY/CY AUC. Because we had previously observed that the ratio of 4HCY/CY AUC was inconsistent in a subset of participants (Figure S2), we wanted to gather preliminary data regarding longitudinal changes in the plasma EMCs before and during CY administration. If an association was found between the plasma EMCs and the ratio of 4HCY/CY AUC, stable EMCs would suggest that further studies can focus on metabolomics-guided CY dosing. The 2-week pre-HCT sample collection time was the earliest feasible time within the final HCT workup (i.e., the period in which the participant undergoes final assessment for HCT, typically up to 2 weeks). The pre-graft sample was obtained immediately before the allograft infusion, which is after all of the conditioning was completed and 72 h before the first PT-CY dose. The pre-CY sample was obtained immediately before the first CY dose. The 24-h post-CY sample was obtained immediately before the second CY dose and may provide insight regarding metabolomic pathways associated with auto-induction of 4HCY formation.

In cohort 1, all 25 participants had samples for three time points, and 14 participants had samples for all four time points. In cohort 2, all 26 participants had samples for three time points, and five participants had samples for all four time points. Total samples at each time point in cohort 1 were: $n = 24$ at pre-HCT; $n = 24$ at pre-graft; $n = 16$ at pre-CY; and $n = 25$ at 24-h post-CY. For cohort 2, there were: $n = 5$ at pre-HCT; $n = 26$ at pre-graft; $n = 26$ at pre-CY; and $n = 26$ at 24-h post-CY.

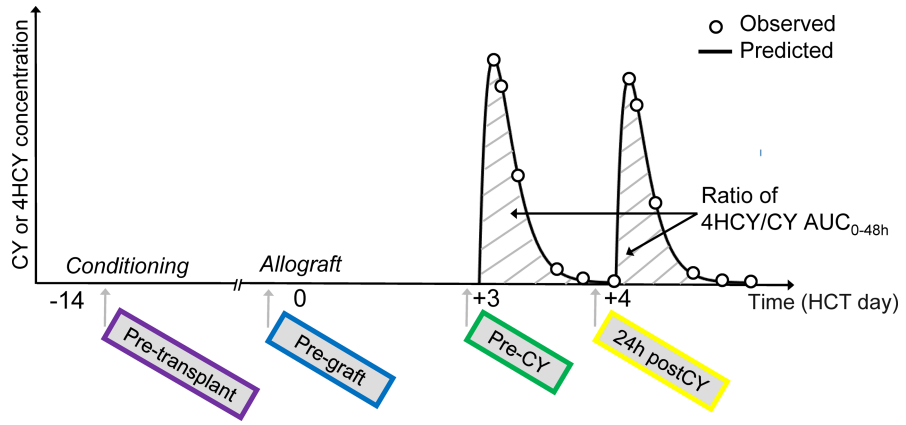

FIGURE 1 Study Design. Plasma EMC samples were obtained at obtained before or with the administration of the post-transplant CY (PT-CY) regimen is associated with the ratio of 4HCY/CY AUC. Gray boxes show the four longitudinal time-points at which plasma EMC samples were obtained. The ratio of the 4HCY/CY $AUC_{0–48\ h}$ was measured after both of the two PT-CY doses; the AUCs are filled with dots or stripes. The HCT conditioning regimen is administered before HCT day −1, the allogeneic graft infusion occurs on HCT day 0, and the PT-CY doses are administered on HCT day +3 and +4 (72 and 96 h, respectively, after infusion of the allogeneic graft). The EMC time points color coding corresponds to that in Figure 2. 4HCY, 4-hydroxycyclophosphamide; AUC, area under the curve; AUC0–48 h, area under the curve from zero to 48 h; EMC, endogenous metabolomics compound; HCT, hematopoietic cell transplantation; PT-CY, post-transplant cyclophosphamide

Pharmacometabonomics of hypothesis generation cohort

To generate an a priori hypothesis regarding metabolomic pathways associated with the ratio of 4HCY/CY AUC, we analyzed an existing dataset with 4HCY/CY AUC[14] and EMCs[15] available. This hypothesis-generation dataset was obtained from a separate cohort of 51 HCT recipients conditioned with CY (60 mg/kg/day i.v. for 2 doses) that received phenytoin during the second CY dose. Phenytoin is well-known to increase the ratio of 4HCY/CY AUC.[16] In this hypothesis-generation cohort, targeted metabolomics analysis was carried out using an LC-MS/MS platform in both positive and negative ion modes for 224 EMCs from 25 metabolic pathways. The results from this hypothesis-generation cohort were used to identify the most promising pathways and subsequently to guide the platform used to analyze these contemporary cohorts. Method S1 contains a more detailed description of this hypothesis-generation cohort and how its results were used to guide the platform chosen to analyze these contemporary cohorts. The untargeted primary metabolites assay from West Coast Metabolomics Center (WCMC; Davis, CA) was chosen.

Untargeted pharmacometabonomics analysis

Profiling of the plasma EMCs was completed at the WCMC. The samples were shipped on dry ice to WCMC and stored at −80°C upon receipt. The samples underwent at most one additional freeze-thaw before metabolomic analysis (i.e., the analysis was conducted after the first or the second thaw). A full description of the untargeted pharmacometabonomic assay[17] is presented in Methods S2. A total of 512 EMCs were detected in at least 90% of samples for cohort 1, 127 annotated and 385 unnamed, whereas 498 were detected in cohort 2, 133 annotated, and an additional 365 unnamed EMCs. There were 18 missing values across the two cohorts: one in cohort 1 and 17 in cohort 2, all within unnamed EMCs. These were imputed with half of the lowest abundance for the EMCs.

Data analysis

Both datasets were used separately to analyze the association of the plasma EMCs with the ratio of the 4HCY/CY AUC. The majority of EMCs were skewed to higher values and therefore centered-log ratio was performed prior to analysis.[18] A univariate linear regression model was used to assess marginal associations for each EMC individually on the ratio of 4HCY/CY AUC (continuous) after pre-HCT, pre-graft, pre-CY, and 24-h post CY, adjusted for age (continuous), sex (0/1), and adjusted ideal body weight (AIBW; continuous). In a previous evaluation of 147 HCT recipients, used to inform the present analysis, age but not sex or weight had an effect on 4HCY formation.[19] However, to ensure that sex or weight did not confound our results, all three covariates were included in univariate models. The common name of the EMC is presented for peaks that have

been identified. For unknown EMCs, the Bin Base identifier is listed.[20] Benjamini-Hochberg methods were used to control for false discovery rate (FDR).[21] Individual EMCs were considered for both p less than 0.05 and FDR less than 0.1. Principal components analysis was performed to assess the variance in EMCs over time. All analyses were conducted in Stata version 16 (College Station, TX). Graphics were generated using the R environment (version 4.0.5).

To evaluate which EMCs coordinately associate with the ratio of 4HCY/CY AUC, pathway analyses using all named EMCs were carried out using MetaboAnalyst 5.0.[22] Within the pathway analysis module, the ratio of 4HCY/CY AUC was evaluated as a dichotomous outcome, below versus above the median. The Global test,[23] which evaluates changes among groups of EMCs, was used to assess statistical significance of each pathway with respect to the dichotomized ratio of 4HCY/CY AUC. A cutoff of FDR less than 0.1 was used for multiple comparisons. Forty-three pathways, containing at least two EMCs from our panel, were evaluated. Betweenness centrality (the shortest path between nodes), based on EMC centrality in a given metabolic network, was used to calculate EMC importance.[24] Pathway impact was calculated as the sum of the importance measures of the pathway-specific EMCs, normalized by the sum of the importance measures of all EMCs in each pathway.[25] A pathway was considered important if the impact value was greater than or equal to 0.1 using MetaboAnalyst.[22]

RESULTS

Patient characteristics and clinical outcomes

Pre-transplant characteristics of the two cohorts are given in Table 1. Overall, the cohorts were similar, with cohort 1 having a younger median age, a lower percentage of men, and a higher proportion of haploidentical grafts than cohort 2. Only one participant received concomitant corticosteroids, which induce CY 4-hydroxylation in vitro[26] and possibly in vivo. None of the participants received concomitant azoles, which affects plasma 4HCY concentrations in HCT recipients.[27]

CY administration and longitudinal plasma EMCs

Principal component analysis of all samples did not reveal any obvious distinction between EMC profiles for the pre-HCT, pre-graft, pre-CY, and 24-h post-CY administration (Figure 2).

TABLE 1 Pre-transplant patient characteristics[a]

Characteristic	Cohort 1 $n = 25$	Cohort 2 $n = 26$
Age, years	39 (18–78)	48 (20–78)
Body mass index, kg/m^2	27 (18–43)	28 (21–53)
Sex, % male	60%	77%
AIBW, kg	71 (57–83)	73 (48–87)
Ratio of 4HCY/CY AUC	0.06 (0.04–0.13)	0.06 (0.02–0.13)
Diagnosis		
Acute myeloid leukemia	10 (40%)	9 (35%)
Acute lymphoblastic leukemia	6 (24%)	6 (23%)
Hodgkin's lymphoma	4 (16%)	4 (15%)
Chronic myeloid neoplasms	2 (8%)	4 (15%)
Non-Hodgkin's lymphoma	1 (4%)	1 (4%)
Sickle cell disease	2 (8%)	0
Other[b]	0	2 (8%)
Donor		
Related (haploidentical)	17 (68%)	12 (46%)
Unrelated	8 (32%)	14 (54%)
Relevant medications		
Ursodiol	25 (100%)	26 (100%)
CY as part of conditioning regimen	4 (16%)	4 (15%)
Corticosteroids with PT-CY	1 (4%)	0 (0%)
Azoles within 7 days of PT-CY	0 (0%)	0 (0%)

Abbreviations: 4HCY/CY AUC, 4 hydroxycyclophosphamide/cyclophosphamide area under the curve; AIBW, adjusted ideal body weight; CY, cyclophosphamide; PT-CY, post-transplant cyclophosphamide.

[a]Data presented as: number (%) or median (range). Cohorts differed because their endogenous metabolomics compounds (EMCs) were quantitated in two separate runs.

[b]Other diagnoses include: myeloid sarcoma ($n = 1$) and multiple myeloma ($n = 1$).

Pharmacometabonomics: Univariate analyses by cohort and longitudinal time point

We hypothesized that the sample obtained at the pre-CY time point, which was drawn immediately before the first PT-CY dose, would have EMCs associated with the ratio of the 4HCY/CY AUC. At the pre-CY time point, cohorts 1 and 2 had 13 and 36 EMCs, respectively, exhibiting unadjusted p values less than 0.05; however, there were no significant EMCs at FDR less than 0.1 (EMCs with $p < 0.05$ are listed in Tables 2 and 3; the full list is given in Table S2 and Table S3). We also evaluated the association of EMCs with the ratio of the 4HCY/CY AUC for the other time

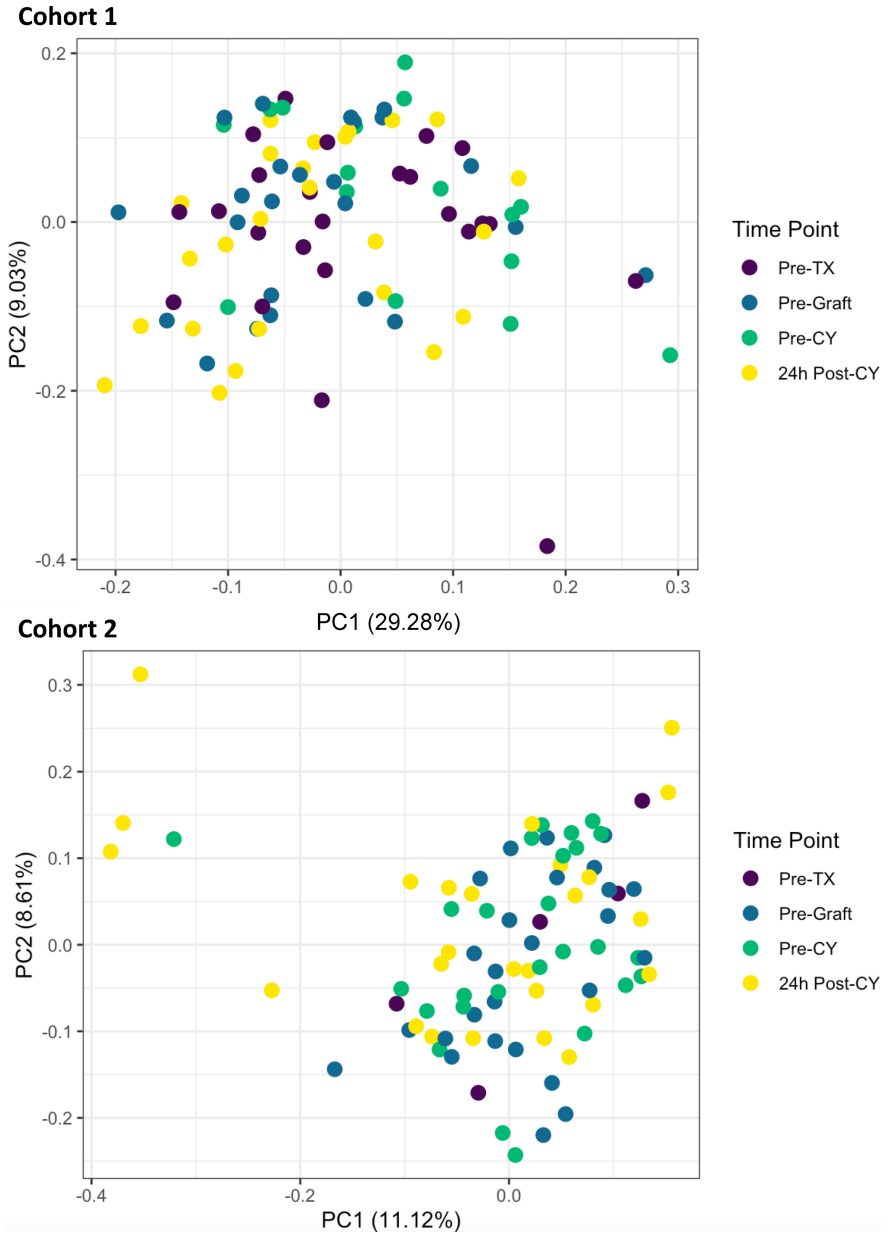

FIGURE 2 Change in plasma EMCs from before post-transplant CY administration (purple, blue, and teal dots) to after administration of the first post-transplant CY dose (yellow dots). Principal Component Analysis showing differences across time points within cohorts. The time points color coding corresponds to that in Figure 1. CY, cyclophosphamid e, administered as post-transplant cyclophosphamide ; TX, hematopoietic cell transplant

points. For the pre-HCT time point, cohort 1 had 49 EMCs which had a raw p value of 0.05; one EMC (i.e., levoglucosan) met the FDR threshold ($p < 0.00006$; FDR = 0.03). Cohort 2 had insufficient samples for analysis at this time point. At the pre-graft time point, cohort 1 and cohort 2 had 20 and 13 EMCs, respectively, exhibiting unadjusted p values less than 0.05, with two unknown EMCs at FDR less than 0.1 in cohort 2. At the 24-h post-CY, cohort 1 and cohort 2 had 48 and 41 EMCs, respectively, with unadjusted p values less than 0.05 with 0 and three EMCs at FDR less than 0.1 in cohorts 1 and 2, respectively. In the 24-h post-CY time point in cohort 2, the 2-ketoisovaleric acid and alpha-ketoglutarate were positively associated, and threitol was inversely associated with the ratio of 4HCY/CY AUC (Tables S4 and S5).

Pathway analyses

Metabolic pathways evaluated for the association of the EMCs with the ratio of the 4HCY/CY AUC are listed in Table S6 and Table S7. There were no pathways significantly associated with the pre-CY ratio for cohort 1 at FDR less than 0.1. For cohort 2, fatty acid biosynthesis had a raw value of $p = 0.04$, but an FDR greater than 0.1. The results from testing at other time points are as follows: for cohort 1, only one pathway, pyrimidine metabolism, had an unadjusted p value less than 0.05 at pre-HCT. For cohort 2, we observed unadjusted p values less than 0.05 for fatty acid biosynthesis at the pre-graft time point, and nine pathways at 24 h-post CY, including pyrimidine metabolism, glycolysis/gluconeogenesis, galactose metabolism,

TABLE 2 Pre-CY plasma EMCs associated ($p < 0.05$) with the ratio of 4HCY/CY AUC in cohort 1 ($n = 16$ samples)

EMCs	Coefficient (SE)[a]	*p* value[b]	FDR
Glutamic acid	−14.04 (4.28)	0.001	0.42
Unknown 382062	−18.24 (5.79)	0.002	0.42
2,3-Dihydroxybutanoic acid	−14.53 (4.98)	0.004	0.60
Methanolphosphate	−22.69 (8.07)	0.005	0.63
3-Hydroxybutyric acid	−32.21 (11.96)	0.01	0.66
Unknown 229949	−18.72 (7.03)	0.01	0.66
Unknown 1981	−15.07 (5.82)	0.01	0.71
Unknown 229935	21.41 (8.79)	0.01	0.95
Unknown 102122	40.66 (18.83)	0.03	0.99
Unknown 31764	−11.34 (5.45)	0.04	0.99
Unknown 3258	−11.15 (5.50)	0.04	0.99
Unknown 191801	−10.90 (5.41)	0.04	0.99

Abbreviations: 4HCY/CY AUC, 4 hydroxycyclophosphamide/cyclophosphamide area under the curve; AIBW, adjusted ideal body weight; EMC, endogenous metabolomics compound; FDR, false discovery rate.

[a]Beta coefficients and standard errors obtained from univariate linear regression models evaluated the association between center-log ratio EMC abundances and the ratio of 4HCY/CY AUC adjusted for age, sex, and AIBW.

[b]The *p*-values listed in descending order. There were no EMCs significant at FDR <0.1. Unknown EMC represent identifiers from Bin Base database.[20]

TABLE 3 Pre-CY plasma EMCs associated ($p < 0.05$) with the ratio of 4HCY/CY AUC in cohort 2 ($n = 26$ samples)

EMCs	Coefficient (SE)[a]	*p* value[b]	FDR
Unknown 87822	−7.45 (2.30)	0.001	0.29
Unknown 4928	13.14 (4.36)	0.003	0.29
Unknown 168793	−10.94 (3.64)	0.003	0.29
Unknown 33142	6.21 (2.12)	0.003	0.29
Unknown 418155	−10.04 (3.44)	0.003	0.29
Unknown 351222	4.50 (1.54)	0.004	0.29
Unknown 105538	−7.24 (2.59)	0.01	0.32
Unknown 41750	−6.86 (2.45)	0.01	0.32
Unknown 380761	−7.15 (2.62)	0.01	0.32
Unknown 346248	8.29 (3.05)	0.01	0.32
Methionine sulfoxide	−4.76 (1.77)	0.01	0.32
Unknown 68	15.67 (5.96)	0.01	0.35
Fructose	−10.45 (4.02)	0.01	0.36
Unknown 26717	5.78 (2.31)	0.01	0.43
Unknown 14736	3.40 (1.39)	0.01	0.43
Unknown 109997	4.42 (1.82)	0.02	0.43
Unknown 1771	9.08 (3.75)	0.02	0.43
Glucose1phosphate	−7.56 (3.13)	0.02	0.43
Glutamine	8.44 (3.57)	0.02	0.48
Unknown 110	7.33 (3.20)	0.02	0.55
Unknown 408463	13.38 (6.04)	0.03	0.61
Unknown 64546	−6.88 (3.14)	0.03	0.61
Unknown 390122	−6.81 (3.13)	0.03	0.61
9-Myristoleate	−9.50 (4.47)	0.03	0.61
Tagatose	−4.28 (2.02)	0.03	0.61
Lauric acid	13.59 (6.53)	0.04	0.61
Unknown 6353	5.10 (2.45)	0.04	0.61
Glutamic acid	6.15 (2.99)	0.04	0.61
Unknown 21817	11.59 (5.63)	0.04	0.61
Unknown 377299	2.84 (1.38)	0.04	0.61
Unknown 2083	−14.33 (7.08)	0.04	0.61
Unknown 408421	−5.34 (2.72)	0.049	0.61

Abbreviations: 4HCY/CY AUC, 4 hydroxycyclophosphamide/cyclophosphamide area under the curve; AIBW, adjusted ideal body weight; CY, cyclophosphamide; EMC, endogenous metabolomics compound; FDR, false discovery rate.

[a]Beta coefficients and standard errors obtained from univariate linear regression models evaluated the association between the center-log ratio EMC abundances and the ratio of 4HCY/CY AUC adjusted for age, sex, and AIBW.

[b]The *p*-valalues listed in descending order. There were no EMCs significant at FDR <0.1. Unknown EMC represent identifiers from Bin Base database.[20]

starch and sucrose metabolism, glutamine and glutamate metabolism, nitrogen metabolism, beta-alanine metabolism, and pantothenate and CoA biosynthesis, all p less than 0.05.

DISCUSSION

In this analysis, we took a first step toward identifying plasma EMCs associated with the ratio of the 4HCY/CY AUC with the long-range goal of furthering our understanding of CY 4-hydroxylation and personalizing CY dosing using pharmacometabonomics. Furthermore, these data demonstrate that changes in the plasma metabolome occur from the pre-HCT time point to 24-h after the first PT-CY dose (an ~21-day time period). Although there were a few EMCs that were significantly associated with the ratio of the 4HCY/CY AUC individually, there was no consistency across time points or between cohorts, and there were no pathways that were significantly associated with any time point.

CY is used to treat a multitude of diseases, ranging from autoimmune disorders[28,29] to cancer to nonmalignant conditions treated with HCT.[9,14] With the increasing use of PT-CY in alternative donor HCT, it is likely that CY use will expand. With the increase in alternative donor HCT, there are more choices in the type of donor, which has made it increasingly difficult for sufficiently

powered PK/pharmacodynamic (PD) studies that evaluate the association of the AUC of CY and its metabolites with clinical outcomes at a single HCT center.[9] Recent preclinical data showed that mid-range PT-CY doses are associated with less GVHD; the precise mechanism(s) regarding how CY and its active metabolites transport into the T-cells of interest, which are Treg and Tcon here, are unknown. Thus, in hopes of minimizing GVHD rates, gaining a mechanistic understanding of factors associated with the ratio of 4HCY/CY AUC is needed. With the short half-life of 4HCY in blood (~3 min), rapid processing at the patient's bedside is needed to sufficiently stabilize 4HCY to characterize its PKs. Conducting PK/PD studies to test this hypothesis would be time- and resource-intensive because a multicenter study would be needed to achieve sufficient power.[9] Thus, we took the first steps toward evaluating if a simple plasma sample can provide insight regarding an individual patient's ratio of 4HCY/CY AUC. A similar approach of obtaining data from one blood sample to evaluate constitutional pharmacogenomics has been taken.[10] Numerous studies have evaluated the constitutional pharmacogenomics of drug-metabolizing enzymes and transporters involved in CY PKs.[10] No consistent genotype-phenotype association has been found with the PKs, efficacy, or toxicity of CY.[10,11] Thus, there is a continued need to identify plasma biomarkers associated with the PKs of CY and its metabolites, especially the resource-intensive ratio of 4HCY/CY AUC.

The plasma AUC of 4HCY is determined by its formation by various cytochrome P450 (CYP) isozymes, and potentially myeloperoxidase, along with its elimination by aldehyde dehydrogenases (ALDH), GST, and ABC transporters (Figure S1).[4] In vitro, CY is metabolized to 4HCY by numerous CYPs (i.e., CYPs 2A6, 2B6, 2C9, 2C18, 2C19, 3A4, and 3A5).[4] In vitro, CY alone and in combination with the often-used antiemetic dexamethasone differentially induces the expression of CYP3A4 and 2B in a concentration-dependent manner, which may be mediated partially through activation of the pregnane X-receptor (PXR).[26] The nuclear receptors PXR and constitutive androstane receptor (CAR) are well-known regulators for xenobiotic biotransformation.[30] CAR has differential roles in the regulation of CY-induced drug-metabolizing enzyme expression and potential drug-drug interactions.[31] With the promiscuity of these orphan nuclear receptors demonstrating broad interactions with a multitude of exogenous and endogenous substances, we hypothesized that the plasma EMCs may provide insight into the 4-hydroxylation of CY.

PK/PD studies have yet to be conducted when CY is used to treat autoimmune diseases, despite the knowledge that the current methods of dosing CY led to considerable interpatient variability in systemic exposure to CY and its metabolites. CY is also susceptible to numerous drug-drug interactions, with patients potentially taking several drugs altering CY PKs at the same time.[9] We had previously demonstrated that approximately half of the patients who undergo HCT have a ratio of 4HCY/CY AUC that is consistent over a couple of weeks (Figure S2).[9] However, there was no clear difference between those patients having a consistent ratio from those having an inconsistent ratio of 4HCY/CY AUC. We hypothesized that plasma EMCs may provide insight for factors associated with the ratio of 4HCY/CY AUC, however, we observed a similar trend with longitudinal changes in the plasma EMCs. Although our data demonstrate the feasibility of longitudinal metabolomics studies, it is limited by the small sample size. Metabolomic studies in preclinical studies with knockout of PXR or CAR may provide insight regarding factors influencing the ratio of 4HCY/CY AUC. Discoveries made using metabolomics are yielding new insights into how EMCs influence organ function, immune function, and gut physiology.[32] Collectively, these works could lead to a system-wide perspective of allogeneic HCT biology wherein EMCs, proteins, and genes are understood to interact synergistically to modify the functions within the allogeneic HCT recipient. These studies, as well as our own evaluating the association of the plasma EMCs with i.v. busulfan clearance,[15,33] highlights the opportunity pharmacometabonomics could offer to improve clinical outcomes. We chose to focus on the alkylating agent CY because of its increasing use of GVHD prophylaxis and preclinical data showing that moderate doses may be effective.

Further studies in larger patient populations are needed to improve the efficacy and toxicity of CY. In addition to evaluating the EMCs, consideration should be given to evaluating the intestinal microbiome and plasma bile acid concentrations. Activation of PXR and CAR suppresses bile acid-metabolizing bacteria in the intestine and modulates bile acid homeostasis in a gut microbiota-dependent manner. Bile acids, particularly lithocholate, inhibit GSTs,[34] which is involved in 4HCY elimination. Thus, variability in bile acid composition may affect GST activity and, in turn, 4HCY elimination. Consideration of drug-drug interactions must also be considered, as the most promising pathways from our hypothesis-generation cohort (Methods S1)—which received phenytoin that is well-known to affect the ratio of 4HCY/CY AUC[16]—were not replicated in these cohorts. Notably, all of our participants were taking ursodiol, which reduces the risk of hepatic complications in patients who undergo HCT.[35] The impact of ursodiol administration on the plasma EMCs or on GST activity in HCT recipients has yet to be characterized. In addition, the composition of the human gut microbiome may directly modulate host gene expression and

detoxifying enzyme activities, such as CYPs.[36] We suggest that future studies involving the PKs of CY and its metabolites also collect plasma EMC and intestinal microbiome samples with the long-range goal of improving the safety and efficacy of CY.

Our dataset was limited in its sample size and thus power, although it does represent a contemporary patient population receiving PT-CY, and the global pharmacometabonomic panel provided high accuracy of EMC identification and relative abundances within relevant pathways. The results show the feasibility of conducting metabolomics studies in allogeneic HCT, with the hope of gaining mechanistic insight into the pathophysiology of relapse and/or GVHD while improving clinical outcomes. Future studies with larger sample sizes (and thus, more relapse and GVHD events) are needed, as they would allow for the inclusion of risk factors for relapse (e.g., cytogenetics) or GVHD (e.g., HLA).

CONFLICT-OF-INTEREST

The authors declared no competing interests for this work.

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AUTHOR CONTRIBUTIONS

J.S.M., R.N., D.O., T.W.R., B.M.S., A.K., D.H., and S.L.N. wrote the manuscript. J.S.M. and T.W.R. designed the research. J.S.M., T.W.R., D.O., A.K., R.N., and S.L.N. performed the research. J.S.M., T.W.R., D.O., A.K., R.N., and S.L.N. analyzed the data.

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SUPPORTING INFORMATION

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