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Impact of methotrexate-dosing regimens for GVHD prophylaxis on clinical outcomes of HLA-matched allogeneic HSCT

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Summary

Severe graft-versus-host disease (GVHD) remains a major complication of allogeneic haematopoietic stem cell transplantation (allo-HSCT), necessitating optimal immunosuppressive strategies. This retrospective study used data from the Japanese Transplant Registry Unified Management Program to compare three methotrexate (MTX)-dosing regimens for GVHD prophylaxis in patients undergoing human leucocyte antigen (HLA)-matched allo-HSCT: a low-dose 3-day regimen (Ld3:10 mg/m^2 on day 1, 7 mg/m^2 on days 3 and 6), a low-dose 4-day regimen (Ld4: Ld3 with an additional 7 mg/m^2 on day 11) and an original-dose 3-day regimen (Od3: 15 mg/m^2 on day 1, 10 mg/m^2 on days 3 and 6). Among 2537 analysed patients, Ld3 was the most commonly used regimen. Multivariate analyses showed no significant differences in the cumulative incidence of grade II–IV acute GVHD among regimens. However, Od3 was associated with an increased risk of grade III–IV acute GVHD, and Ld4 was linked to delayed neutrophil engraftment. This study is the first large-scale retrospective analysis of the impact of different MTX-dosing regimens on the outcomes of HLA-matched allo-HSCT, providing valuable insights into optimal MTX-dosing strategies in clinical practice.

KEYWORDS

allo-HSCT, dosing regimens, graft-versus-host disease, GVHD prophylaxis, methotrexate

INTRODUCTION

Severe graft-versus-host disease (GVHD) is one of the major complications of allogeneic haematopoietic stem cell transplantation (allo-HSCT) and is associated with increased non-relapse mortality (NRM) and reduced quality of life.[1] However, excessive immunosuppression for GVHD prevention can weaken donor-derived antitumor immunity[2,3] and increase the risk of infections. Therefore, optimal control of immunosuppression is crucial to improve transplantation outcomes.

Since the efficacy of GVHD prophylaxis with methotrexate (MTX) and ciclosporin (CyA) was initially reported,[4] a combination of MTX and calcineurin inhibitor (CNI) such as CyA or tacrolimus (Tac)[5] has become the standard approach. MTX dosing was originally developed at 15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6 and 11 (referred to as the original dose in this study)[4]; however, the adverse effects

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of MTX, such as mucositis, nephrotoxicity and delayed engraftment, have prompted efforts to replace MTX with other agents, such as mycophenolate mofetil[6] or to optimize the dosing of MTX.[7–11] Specific strategies include omitting the day 11 dose of MTX, reducing the dose per administration or a combination of both.

In Japan, a lower dose, 3-day MTX-dosing regimen of 10 mg/m^2 on days 1 and 7 mg/m^2 on days 3 and 6 has become the most common regimen for human leucocyte antigen (HLA)-matched transplantation, although other MTX-dosing regimens are also in use, leading to variability in clinical practice. However, this regimen lacks sufficient evidence. Furthermore, there are no prospective data comparing the effectiveness of different MTX-dosing regimens in preventing GVHD, and retrospective studies are limited.

Moreover, the development of novel GVHD prophylaxis strategies, such as post-transplant cyclophosphamide (PTCy)[12] and other emerging agents,[13–16] has made designing prospective clinical trials to investigate MTX dosing increasingly challenging. Consequently, robust evidence regarding MTX dosing derived from real-world practice, albeit retrospective, has become increasingly essential.

This study aimed to understand the current practices of MTX-based GVHD prophylaxis in Japan by retrospectively evaluating the impact of different MTX-dosing regimens on clinical outcomes. Furthermore, to evaluate the effects of MTX dosing on clinical outcomes while minimizing confounding factors, in this study, we only included patients who underwent HLA-matched allo-HSCT between 2019 and 2022. By restricting our cohort to this recent and concise timeframe, we reduced variability in factors such as conditioning regimen trends and supportive care, thereby enhancing the applicability of our findings to current practice.

METHODS

Patient data were obtained from the Transplant Registry Unified Management Program (TRUMP) of the Japanese Data Centre for Haematopoietic Cell Transplantation sponsored by the Japanese Society for Transplantation and Cellular Therapy.[17,18] This study was approved by the data management committee of the TRUMP and the Institutional Review Board of Nagoya City University Hospital.

Patients meeting the following criteria were included from the TRUMP database: (i) received allo-HSCT from a fully HLA-matched donor for the treatment of haematological malignancies between 2019 and 2022, (ii) used either bone marrow (BM) or peripheral blood stem cells (PBSC) as a stem cell source and (iii) received MTX and a CNI as GVHD prophylaxis. Patients were excluded if they satisfied any of the following criteria: (i) 15 years old or younger, (ii) history of prior allo-HSCT, (iii) received GVHD prophylaxis in addition to MTX and CNI or (iv) died within 11 days after allo-HSCT. HLA matching was assessed using serological data for related donors at the HLA-A, -B and -DRB1 antigens and allele-level data for unrelated donors at the HLA-A, -B, -C and -DRB1 loci.[19]

In this study, the MTX-dosing regimens were defined as follows: (i) low-dose 3 (Ld3): 10 mg/m^2 on day 1, followed by 7 mg/m^2 on days 3 and 6; (ii) low-dose 4 (Ld4): 10 mg/m^2 on day 1, followed by 7 mg/m^2 on days 3, 6 and 11; and (iii) original dose 3 (Od3): 15 mg/m^2 on day 1, followed by 10 mg/m^2 on days 3 and 6. The other MTX-dosing regimens were not specifically defined in this study.

Definitions of end-points

The main end-points of this study were the cumulative incidence rates of acute and extensive chronic GVHD, followed by cumulative engraftment rates of neutrophils and platelets, NRM, relapse rate, overall survival (OS) and incidence of severe infections.

The diagnosis and grading of acute and chronic GVHD followed the standard criteria[20,21] and were performed by clinicians at each institution. Neutrophil engraftment was defined as achieving an absolute neutrophil count of 0.5×10^9/L for three consecutive days. Platelet engraftment was defined as achieving a platelet count of 50×10^9/L × without transfusion for seven consecutive days. Severe infections were defined as bacteraemia confirmed by blood culture tests or invasive fungal infections caused by *Candida*, *Toxoplasma*, *Aspergillus*, *Mucor*, *Cryptococcus*, *Fusarium* or *Trichosporon* that occurred within 100 days of allo-HSCT.

Statistical methods

Clinical characteristics among MTX regimen groups were compared using Fisher's exact test for categorical variables and the Kruskal–Wallis test for continuous variables. The probabilities of acute and extensive chronic GVHD, and of neutrophil and platelet engraftment, were estimated using cumulative incidence curves. Competing risks were defined as follows: death or relapse before GVHD for GVHD, death before engraftment for engraftment, death without relapse for relapse and relapse for NRM.

To evaluate the appropriateness of pooling BM and PBSC recipients (defined as BM and PBSC groups respectively), we fitted multivariable models for GVHD incidence and engraftment outcomes that included a two degree of freedom interaction term between graft source (BM and PBSC) and MTX-dosing regimen and assessed the statistical significance of this interaction. If the interaction was not significant, subsequent analyses stratified by graft source were presented as subgroup analyses. The detailed methodology for the multivariate analyses is presented in Supporting Methods S1.

Subgroup analyses for GVHD within clinically relevant factors were conducted in cases where the overall comparison of the three MTX-dosing regimens was statistically

TABLE 1 Baseline clinical characteristics.

		MTX-dosing subgroup			
	Total	**Ld3**	**Ld4**	**Od3**	
	***N*=2537**	***N*=1473**	***N*=613**	***N*=451**	***p*-value***
Median age, years (IQR)	51.0 (41.0-60.0)	51.0 (40.0-60.0)	52.0 (43.0-60.0)	50.0 (41.0-60.0)	0.029
Male:female ratio (%)	58:42	59:41	59:41	55:45	0.45
Performance status, *n* (%)					0.66
0 or 1	2439 (96.1)	1417 (96.2)	586 (95.6)	436 (96.7)	
≥2	96 (3.8)	54 (3.7)	27 (4.4)	15 (3.3)	
Missing	2 (0.1)	2 (0.1)	0 (0.0)	0 (0.0)	
HCT-CI score, *n* (%)					0.22
<3	2163 (85.3)	1242 (84.3)	536 (87.4)	385 (85.4)	
≥3	369 (14.5)	228 (15.5)	77 (12.6)	64 (14.2)	
Missing	5 (0.2)	3 (0.2)	0 (0.0)	2 (0.4)	
Disease risk, *n* (%)					0.7
Standard risk	1547 (61.0)	911 (61.8)	359 (58.6)	277 (61.4)	
Advanced risk	984 (38.8)	559 (37.9)	252 (41.1)	173 (38.4)	
Missing	6 (0.2)	3 (0.2)	2 (0.3)	1 (0.2)	
Diagnosis, *n* (%)					0.32
AML	1100 (43.4)	652 (44.3)	257 (41.9)	191 (42.4)	
ALL	491 (19.4)	291 (19.8)	111 (18.1)	89 (19.7)	
MDS	455 (17.9)	260 (17.7)	105 (17.5)	90 (20.0)	
Lymphoid malignancy	311 (12.3)	174 (11.8)	85 (13.9)	52 (11.5)	
CML	79 (3.1)	47 (3.2)	24 (3.9)	8 (1.8)	
MPD/MPN	101 (4.0)	49 (3.3)	31 (5.1)	21 (4.7)	
Intensity of conditioning regimen, *n* (%)					0.24
Myeloablative	1654 (65.2)	966 (65.6)	380 (62.0)	308 (68.3)	
Non-myeloablative	882 (34.8)	506 (34.4)	233 (38.0)	143 (31.7)	
Missing	1 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	
TBI for conditioning, *n* (%)	1367 (53.9)	834 (56.6)	331 (54.0)	202 (44.8)	<0.001
Donor type (%)					<0.001
Related:unrelated	43.0:57.0	51.2:48.8	24.1:75.9	42.1:57.9	
Blood type combination, *n* (%)					0.12
Match or minor mismatch	1844 (72.7)	1091 (74.1)	442 (72.1)	311 (69.0)	
Major or major/minor mismatch	689 (27.2)	381 (25.9)	170 (27.7)	138 (30.6)	
Missing	4 (0.2)	1 (0.1)	1 (0.2)	2 (0.4)	
Sex combination, *n* (%)					0.051
Female to male	502 (19.8)	313 (21.2)	100 (16.3)	89 (19.7)	
Other combination	2033 (80.1)	1160 (78.8)	512 (83.5)	361 (80.0)	
Missing	2 (0.1)	0 (0.0)	1 (0.2)	1 (0.2)	
Graft source, (%)					<0.001
BM:PBSC	54:46	50:50	64:36	54:46	
Calcineurin inhibitor (%)					<0.001
Tac:CyA	68.3:31.7	66.5:33.5	76.8:23.2	62.3:37.7	

Note: Ld3, 3-day methotrexate (MTX)-dosing regimen of 10 mg/m^2 on day 1, 7 mg/m^2 on days 3 and 6. Ld4, 4-day MTX-dosing regimen of 10 mg/m^2 on day 1, 7 mg/m^2 on days 3, 6 and 11. Od3, 3-day MTX-dosing regimen of 15 mg/m^2 on day 1, 10 mg/m^2 on days 3 and 6.

Abbreviations: ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; BM, bone marrow; CML, chronic myeloid leukaemia; CyA, ciclosporin; HCT-CI, haematopoietic cell transplantation-specific comorbidity index; IQR, interquartile range; MDS, myelodysplastic syndrome; MPD/MPN, myeloproliferative disorder/myeloproliferative neoplasm; MTX, methotrexate; PBSC, peripheral blood stem cell; Tac, tacrolimus; TBI, total body irradiation.

*Patients were compared across the three MTX-dosing regimens.

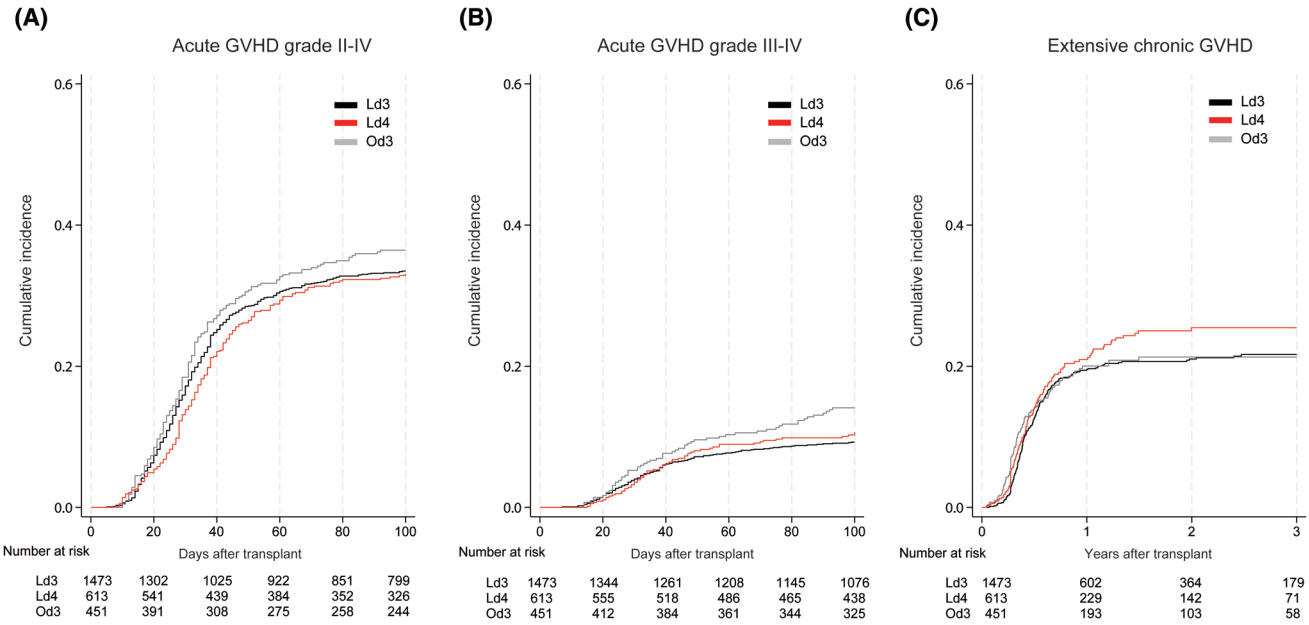

FIGURE 1 Cumulative incidence of graft-versus-host disease (GVHD). (A) Acute GVHD grade II–IV. (B) Acute GVHD grade III–IV. (C) Extensive chronic GVHD.

significant in the multivariable model (omnibus Wald test), and when a pairwise comparison with Ld3 yielded a hazard ratio (HR) whose 95% confidence interval (CI) did not include 1.0. A two-sided p-value of <0.05 was considered statistically significant. All analyses were conducted using Stata version 18 (Stata Corp., Texas, USA).

RESULTS

Patient characteristics

Between 2019 and 2022, 2913 patients who met the eligibility criteria were included in this study. Among them, 1473 (50.5%), 613 (21.0%) and 451 (15.5%) patients received the Ld3, Ld4 and Od3 MTX regimens, respectively, while the remaining patients received other MTX-dosing regimens (Figure S1). Further analyses were performed on only the patients who received the Ld3, Ld4 or Od3 regimens ($n = 2537$ in total). The median follow-up time among survivors at the time of analysis was 1.91 years (interquartile range [IQR] 0.95–3.04). Baseline patient characteristics are shown in Table 1. The median age was 51.0 years (IQR, 41.0–60.0), 58% were men, 65% received myeloablative conditioning, 43% received stem cell grafts from a related donor and 54% received a BM graft. The three most frequent haematological malignancies in this cohort were acute myeloid leukaemia (43.4%), acute lymphoblastic leukaemia (19.4%) and myelodysplastic syndrome (17.9%).

Tables S1 and S2 present the detailed patient characteristics according to the graft source. Notably, the distribution of donor types (related versus unrelated) differed significantly between the BM and PBSC groups. Furthermore, among the MTX-dosing regimens, a higher proportion of patients in the Od3 and Ld4 groups received stem cell grafts from unrelated donors than those in the Ld3 group. Specifically, in the BM group, 77.8%, 79.8% and 96.9% of patients in the Ld3, Od3 and Ld4 groups had unrelated donors, respectively. The corresponding proportions in the PBSC group were 19.4%, 32.5% and 39.0% respectively.

Incidence and risk of GVHD

Because the interaction between graft source and MTX dosing was not significant for any end-point (acute or extensive chronic GVHD and neutrophil or platelet engraftment; Table S3), BM and PBSC recipients were analysed together in the primary analyses. Stratified analyses by graft source were conducted as subgroup analyses. The cumulative incidence rates of acute GVHD at 100 days and extensive chronic GVHD at 3 years are shown in Figure 1. Data stratified by graft source are presented in Figure S2. Table S4 summarizes GVHD incidence stratified by graft source and MTX regimen groups, including the cumulative incidence rates of acute GVHD with the corresponding organ stage details, as well as that of extensive chronic GVHD. The cumulative incidence (%) of acute GVHD grade II–IV/grade III–IV at 100 days was 33.4/9.3, 33.0/10.7 and 36.4/14.1 in Ld3, Ld4 and Od3 groups respectively. The cumulative incidence (%) of extensive chronic GVHD at 3 years was 21.7, 25.4 and 21.3 in the Ld3, Ld4 and Od3 groups respectively. Gut GVHD accounted for most cases of severe acute GVHD, and this trend was consistent across all MTX regimen groups (Table S4).

Multivariate analyses of the cumulative incidence of GVHD are shown in Table 2 (acute GVHD) and Table S5 (extensive chronic GVHD) respectively. No significant

TABLE 2 Multivariate analyses of severe acute GVHD.

	Acute GVHD grade II–IV			Acute GVHD grade III–IV		
Factors	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
MTX dose group[a]			0.289			0.013
Ld3	Ref	—		Ref	—	
Od3	1.139	0.949–1.367		1.543	1.148–2.074	
Ld4	0.974	0.827–1.149		1.262	0.948–1.680	
Age	Not selected			Not selected		
<60 years						
≥60 years						
Sex			0.112			0.002
Male	Ref	—		Ref	—	
Female	0.884	0.760–1.029		0.679	0.528–0.872	
PS			0.170	Not selected		
<2	Ref	—				
2 or more	0.760	0.513–1.125				
HCT-CI score			0.400	Not selected		
<3	Ref	—				
3 or more	0.919	0.756–1.118				
Disease risk			0.126			0.001
Standard	Ref	—		Ref	—	
High	1.115	0.970–1.282		1.490	1.173–1.892	
Intensity of conditioning	Not selected					0.132
Myeloablative				Ref	—	
Non-myeloablative				1.208	0.945–1.544	
TBI for conditioning			0.108	Not selected		
Not used	Ref	—				
Used	1.120	0.975–1.286				
Donor	Not selected			Not selected		
Related donor						
Non-related donor						
Graft source			0.846			0.015
BM	Ref	—		Ref	—	
PBSC	0.986	0.859–1.133		1.347	1.059–1.714	
Blood type combination			0.424			0.284
Minor or no mismatch	Ref	—		Ref	—	
Major or major minor mismatch	1.063	0.915–1.237		1.150	0.891–1.483	
Sex combination			0.556	Not selected		
Female to male	Ref	—				
Other combination	1.059	0.876–1.279				
Calcineurin inhibitor	Not selected			Not selected		
Tac						
CyA						

Note: Not selected: variables excluded from the final model by stepwise selection based on the Akaike information criterion. Ld3, 3-day methotrexate (MTX)-dosing regimen of 10 mg/m^2 on day 1, 7 mg/m^2 on days 3 and 6. Ld4, 4-day MTX-dosing regimen of 10 mg/m^2 on day 1, 7 mg/m^2 on days 3, 6 and 11. Od3, 3-day MTX-dosing regimen of 15 mg/m^2 on day 1, 10 mg/m^2 on days 3 and 6.

Abbreviations: BM, bone marrow; CI, confidence interval; CyA, ciclosporin; GVHD, graft-versus-host disease; HCT-CI, haematopoietic cell transplantation-specific comorbidity index; MTX, methotrexate; PBSC, peripheral blood stem cell; PS, performance status; Ref, reference; Tac, tacrolimus; TBI, total body irradiation.

[a]Omnibus Wald test for MTX-dosing groups (Ld3, Ld4, Od3): $\chi^2(2) = 2.48$, $p = 0.289$ (acute GVHD grade II–IV); $\chi^2(2) = 8.73$, $p = 0.013$ (acute GVHD grade III–IV).

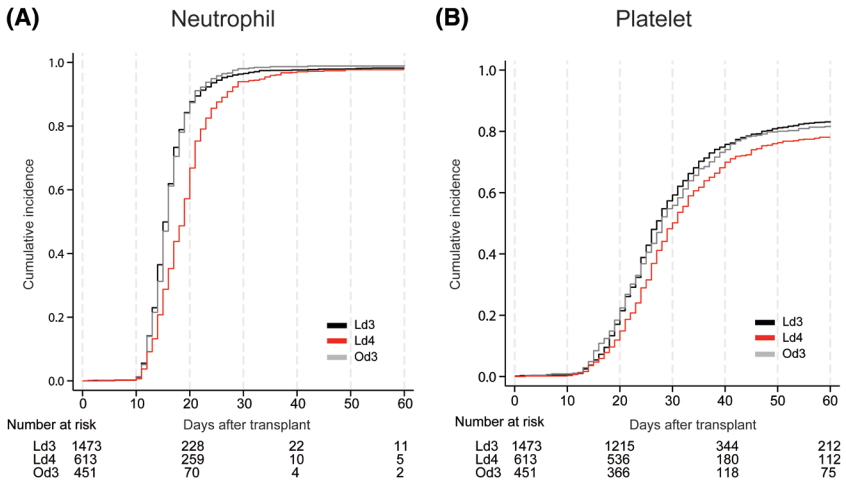

FIGURE 2 Cumulative incidence of engraftment. (A) Neutrophil engraftment. (B) Platelet engraftment.

associations were found between the MTX-dosing regimen and the cumulative incidence of acute GVHD grade II–IV or with extensive chronic GVHD. However, MTX-dosing regimens were significantly associated with the incidence of acute GVHD grade III–IV (omnibus Wald test, $p = 0.013$); specifically, the Od3 regimen was associated with an increased risk compared with Ld3 (HR 1.54, 95% CI 1.15–2.07). This association was consistent across both the BM and PBSC groups (Table S6). Subgroup analyses of acute GVHD grade III–IV showed that the higher risk associated with Od3 compared to that associated with Ld3 was consistent across all clinical factors, including patient age, sex, disease risk, intensity of conditioning, graft source and donor type (Figure S3).

Engraftment and other end-points

Cumulative probabilities of neutrophil and platelet engraftment are shown in Figure 2. The median duration between stem cell infusion and neutrophil engraftment was 16 (IQR 14–18), 19 (IQR 15–21) and 16 (IQR 14–18) days for Ld3, Ld4 and Od3 respectively. The corresponding duration for platelet engraftment was 27 (IQR 21–38), 30 (IQR 24–45) and 28 (IQR 21–41) days for Ld3, Ld4 and Od3 respectively. Data stratified by graft source are presented in Figure S4.

Table 3 summarizes the results of the multivariate analyses for the engraftment of neutrophils and platelets. In multivariate analyses, the MTX-dosing regimens showed a significant effect on neutrophil engraftment (omnibus Wald test, $p < 0.001$). Specifically, the Ld4 regimen was associated with poor engraftment compared with Ld3 (HR 0.69, 95% CI 0.63–0.75); this association was consistent across both the BM and PBSC groups (Table S7). Overall, MTX-dosing regimens were not significantly associated with poor platelet engraftment. However, in an overall comparison, MTX-dosing regimens were significantly associated with poor platelet engraftment within the BM group (omnibus Wald test, $p = 0.036$). In this group, the Ld4 regimen was associated with poor platelet engraftment compared with Ld3 (HR 0.84, 95% CI 0.73–0.96); however, no such association was observed in the PBSC group (Table S7). No significant differences in the incidence of severe infections were observed among the three MTX regimen groups (2.1%, 2.8% and 2.9% of patients in the Ld3, Ld4 and Od3 groups respectively; $p = 0.51$). This trend was consistent across both BM and PBSC groups (data not shown).

Next, we evaluated the impact of MTX dosing on engraftment among selected patients who received BM graft with a sufficient total nucleated cell count ($\geq 2 \times 10^8$/kg) at BM collection and received Ld3 or Ld4 regimen. Ld4 remained a significant risk factor for poor neutrophil engraftment compared to Ld3 in this subgroup (HR 0.61, 95% CI 0.53–0.71) (Table S8).

Finally, we evaluated the effects of the MTX-dosing regimen on OS, relapse and NRM. Multivariate analyses revealed no significant differences in post-transplantation outcomes among the three MTX regimen groups (Figure S5; Tables S9–S11).

DISCUSSION

To the best of our knowledge, this study is one of the largest retrospective studies comparing the clinical outcomes of different MTX-dosing regimens in patients undergoing fully HLA-matched transplantation. The most important finding of the current study was that neither an increased MTX dose as in Od3 nor extended administration as in Ld4 clearly demonstrated a risk reduction of GVHD compared to Ld3.

A key challenge in MTX-based GVHD prophylaxis is the limited evidence on how different MTX-dosing regimens affect clinical outcomes. Originally, MTX prophylaxis for GVHD was introduced at a dose of 15 mg/m^2 on day 1, followed by 10 mg/m^2 on days 3, 6 and 11.[4] Several MTX-dosing regimens with lower doses and/or shorter courses have been developed to mitigate MTX-associated toxicities. However, the significance of MTX administration on day 11 remains controversial. Some studies have reported increased

TABLE 3 Multivariate analyses of neutrophil and platelet engraftment.

	Neutrophil engraftment			Platelet engraftment		
Factors	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
MTX-dosing group[a]			<0.001			0.308
Ld3	Ref	—		Ref	—	
Od3	1.052	0.949–1.167		1.005	0.895–1.130	
Ld4	0.689	0.630–0.754		0.927	0.837–1.027	
Age	Not selected			Not selected		
<60 years						
≥60 years						
Sex			0.088	Not selected		
Male	Ref	—				
Female	0.927	0.850–1.011				
PS			0.001			<0.001
<2	Ref	—		Ref	—	
2 or more	0.671	0.534–0.843		0.506	0.389–0.658	
HCT-CI score			0.285			0.025
<3	Ref	—		Ref	—	
3 or more	0.937	0.831–1.056		0.859	0.753–0.981	
Disease risk			<0.001			<0.001
Standard	Ref	—		Ref	—	
High	0.814	0.750–0.884		0.690	0.628–0.757	
Intensity of conditioning			<0.001			0.046
Myeloablative	Ref	—		Ref	—	
Non-myeloablative	0.837	0.771–0.909		0.909	0.828–0.998	
TBI for conditioning	Not selected			Not selected		
Not used						
Used						
Donor			0.064			<0.001
Related	Ref	—		Ref	—	
Unrelated	0.892	0.790–1.007		0.750	0.671–0.837	
Graft source			<0.001			<0.001
BM	Ref	—		Ref	—	
PBSC	1.691	1.529–1.870		1.481	1.331–1.648	
Blood type combination			0.018			0.003
Minor or no mismatch	Ref	—		Ref	—	
Major or major minor mismatch	0.899	0.823–0.982		0.863	0.785–0.950	
Sex combination			0.003			<0.001
Female to male	Ref	—		Ref	—	
Other combination	1.184	1.060–1.322		1.253	1.120–1.402	
Calcineurin inhibitor			0.002	Not selected		
Tac	Ref	—				
CyA	0.841	0.756–0.936				

Note: Not selected: variables excluded from the final model by stepwise selection based on the Akaike information criterion. Ld3, 3-day methotrexate (MTX)-dosing regimen of 10 mg/m^2 on day 1, 7 mg/m^2 on days 3 and 6. Ld4, 4-day MTX-dosing regimen of 10 mg/m^2 on day 1, 7 mg/m^2 on days 3, 6 and 11. Od3, 3-day MTX-dosing regimen of 15 mg/m^2 on day 1, 10 mg/m^2 on days 3 and 6.

Abbreviations: BM, bone marrow; CI, confidence interval; CyA, ciclosporin; GVHD, graft-versus-host disease; HCT-CI, haematopoietic cell transplantation-specific comorbidity index; MTX, methotrexate; PBSC, peripheral blood stem cell; PS, performance status; Ref, reference; Tac, tacrolimus; TBI, total body irradiation.

[a]Omnibus Wald test for MTX-dosing groups (Ld3, Ld4, Od3): $\chi^2(2) = 83.45$, $p < 0.001$ (neutrophil engraftment); $\chi^2(2) = 2.35$, $p = 0.308$ (platelet engraftment).

acute GVHD with its omission,[22] while others have not.[23] These studies are limited by small, retrospective cohorts and confounding due to toxicity-driven MTX omission. The interpretation of prospective trials is also difficult owing to concurrent GVHD prophylactic agents.[14]

Because no significant interaction was found between MTX-dosing regimen and graft source with respect to GVHD outcomes, the primary analyses pooled BM and PBSC groups. However, differences in GVHD risk between BM and PBSC grafts—particularly for chronic GVHD—have been well documented.[24–26] Given the critical role of graft source in shaping GVHD prophylaxis strategies, comprehensive subgroup analyses stratified by graft source were also conducted. In the current study, the Od3 regimen, which involves a higher dose of MTX than the most commonly used Ld3 regimen in Japan, tended to increase the frequency of acute GVHD grade III–IV. This trend was consistently observed across subgroups defined by key clinical factors (Figure S3). This finding was contrary to our expectation that a higher dose of MTX could effectively prevent GVHD.[14] One possible explanation is that the relatively higher MTX dose used in Od3 may cause greater tissue damage, indirectly triggering GVHD,[27–29] compared to the Ld3 regimen. However, in this study, we did not collect data on MTX-related tissue damage—such as mucositis, diarrhoea or hepatic injury—during the period from transplantation to engraftment, which prevented a direct evaluation of this hypothesis. Moreover, no significant advantages were observed in terms of engraftment, relapse rates, NRM or OS. Based on these findings, increasing the MTX dose, as in Od3, may offer little advantage for allo-HSCT from HLA-matched donors.

The Ld4 regimen, with a longer MTX administration period than the Ld3 regimen, was not significantly associated with changes in the risk of acute or extensive chronic GVHD. As expected, haematopoietic engraftment was delayed in the Ld4 group, particularly in the BM group. Notably, even when the number of infused nucleated cells was sufficient, neutrophil engraftment was significantly delayed in the BM group. While the delay in neutrophil engraftment did not clearly translate into increased severe infectious events, in patients who were considered to have a high risk of infection during the nadir phase, the benefit of extending MTX administration, as in the Ld4 regimen, may be limited.

This study also showed that Ld3 was the most commonly used MTX-dosing regimen across graft sources, despite dosing variations in Japan. Unrelated donors more often provided BM grafts, while related donors commonly provided PBSC grafts (Table S2), reflecting Japan's delayed adoption of PBSC transplants from unrelated donors. Since the Japan Marrow Donor Program began facilitating such transplants in 2010, their use has remained limited but gradually increased.

A previous meta-analysis of randomized controlled trials demonstrated that the addition of anti-thymocyte globulin (ATG) to CNI and MTX significantly reduced the incidence of both severe acute and chronic GVHD, without adversely affecting OS or increasing NRM.[30] While these findings support the efficacy of ATG, its optimal dosing and timing remain unclear and may vary depending on institutional practices and patient characteristics. In the present study, we excluded patients who received ATG for GVHD prophylaxis to minimize heterogeneity and to better isolate the effects of the MTX-dosing regimen under investigation. Importantly, based on its success in HLA-haploidentical transplantation, the role of PTCy for GVHD prophylaxis in HSCT from HLA-matched donors has been increasingly explored.[12,31] Although PTCy may emerge as one of the new standards for GVHD prophylaxis in this setting, further validation is required. Furthermore, new agents for GVHD prophylaxis have been developed, in addition to the backbone regimen of MTX plus CNI.[14,16] Therefore, optimization of MTX-based GVHD prophylaxis requires continuous evaluation.

This study has some limitations. First, patient backgrounds differed significantly between MTX-dosing groups. Although adjusted by multivariate analyses, residual confounding may remain: for example, institutional policies likely influenced MTX-dosing selection, introducing facility-level bias. Second, some patients initially assigned to a 4-day regimen (Ld4 or original dose) may have switched to a 3-day regimen (Ld3 or Od3) owing to adverse events, potentially biasing group comparisons. Patients receiving the original dose were excluded owing to small numbers, but comparisons between Ld3 versus Od3 and Ld3 versus Ld4 suggest that Ld3 is not inferior in preventing GVHD or facilitating engraftment. Third, although factors other than MTX-dosing regimens were significant in the multivariate analyses, they were beyond this study's scope and are not discussed in detail; most aligned with clinical expectations. Lastly, the observation period may have been too short to fully assess chronic GVHD, warranting cautious interpretation of related findings.

In conclusion, this study provides a large-scale evaluation of different MTX-dosing regimens for GVHD prophylaxis. In Japan, Ld3 is the most commonly used GVHD prophylaxis regimen. When comparing Ld3 with Ld4 and Od3 across various end-points, no clear disadvantages of Ld3 were identified, supporting its widespread clinical use.

AUTHOR CONTRIBUTIONS

TS and TJ analysed data and drafted the manuscript. JK supervised the study. ND, YK, TN, YO, NA, TF, MS, YH, KS, SO and MT provided patient data. MY and YA managed data from the Transplant Registry Unified Management Program. KY, TK and JK reviewed the initial draft and offered critical feedback. All authors reviewed the manuscript thoroughly and approved the final version for submission.

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CONFLICT OF INTEREST STATEMENT

Dr. Suzuki had honoraria from Sanofi, Chugai Pharmaceutical, Genmab, Astellas Pharma, Janssen Pharmaceutical, Nippon Shinyaku and Pfizer. Dr. Yoshifuji had honoraria from Chugai Pharmaceutical, Novartis, Meiji Seika Pharma and Janssen Pharmaceutical. Dr. Sawa had honoraria from Kyowa Kirin, Chugai, Pfizer, Astellas, Nippon-Shinyaku, Ono, MSD, Bristol-Myers-Squibb, Asahi-kasei, Novartis, Eisai, Otsuka, Sumitomo-Dainippon, Sanofi, Takeda, Mundipharma, abbvie, CSL Behring, SymBio, Janssen, AstraZeneca, DAIICHI SANKYO, Amgen, Novo Nordisk and Nippon Kayaku, had payment for expert testimony from Kyowa Kirin, Takeda, DAIICHI SANKYO, Asahi-kasei and abbvie. Dr. Serizawa had honoraria from Sanofi, Takeda Pharmaceutical and Janssen Pharmaceutical. Dr. Tanaka had grants from Chugai Pharmaceutical, honoraria from Abbvie, Daiichi-Sankyo, Astellas Pharma, Otsuka Pharmaceutical, Asahi Kasei Pharma, Amgen, Nippon Shinyaku, Meiji Seika, Kyowa-kirin, Sumitomo Pharma, Pfizer, MSD, Chugai Pharmaceutical, Janssen Pharmaceutical, Novartis and Takeda Pharmaceutical, participated on Advisory Board of Abbvie and Mochida Pharmaceutical. Dr. Yoshimitsu had honoraria from Takeda Pharmaceutical, Sanofi, Novartis, Chugai Pharmaceutical, PharmaEssentia, Astellas Pharma, Genmab, Ono Pharmaceutical, Daiichi Sankyo, Nippon Shinyaku, Bristol-Myers Squibb, Eisai, Kissei Pharmaceutical, Nippon Kayaku and Meiji Seika Pharma.

DATA AVAILABILITY STATEMENT

The data from this study are not publicly available because of the ethical restrictions that stipulate that providing such data would exceed the scope of the recipient/donor's consent for research use in the registry.

PATIENT CONSENT STATEMENT

All participating patients provided written informed consent for the JSTCT upon registration. Our observational study used data that were de-identified by the JSTCT before being shared with us.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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