haematologica
Journal of the Ferrata Storti Foundation

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Received: June 10, 2026.
Accepted: November 9, 2026.

Citation: Pleun Schonewille, Bram Kottink, Sharon Berendsen, Yousra van der Leest, Klaartje Nijssen, Lotte van der Wagen, Laura Daenen, Viola Kraak, Jan Willem Dankbaar, Arief Lalmohamed, Aurelia de Vries Schultink, Maaike Dirks, Jürgen Kuball and Moniek A. de Witte. Severe and fatal central nervous system toxicity after fludarabine, thiotepa and melphalan conditioning for allogeneic stem cell transplantation.
Haematologica. 2026 Nov 19. doi: 10.3324/haematol.2026.301403 [Epub ahead of print]

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Severe and fatal central nervous system toxicity after fludarabine, thiotepa and melphalan conditioning for allogeneic stem cell transplantation

Pleun Schonewille[1], Bram Kottink[2], Sharon Berendsen[3], Yousra van der Leest[1], Klaartje Nijssen[1], Lotte van der Wagen[1], Laura Daenen[1], Viola Kraak[1], Jan Willem Dankbaar[4], Arief Lalmohamed[2], Aurelia de Vries Schultink[2], Maaike Dirks[3], Jürgen Kuball[1,5], Moniek A. de Witte[1]

[1]Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands
[2]Department of Clinical Pharmacy, University Medical Center Utrecht, Utrecht, The Netherlands
[3]Department of Neurology, University Medical Center Utrecht, Utrecht, The Netherlands
[4]Department of Radiology, University Medical Center Utrecht, Utrecht, The Netherlands
[5]Center for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Corresponding author: Moniek A. de Witte; University Medical Center Utrecht; M.A.deWitte-7@umcutrecht.nl

Author contributions
PS, YL and MAW collected clinical data and neurological complications. SB and MD evaluated neurological complications. BK, AL and AVS provided fludarabine exposure data and developed hypotheses regarding potential pharmacological explanations for the findings. KN contributed to data collection. PS performed analyses on clinical and exposure data and prepared tables and figures. LW, LD, VK, YL and MAW contributed to the patient care. JWD evaluated and provided the radiological data. MAW, JK, PS and YL designed the study. PS and MAW wrote the manuscript. All co-authors revised the manuscript.

Data-sharing agreement
The raw data are not publicly available due to patient privacy and institutional restrictions. Data may be available from the corresponding author upon reasonable request.

Conflict of interest disclosures
JK is inventor on multiple patents dealing with gamma delta-TCRs, ligands, and isolation strategies of engineered immune cells. JK is cofounder and shareholder of Gadeta. JK received research, advisor and clinical study support from Miltenyi Biotec. JK received further research support from Novartis and Gadeta. MAW received grant support from Leukemiefonds.nl and Oncode Accelerator. MAW received research support from Miltenyi Biotec and Novartis. MAW received speakers fee from Miltenyi Biotec, Novartis and AOP Pharma. PS, BK, SB, YL, KN, LW, LD, VK, JWD, AL, AVS and MD have no conflicts of interest to disclose.

Artificial Intelligence
Artificial intelligence (AI) large language models (Claude Opus 5.5 and Fable 5.1, Anthropic; Microsoft 365 Copilot) were used to improve the language and readability of the manuscript and to assist with R coding. The authors reviewed and edited the AI-assisted text and code and take full responsibility for the content of the published article.

Short Title: Severe and fatal central nervous system toxicity after fludarabine, thiotepa and melphalan conditioning for allogeneic stem cell transplantation

Abstract

Neurological complications (NC) are a known risk after allogeneic hematopoietic stem cell transplantation (allo-HSCT). In routine clinical care, we observed unexpected severe noninfectious central nervous system (CNS) events following conditioning with fludarabine, thiotepa and melphalan (Flu-Thio-Mel). To assess the potential association between NC and Flu-Thio-Mel conditioning, we conducted a systematic retrospective evaluation of the frequency, type, location and etiology of NC in adult patients who received $\alpha\beta TCR^+/CD19^+$-depleted allo-HSCT. Only NC considered conditioning-related, based on clinical and diagnostic findings, were included. Outcomes were compared between patients receiving Flu-Thio-Mel (N=55) and patients receiving fludarabine and busulfan (Flu-Bu; N=47) as conditioning regimen for allo-HSCT. Overall, NC were frequent in both cohorts (31% after Flu-Thio-Mel and 30% after Flu-Bu) and most often clinically mild. Peripheral neuropathy was the most common manifestation. In contrast, CNS toxicity was observed exclusively in the Flu-Thio-Mel cohort (n=7), presenting as fatal leukoencephalopathy (n=3, CTCAE grade 5), severe encephalomyelopathy (n=2, CTCAE grade 3−4), and myeloneuropathy (n=2, CTCAE grade 2). Older age, impaired renal function and higher fludarabine exposure may have increased susceptibility to Flu-Thio-Mel–associated CNS toxicity. The occurrence of severe CNS toxicity (CTCAE grade 3−5) in 9% of patients after Flu-Thio-Mel warrants caution and further evaluation of this conditioning regimen, particularly in vulnerable patients.

Introduction

Neurological complications (NC) are a heterogeneous group of adverse events following allogeneic hematopoietic stem cell transplantation (allo-HSCT). They can involve the central nervous system (CNS), peripheral nervous system (PNS), or both.[1-3] Reported incidences vary across studies, from 3% to 44% or more.[1, 2, 4-6]

Common etiologies of NC include infections, inflammatory or immune-mediated processes, neoplasia, and drug-related toxicities.[1, 2, 4, 5] NC caused by toxicity, especially those affecting the CNS, have been linked to morbidity and poorer survival.[1, 3, 5] Manifestations range from mild peripheral neuropathies (3% 5-year cumulative incidence) to headaches (2%) and encephalopathies (5%), among others.[2-4] Reported risk factors for toxicity-related NC include age over 40 years, use of donors other than matched siblings, and graft-versus-host disease (GVHD).[3, 5] Several drugs commonly used in the allo-HSCT setting, such as calcineurin inhibitors, fludarabine, and busulfan are recognized contributors to neurotoxicity.[1, 4, 7, 8]

In our center, we unexpectedly observed several fatal cases of non-infectious NC between 2022 and 2024 after allo-HSCT conditioning using fludarabine, thiotepa and melphalan (Flu-Thio-Mel) during routine clinical care. Flu-Thio-Mel was implemented as a conditioning regimen at our institute for patients with a haploidentical stem cell donor, contraindications to busulfan, or non-malignant diseases. This regimen, in combination with an $\alpha\beta TCR^+/CD19^+$-depleted allograft, has previously demonstrated favorable outcomes and low rates of GVHD.[9]

To address the potential association between Flu-Thio-Mel and conditioning-related NC, we conducted a single-center retrospective study in which we evaluated the occurrence, clinical manifestation and etiology of NC following Flu-Thio-Mel conditioning for allo-HSCT. We compared outcomes with a cohort receiving fludarabine and busulfan (Flu-Bu) conditioning.

Methods

Study design and population

This retrospective study included adult patients who received αβTCR$^+$/CD19$^+$-depleted allo-HSCT at the University Medical Center Utrecht. The Flu-Thio-Mel cohort included all consecutive patients transplanted with Flu-Thio-Mel conditioning in 2018-2024 (N=55). Conditioning consisted of fludarabine (40 mg/m^2 daily, days −8 to −5), thiotepa (5 mg/kg twice, day −4), melphalan (70 mg/m^2 daily, days −3 to −2) and anti-thymocyte globulin (ATG; Grafalon, Neovii; 30 mg/kg divided over days −12 to −9), as previously described.[9] Two patients received a personalized dose of ATG. The Flu-Bu cohort included all patients from the control arm of a prospective cohort, transplanted in 2019-2022 (N=47). Conditioning consisted of fludarabine (40 mg/m^2 daily, days −5 to −2), busulfan (cumulative target AUC 90 mg*h/L, days −5 to −2), and ATG (Thymoglobulin; 1.5 mg/kg daily, days −12 to −9), as previously described.[10] All patients with myelofibrosis received peri-transplant ruxolitinib (5 mg twice daily, days −5 to +14) as GVHD prophylaxis. Patients provided written informed consent for using their data for scientific purposes, in the form of broad informed consent (Flu-Thio-Mel cohort) or study-specific informed consent (Flu-Bu cohort). Both protocols were approved by the ethics committee (reference numbers METC 21-323 and METC 18-382, respectively).

Definitions and data collected

The primary outcome was a potentially conditioning-related NC that was new, recurring or worsening within 1 year after allo-HSCT. Medical records were screened for neurological events by a hematology researcher, consulting a second researcher when needed. A neurologist independently reviewed identified events and established the underlying diagnosis, based on all available diagnostic and clinical data, including neurological examination, MRI, and serum and cerebrospinal fluid (CSF) analysis. Diagnoses inherently indicative of an alternative cause (e.g., infection) and pre-existing stable neurological conditions were classified as unlikely conditioning-related and excluded. NC for which

conditioning could reasonably have contributed to development or aggravation (e.g., neuropathy) were classified unlikely when an alternative cause was documented, probable when conditioning toxicity was documented as the (likely) cause, and possible when neither was documented. Possible and probable NC were included. CNS toxicity was defined as a subset of probable conditioning-related NC affecting the CNS. All patients with CNS toxicity had been evaluated by a consulting neurologist at symptom onset, and had extensive diagnostic work-up available. The CNS events prompted additional review by a second neurologist researcher with discussion until consensus.

For included NC, time of onset, anatomical location, Common Terminology Criteria for Adverse Events (CTCAE) v.5.0 grade, diagnostic tests, treatments received, and neurological outcome at last clinical visit were collected. Standard patient, donor, and transplant characteristics (e.g., age, sex, estimated glomerular filtration rate at time of conditioning (eGFR, Supplementary Methods), donor type, and post-transplant GVHD) and additional variables of interest (e.g., previous use of chemotherapy and potentially neurotoxic co-medication[11]) were obtained from institutional databases and health records.

Fludarabine exposure

Individual fludarabine exposure (F-ara-A $AUC_{0\text{-}192h}$) was calculated using a previously published population pharmacokinetic model based on patient characteristics[12], with sensitivity analyses incorporating available blood samples (Supplementary Methods).

Statistical analysis

Statistical analyses were performed using R v.4.4.1. P-values <0.05 were considered statistically significant.

Results

Patient characteristics

The Flu-Thio-Mel cohort included 55 patients (median age 55 years), and the Flu-Bu cohort included 47 patients (median age 61 years, **Table 1**). Age and eGFR, parameters generally associated with toxicity, were statistically comparable between the cohorts. The proportions of disease indications differed between Flu-Thio-Mel and Flu-Bu cohorts (P=0.001), as a non-malignant disease such as inborn errors of immunity was an indication for Flu-Thio-Mel-based conditioning. Donor match grades differed between cohorts as well (P<0.001). A relatively large proportion of patients in the Flu-Thio-Mel cohort was transplanted with a haploidentical donor, as this was also an indication for Flu-Thio-Mel conditioning. Other characteristics, including previous treatment with chemotherapy, were similar between patients treated with Flu-Thio-Mel and Flu-Bu.

Neurological complications overall

Overall, the presence of NC was similar in both cohorts (**Table 2**; flow diagram of NC attribution in **Supplementary Figure 1**). 17 patients (31%) in the Flu-Thio-Mel cohort and 14 patients (30%) in the Flu-Bu cohort had an NC within one year post-transplant. In contrast, the anatomical locations affected by NC differed between cohorts (P=0.007, **Table 2**). The PNS was affected most frequently, with (poly)neuropathy as the most prevalent type (seven cases after Flu-Thio-Mel (13% of the cohort); nine after Flu-Bu (19%)). Other NC of the PNS included immune-mediated or inflammatory moderate to severe radiculoplexoneuropathy (Flu-Thio-Mel, n=2; Flu-Bu, n=1). In the Flu-Bu cohort, there were two cases with clinical Lhermitte's sign (with undetermined etiology) and single cases of muscle cramps and restless leg syndrome (with undetermined etiology but possibly conditioning-related); all were clinically mild. The CNS was affected only after Flu-Thio-Mel (n=8 (15%)). Complications affecting the CNS presented as lethal leukoencephalopathy or encephalopathy (CNS, n=4), encephalomyelopathy (CNS, n=2), and myeloneuropathy (CNS/PNS, n=2).

Additionally, the severity of NC differed between cohorts (*P*=0.009), as there were more severe events after Flu-Thio-Mel, while all NC after Flu-Bu were mild or moderate (**Table 2**). In the Flu-Thio-Mel cohort, three NC were CTCAE grade 3−4 (5%); two involving the CNS and one involving the PNS. Five NC were grade 5 (9%), four involving the CNS and one involving the PNS. Generally, most NC were mild-moderate with grade 1−2 (Flu-Thio-Mel, 16%; Flu-Bu, 30% of the cohort).

Central nervous system toxicity after fludarabine, thiotepa and melphalan conditioning

We observed seven cases of CNS toxicity among 55 patients in the Flu-Thio-Mel cohort (13%), whereas no such cases were identified in the Flu-Bu cohort (0%; *P*=0.014, Fisher's exact test). As all Flu-Bu recipients had a non-haploidentical donor and a malignant indication, we repeated the comparison in these subgroups. The difference remained among patients without a haploidentical donor (7/35 versus 0/47, *P*=0.002) and those with a malignant indication (5/42 versus 0/47, *P*=0.020). The clinical presentations of CNS toxicity included leukoencephalopathy (n=3), encephalomyelopathy (n=2) and myeloneuropathy (n=2) (clinical details and diagnostic findings are summarized in **Table 3).** In all cases, MRI, CSF (n=5; **Supplementary Table**) and serum laboratory findings did not indicate an infectious, structural, vascular, malignant, metabolic or immune-mediated cause, and there were no signs of thrombotic microangiopathy. The remaining patient with encephalopathy (**Table 2**) was not classified as CNS toxicity, since the etiology was multifactorial with suspected infection and sepsis. In six of the seven patients, CNS toxicity-related symptoms were entirely new. One patient with encephalomyelopathy had pre-existing neuropathy, most likely related to earlier cytarabine treatment, and experienced both worsening and new neurological symptoms after allo-HSCT.

Severe toxic leukoencephalopathy (n=3) was characterized by a subacute onset (6−32 days after allo-HSCT) and progressive deterioration of consciousness, leading to a comatose state and death in all three patients (20−25 days after symptom onset, **Table 3**). The first patient developed an akinetic-

mutism syndrome with bilateral limb spasticity. The second patient experienced agitation, restlessness and visual hallucinations during pneumonia treatment, initially attributed to delirium, followed by a decline in consciousness. Electroencephalography showed a reactive pattern with excess delta activity, suggesting encephalopathy, without epileptiform abnormalities. Intravenous methylprednisolone (1000 mg once) had no demonstrable effect. The third patient presented with disorientation and pronounced myoclonus of the arms and legs. Intravenous methylprednisolone (1000 mg daily for three days) had no demonstrable effect. All patients had a bilateral Babinski sign, and none had seizures. Brain MRI showed confluent T2-hyperintense periventricular white matter abnormalities in all three patients (**Figure 1A**). Diffusion-weighted imaging and apparent diffusion coefficient mapping (performed in the second patient) showed decreased diffusion in the white matter abnormalities, indicative of cytotoxic edema (**Figure 1B, C**). There were no contrast-enhancing lesions, no cortical or deep gray matter involvement and no signs of hemorrhage.

Toxic encephalomyelopathy (n=2) developed 39−67 days after allo-HSCT (**Table 3**). The first patient developed progressive sensory deficits of the legs and trunk and paraparesis. In addition, the patient developed sphincter dysfunction, hyperreflexia and a bilateral Babinski sign. Symptoms reached CTCAE grade 4 one month after onset. Intravenous methylprednisolone (1000 mg daily for three days) seven months after symptom onset had no demonstrable effect. The patient showed partial recovery after eight months and remained care- and wheelchair-dependent after three years. The second patient developed sensory deficits progressing over weeks from feet to the legs, trunk, sacral region and fingers of both hands, accompanied by urge incontinence, Lhermitte's sign, mild paraparesis and hyperreflexia. Symptoms reached CTCAE grade 3 one month after onset. Weeks after onset, the patient developed a right visual field deficit. Two courses of methylprednisolone (1000 mg daily for five days) possibly had a modest effect. Symptoms stabilized and improved after two months, but gait instability and visual complaints persisted after 2.5 years. In both patients, spinal cord MRI showed no clear abnormalities in the acute setting but showed T2-hyperintense lesions indicative of myelopathy on

follow-up (**Supplementary Figure 2**). Brain MRI showed periventricular white matter abnormalities indicative of leukoencephalopathy in both patients (**Figure 1A**).

Toxic myeloneuropathy (n=2) started 25 days and 139 days after allo-HSCT (**Table 3**). It was characterized by sensory deficits and autonomic symptoms. The first patient developed distal to proximal progressive sensory deficits, and additional urge incontinence. Symptoms reached CTCAE grade 2 two months after onset. Electromyography showed signs of axonal polyneuropathy. Symptoms stabilized and improved from 3.5 months, with persistent sensory symptoms at four years. The second patient developed radiating pain from the neck towards both arms. Additionally, the patient experienced erectile dysfunction and urge incontinence. Neurological examination showed Lhermitte's sign, decreased gnostic sensibility in the distal legs and hyperreflexia. Symptoms reached CTCAE grade 2 two months after onset. Electromyography showed no signs of polyneuropathy, except for ulnar compression neuropathy and carpal tunnel syndrome, probably unrelated to the myeloneuropathy. The patient experienced near-complete spontaneous resolution after six months. Spinal cord (both patients) and brain MRI (first patient) showed no signs of myelopathy or abnormalities; clinical syndromes suggested myelopathy.

Factors associated with central nervous system toxicity

Patients with CNS toxicity were older than other patients treated with Flu-Thio-Mel (median age 66 [IQR 59−68] versus 54.50 [IQR 36−62] years, *P*=0.025) (**Supplementary Figure 3A**). Additionally, patients with CNS toxicity had a lower eGFR than other patients in the cohort (median eGFR 72.57 mL/min/1.73m^2 [IQR 71−93] versus 105.13 [IQR 89−122], *P*=0.024) (**Supplementary Figure 3B**). Sex, diagnosis, donor match grade, prior neurological disease, previous systemic or intrathecal chemotherapy, and previous cranial radiotherapy were similarly distributed between Flu-Thio-Mel recipients with and without CNS toxicity. None of the patients with CNS toxicity developed GVHD. The use of frequently prescribed potentially neurotoxic co-medications[11] (foscarnet, mold-active triazoles

and carbapenems) from day -4 up to the latest observed onset of CNS toxicity (day +139) was not increased in patients with NC or CNS toxicity (data not shown).

Fludarabine exposure

Fludarabine exposure was predicted a priori, based on patient characteristics, in all patients (N=102). A priori predicted fludarabine exposure did not differ significantly between cohorts, with a median AUC of 28.7 mg·h/L (IQR 26.0−32.8) in the Flu-Bu cohort and 27.9 mg·h/L (IQR 25.2−30.2) in the Flu-Thio-Mel cohort (*P*=0.157, **Figure 2**).

Within the Flu-Bu cohort, fludarabine AUC did not differ between patients with and without NC (*P*=0.863, **Figure 2A**). In the Flu-Thio-Mel cohort, however, the median fludarabine AUC was 26.6 mg·h/L in patients without NC, 29.2 mg·h/L in patients with NC without CNS toxicity, and 30.8 mg·h/L in patients with CNS toxicity. Overall differences across the three groups were significant (Kruskal-Wallis, *P*=0.009). Post-hoc pairwise comparisons demonstrated significantly higher fludarabine exposure in patients with CNS toxicity compared with patients without NC (Holm-adjusted Dunn's test *P*=0.021, **Figure 2B**).

As a sensitivity analysis, plasma fludarabine concentrations (F-ara-A) were incorporated into exposure predictions (sample-informed) for 12 patients in the Flu-Thio-Mel cohort (no NC, n=5; CNS toxicity, n=7, **Supplementary Figure 4**). In these patients, the median sample-informed AUC was 25.2 mg·h/L, compared with 27.9 mg·h/L from a priori predictions. Among sample-informed predictions, fludarabine exposure was also higher in patients with CNS toxicity than in the comparator patients without NC (*P*=0.010).

Discussion

In the current study, we systematically characterized neurological complications (NC) after Flu-Thio-Mel and Flu-Bu conditioning for allo-HSCT. We report two main findings. First, conditioning-related NC were frequent in both cohorts. Second, CNS toxicity occurred only after Flu-Thio-Mel conditioning and was mostly severe or fatal.

Overall, NC within the first year after allo-HSCT were common, affecting 30% of patients. NC were predominantly clinically mild, with peripheral (poly)neuropathy as the most frequent manifestation (16%). Although this rate falls within the wide range reported previously (3–44%), it appears relatively high compared with most studies.[1, 2, 4, 5] This variation likely reflects differences in study design, patient populations, and approaches to NC identification and attribution. By systematically and manually reviewing patient records and capturing all mild documented neurological complaints, we may have identified events that are underreported in routine practice. These findings suggest that NC, especially mild ones, after allo-HSCT may be more common than currently appreciated.

CNS toxicity, a subset of probable conditioning-related NC affecting the CNS, occurred in 7 of 55 patients (13%) treated with Flu-Thio-Mel and was severe or fatal in 9%. CNS toxicity was not observed in patients treated with Flu-Bu. It presented as fatal leukoencephalopathy (n=3), severe encephalomyelopathy (n=2), and moderate myeloneuropathy (n=2). Death due to CNS toxicity following allo-HSCT was evaluated in a large EBMT multicenter registry study, using data from 2010-2019.[7] The proportion of fatal CNS toxicity after Flu-Thio-Mel in our study (5%) appears high compared with the EBMT study in which 1% of deaths were attributed to CNS toxicity, although, importantly, differences in study design limit direct comparison of these rates.[7] Encephalopathy and leukoencephalopathy are rare but well-recognized severe complications after allo-HSCT.[2, 3, 13] In contrast, encephalomyelopathy or myeloneuropathy have rarely been described previously.[14] In our

cohort, these presentations were accompanied by autonomic symptoms, consistent with myelopathy. Spinal cord involvement is not a typical feature of fludarabine-associated toxic leukoencephalopathy. Patients who developed CNS toxicity were older and more frequently had impaired renal function than Flu-Thio-Mel patients without CNS toxicity, consistent with previous reports of fludarabine-associated leukoencephalopathy.[13] However, age, renal function, and fludarabine dose were comparable between the Flu-Thio-Mel and Flu-Bu cohorts, with a trend towards older age in the Flu-Bu cohort (*P*=0.080), suggesting that these factors alone may not fully explain the difference in CNS toxicity occurrence. Calcineurin inhibitors including cyclosporine and tacrolimus can cause neurotoxicity[8] but were not used in this allo-HSCT platform, and infectious causes were excluded during neurological review. This points to a possible regimen-associated factor, as discussed further below.

Within the Flu-Thio-Mel cohort there seemed to be a fludarabine exposure−response gradient, with increased exposure from patients without NC, to those with non-CNS toxicity NC, and highest exposure in those with CNS toxicity. This is in contrast to the Flu-Bu cohort, in which fludarabine exposure did not differ between patients with and without NC, and no CNS toxicity occurred despite similar fludarabine exposure to that in the Flu-Thio-Mel cohort. These findings suggest that higher fludarabine exposure may be associated with CNS toxicity in the context of Flu-Thio-Mel conditioning, possibly in combination with thiotepa and/or melphalan. Exposure alone is unlikely to predict CNS toxicity, given the substantial overlap in exposure between patients without NC and with NC or CNS toxicity. Future pharmacokinetic and mechanistic studies are needed to validate these findings and to determine whether fludarabine exposure contributes meaningfully to CNS toxicity risk alongside other patient and treatment factors.

Several lines of evidence could support a potential mechanistic role for the conditioning agents. Fludarabine is an antimetabolite with well-described, exposure-dependent neurotoxicity.[13, 15-17] The brain MRI abnormalities in patients with leukoencephalopathy and encephalomyelopathy resembled

previously reported cases of fludarabine-associated acute toxic leukoencephalopathy.[13] Encephalopathy in our study was clinically and radiologically distinct from fludarabine-associated posterior reversible encephalopathy syndrome.[13] Thiotepa is an alkylating agent that readily penetrates the blood-brain barrier.[18] It has been associated with neurotoxicity, especially at high doses, including rare reports of encephalopathy and myelopathy.[19, 20] Neurotoxic effects of melphalan and busulfan have been described, but the reported associated clinical presentations differ from those observed in our cohort.[4, 21-23]

The clearance of fludarabine, thiotepa's active metabolite TEPA, and, to a lesser extent, melphalan is influenced by renal function, whereas busulfan elimination is largely independent of renal function.[24] We hypothesize that both conditioning regimens contribute to mild peripheral neurotoxicity, while the combination of fludarabine and thiotepa, and possibly melphalan, may exert a synergistic neurotoxic effect within the CNS. One possible mechanism is cumulative axonal or myelin damage, related to increased CNS drug exposure due to altered blood-brain barrier penetration, and/or impaired clearance. The phase I/II trial of Flu-Thio-Mel in a pediatric and an adult population did not report a clear neurotoxicity signal, although one patient (2%) had a demyelinating neuropathy as cause of death.[9] Interestingly, in the EBMT registry study a relatively large proportion of patients with fatal CNS toxicity had received Flu-Thio-Mel conditioning.[7] Together with our own findings, these observations are consistent with our hypothesis of a potential contribution of the Flu-Thio-Mel combination to CNS toxicity.

Based on our results, we have two recommendations for future research and clinical practice. First, it would be valuable to systematically and uniformly report NC, including significant neuropathy and autonomic dysfunction, following allo-HSCT in large transplant registries such as EBMT and CIBMTR.[25] This requires systematic assessment of neurological symptoms, especially mild complaints, during routine clinical visits. Second, since the Flu-Thio-Mel regimen is used in clinical practice, CNS

toxicity after Flu-Thio-Mel deserves further evaluation.[26, 27] This is especially relevant as a growing proportion of allo-HSCT recipients are aged ≥65 years, a group possibly more susceptible to CNS toxicity.[28, 29] Whether modification of the conditioning regimen, such as lowering the thiotepa dose[30] or pharmacokinetic-guided dosing of fludarabine, can reduce CNS toxicity while maintaining efficacy may warrant further investigation.

This study has several limitations. First, its retrospective, single-center design may limit generalizability and introduces the potential for selection bias and ascertainment bias. Moreover, mild and autonomic symptoms could have been underreported, and because onset was based on first documentation, the time to onset of mild symptoms may have been overestimated. Attribution to conditioning also remains partly subjective, despite review by experienced neurologists. Second, the relatively small sample size and low number of CNS toxicity events limited statistical power and precluded robust multivariable adjustment. Third, baseline characteristics differed between cohorts, reflecting differences in conditioning indications (underlying diagnosis and donor type). There were trends toward older age in the Flu-Bu cohort and a higher prevalence of prior neurological disorders in the Flu-Thio-Mel cohort. Although variables associated with CNS toxicity within the Flu-Thio-Mel cohort (older age and lower eGFR) did not appear to be more prevalent in the Flu-Thio-Mel than in the Flu-Bu cohort overall, residual confounding by measured or unmeasured characteristics cannot be excluded.

Additionally, individual fludarabine exposure was calculated using a population pharmacokinetic model rather than predicted using rich individual pharmacokinetic sampling. Because the model incorporates patient characteristics such as renal function, which was itself associated with CNS toxicity, the observed exposure association could not be fully separated from these characteristics. Therefore, findings should be interpreted as descriptive rather than as evidence of an independent exposure–toxicity relationship. Exposure data for thiotepa and melphalan were unavailable, limiting

assessment of their contribution to CNS toxicity. Finally, renal function was estimated using creatinine-based eGFR rather than cystatin C-based eGFR, which may be more accurate in patients with altered muscle mass. Overall, our findings cannot establish causality between Flu-Thio-Mel conditioning, fludarabine exposure, and CNS toxicity, but they support increased awareness of this potential complication. Validation in larger, preferably multicenter, cohorts is required.

In conclusion, conditioning-related NC after allo-HSCT were common in both Flu-Thio-Mel and Flu-Bu recipients, most often mild, and may still be underreported in the literature. CNS toxicity occurred exclusively among Flu-Thio-Mel recipients (13%), with severe or fatal cases in 9%. To our knowledge, we are the first to describe in detail cases of toxic encephalomyelopathy and myeloneuropathy following allo-HSCT. Heightened awareness of the potential risk of CNS toxicity is warranted when considering Flu-Thio-Mel conditioning, particularly in vulnerable patients.

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Tables

Table 1: Baseline characteristics of patients with fludarabine, thiotepa and melphalan versus fludarabine and busulfan conditioning

	Flu-Thio-Mel, N=55*	**Flu-Bu, N=47***	***P***
Age	55.00 [40.00, 63.50]	61.00 [50.00, 67.50]	0.080†
Sex (female)	25 (45.5)	21 (44.7)	1.000‡
Indication			0.001§
AML/high-risk MDS	19 (34.5)	14 (29.8)	
MDS/MPN	8 (14.5)	15 (31.9)	
Myelofibrosis	12 (21.8)	8 (17.0)	
Lymphoma	2 (3.6)	5 (10.6)	
ALL	0 (0.0)	5 (10.6)	
Inborn errors of immunity	8 (14.5)	0 (0.0)	
VEXAS syndrome	1 (1.8)	0 (0.0)	
Severe aplastic anemia	3 (5.5)	0 (0.0)	
Plasma cell leukemia	1 (1.8)	0 (0.0)	
Bone marrow failure	1 (1.8)	0 (0.0)	
eGFR	101.52 [88.00, 121.04]	97.28 [79.14, 115.56]	0.319†
Neurological history	21 (38.2)	10 (21.3)	0.090‡
Previous chemotherapy	36 (65.5)	27 (57.4)	0.532‡
Previous intrathecal chemotherapy	2 (3.6)	5 (10.6)	0.317‡
Previous cranial radiotherapy	2 (3.6)	0 (0.0)	0.499§
Donor match			<0.001‡
MRD 10/10	2 (3.6)	12 (25.5)	
MUD 10/10	27 (49.1)	26 (55.3)	
MUD 9/10	6 (10.9)	9 (19.1)	
Haploidentical	20 (36.4)	0 (0.0)	

ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, eGFR: estimated glomerular filtration rate, Flu-Bu: fludarabine and busulfan, Flu-Thio-Mel: fludarabine, thiotepa and melphalan, MDS: myelodysplastic syndrome; MPN: myeloproliferative neoplasm, MRD: matched related donor, MUD: matched unrelated donor, VEXAS: vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic. *: Median [Q1, Q3]; n (%). †: Wilcoxon rank-sum test, ‡: Chi-squared test, §: Fisher's exact test. AML/high-risk MDS includes AML, MDS with excess of blasts-2 (MDS-EB-2), and mixed phenotype leukemia (MPAL). MDS/MPN includes MDS other than MDS-EB-2, MDS/myeloproliferative neoplasms and chronic myelomonocytic leukemia (CMML).

Table 2: Counts and characteristics of neurological complications in patients with fludarabine, thiotepa and melphalan versus fludarabine and busulfan conditioning

	Flu-Thio-Mel, N=55*	**Flu-Bu, N=47***	***P***
Neurological complication	17 (30.9)	14 (29.8)	1.000‡
Onset (days after allo-HSCT)	46.00 [18.00, 100.00]	99.50 [68.25, 128.50]	0.054†
Location affected			0.007§
PNS	9 (16.4)	14 (29.8)	
Neuropathy	*7 (12.7)*	*9 (19.1)*	
Radiculoplexoneuropathy	*2 (3.6)*	*1 (2.1)*	
Clinical Lhermitte's sign¶	*0 (0.0)*	*2 (4.3)*	
Muscle cramps	*0 (0.0)*	*1 (2.1)*	
Restless leg syndrome	*0 (0.0)*	*1 (2.1)*	
PNS/CNS both	2 (3.6)	0 (0.0)	
Myeloneuropathy	*2 (3.6)*	*0 (0.0)*	
CNS	6 (10.9)	0 (0.0)	
Leukoencephalopathy/Encephalopathy	*4 (7.3)*	*0 (0.0)*	
Encephalomyelopathy	*2 (3.6)*	*0 (0.0)*	
Present before transplant	4 (7.3)	3 (6.4)	1.000§
CTCAE grade			0.009§
1-2	9 (16.4)	14 (29.8)	
3-4	3 (5.5)	0 (0.0)	
5	5 (9.1)	0 (0.0)	
Outcome			0.111§
Resolved	3 (5.5)	4 (8.5)	
Improved	5 (9.1)	3 (6.4)	
Persistent	4 (7.3)	7 (14.9)	
Death	5 (9.1)	0 (0.0)	

Allo-HSCT: allogeneic hematopoietic stem cell transplantation, CNS: central nervous system, CTCAE: common terminology criteria for adverse events v5.0, Flu-Bu: fludarabine and busulfan, Flu-Thio-Mel: fludarabine, thiotepa and melphalan, PNS: peripheral nervous system. *: Median [Q1, Q3]; n (% of whole cohort). †: Wilcoxon rank-sum test, ‡: Chi-squared test, §: Fisher's exact test, ¶: Undetermined location; absence of myelopathy (n=1) and absence of other complaints (n=1) favored PNS involvement.

Table 3: Patient and clinical details of central nervous system toxicity presentations after fludarabine, thiotepa and melphalan conditioning

	Age; eGFR	Indication; Donor type, HLA match	Onset (range in days)	Neurological symptoms and tests	CTCAE grade	Diagnostic tests	CSF analysis	MP treatment	Outcome
Leukoencephalopathy (n=3)	64-72 years, eGFR 65–90	MF (n=2), MDS-EB-1 (n=1) MUD 10/10	6–32	Severe decline in consciousness to coma in <1 month. Akinetic mutism, limb spasticity (n=1), agitation, visual hallucinations (n=1), myoclonus (n=1). Bilateral Babinski sign, no seizures (n=3).	5	MRI: extensive periventricular WM abnormalities. EEG: reactive pattern with excess delta activity, no epileptiform abnormalities (n=1).	No pleocytosis (n=2), mildly elevated protein (n=1, 84 mg/dL), normal protein (n=1, 39 mg/dL). Viral PCR panel negative (n=2), culture negative (n=1).	No effect (n=2)	Death, 20–25 days after onset
Encephalomyelopathy (n=2)	53-66 years, eGFR 73–96	AML, SAA MUD 10/10	39–67	Progressive sensory deficits (within 1 month) from distal to proximal and the trunk, autonomic dysfunction. Elevated tendon reflexes (n=2), Babinski sign (n=1), Lhermitte's sign (n=1).	3–4	Repeated MRI: WM abnormalities as in leukoencephalopathy, myelopathy of spinal cord. EMG: pre-existing axonal polyneuropathy (n=1).	No pleocytosis (n=2), elevated protein (n=1, 112 mg/dL), normal protein (n=1, 28 mg/dL). Viral PCR panel and culture negative (n=2).	No effect (n=1), possible effect (n=1)	Stabilization, improvement 2-8 months after onset, remaining deficits
Myeloneuropathy (n=2)	51-66 years, eGFR 70–122	MF, VEXAS syndrome MUD 9/10, 10/10	25–139	Distal to proximal sensory deficits, autonomic dysfunction (progression over 2 months). Normal plantar reflexes (n=2), Lhermitte's sign, elevated tendon reflexes (n=1).	2	MRI: no abnormalities. EMG: axonal polyneuropathy (n=1), unrelated ulnar compression neuropathy and CTS (n=1).	No pleocytosis (n=1), almost normal protein (n=1, 45 mg/dL). Viral PCR panel and culture negative (n=1).		Improvement 4-6 months after onset, persisting deficits (n=1), near-complete resolution (n=1)

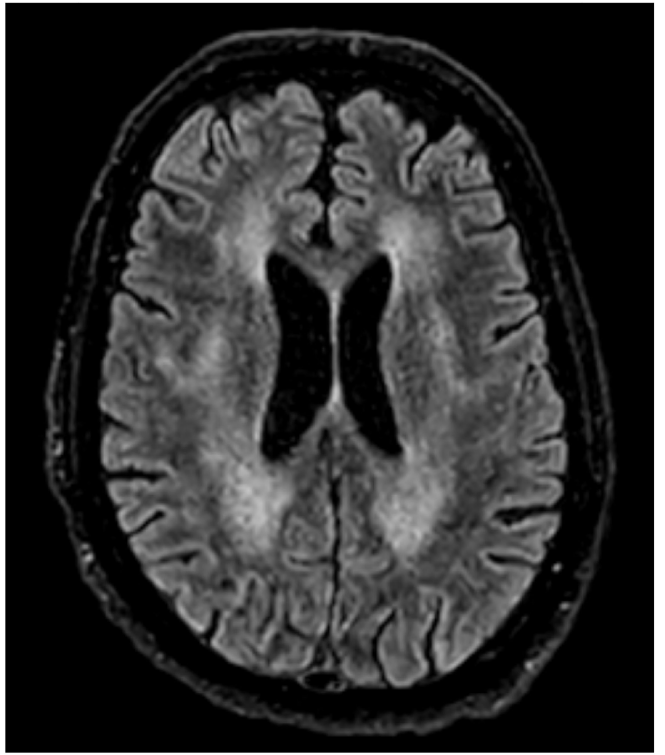
AML: acute myeloid leukemia, CSF: cerebrospinal fluid, CTCAE: common terminology criteria for adverse events v5.0, CTS: carpal tunnel syndrome, EEG: electroencephalography, eGFR: estimated glomerular filtration rate (mL/min/1.73m^2), EMG: electromyography, MDS-EB-1: myelodysplastic syndrome with excess of blasts-1, MF: myelofibrosis, MUD: matched unrelated donor, MP: methylprednisolone (none of the patients was treated with IVIg), MRI: magnetic resonance imaging, SAA: severe aplastic anemia, VEXAS: vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic, WM: white matter.

Figure legends

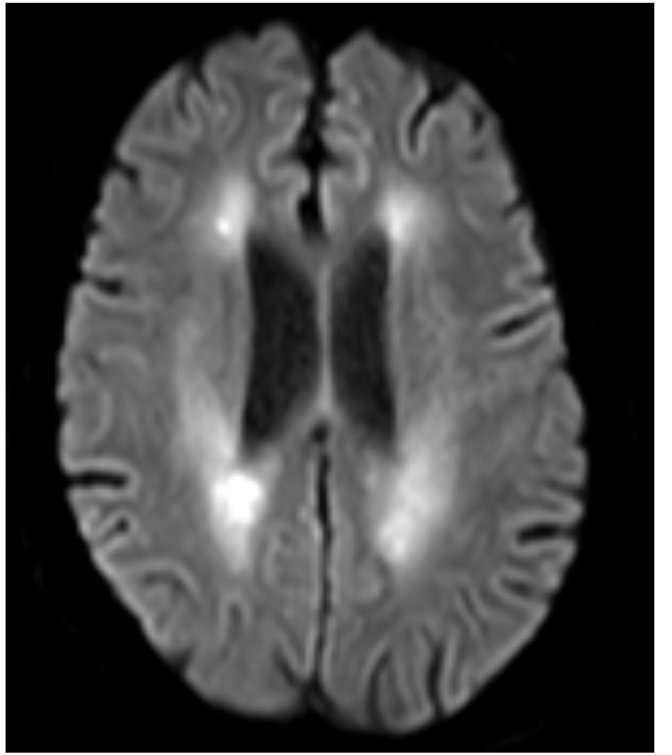
Figure 1: Representative MRI images of toxic leukoencephalopathy. Periventricular abnormalities in the white matter were observed as **A**) symmetric, diffusely increased signal intensity on T2-weighted FLAIR images, **B**) mildly decreased diffusion with increased signal on diffusion-weighted imaging (DWI), and **C**) lowered signal on apparent diffusion coefficient (ADC). There were no contrast-enhancing lesions, no cortical or deep grey matter involvement and there were no signs of hemorrhage. Radiology images of the brain were comparable between the patients with toxic leukoencephalopathy (n=3) and encephalomyelopathy (n=2) in the current study.

Figure 2: Fludarabine exposure in Flu-Bu and Flu-Thio-Mel cohorts. Cumulative fludarabine exposure (expressed as F-ara-A $AUC_{0\text{-}192h}$ in mg·h/L) was predicted a priori, comparing **A**) those with and without NC within the Flu-Bu cohort, and **B**) those without NC, those with NC but no CNS toxicity, and those with CNS toxicity within the Flu-Thio-Mel cohort. Overall fludarabine exposure was comparable across Flu-Bu and Flu-Thio-Mel cohorts (*P*=0.157, Wilcoxon rank-sum test). Within the Flu-Bu cohort, exposure did not differ between patients with and without NC (*P*=0.863, Wilcoxon rank-sum test). Within the Flu-Thio-Mel cohort, exposure was significantly higher in the subset with CNS toxicity than in the subset without NC (*P*=0.021, Holm-adjusted Dunn's test). Data points represent individuals; boxes represent the interquartile range with the median; whiskers represent the range of values. CNS: central nervous system, Flu-Bu: fludarabine-busulfan conditioning, Flu-Thio-Mel: fludarabine-thiotepa-melphalan conditioning, NC: neurological complication.

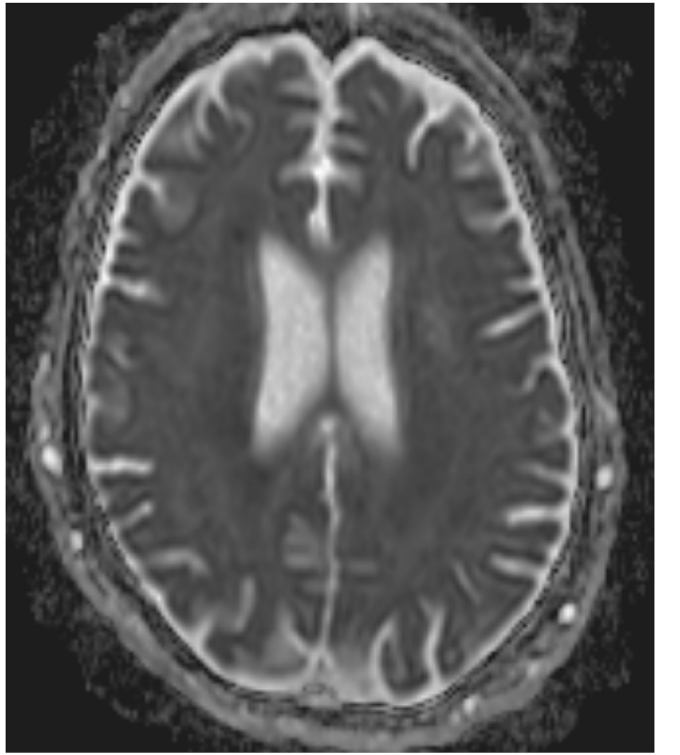
A

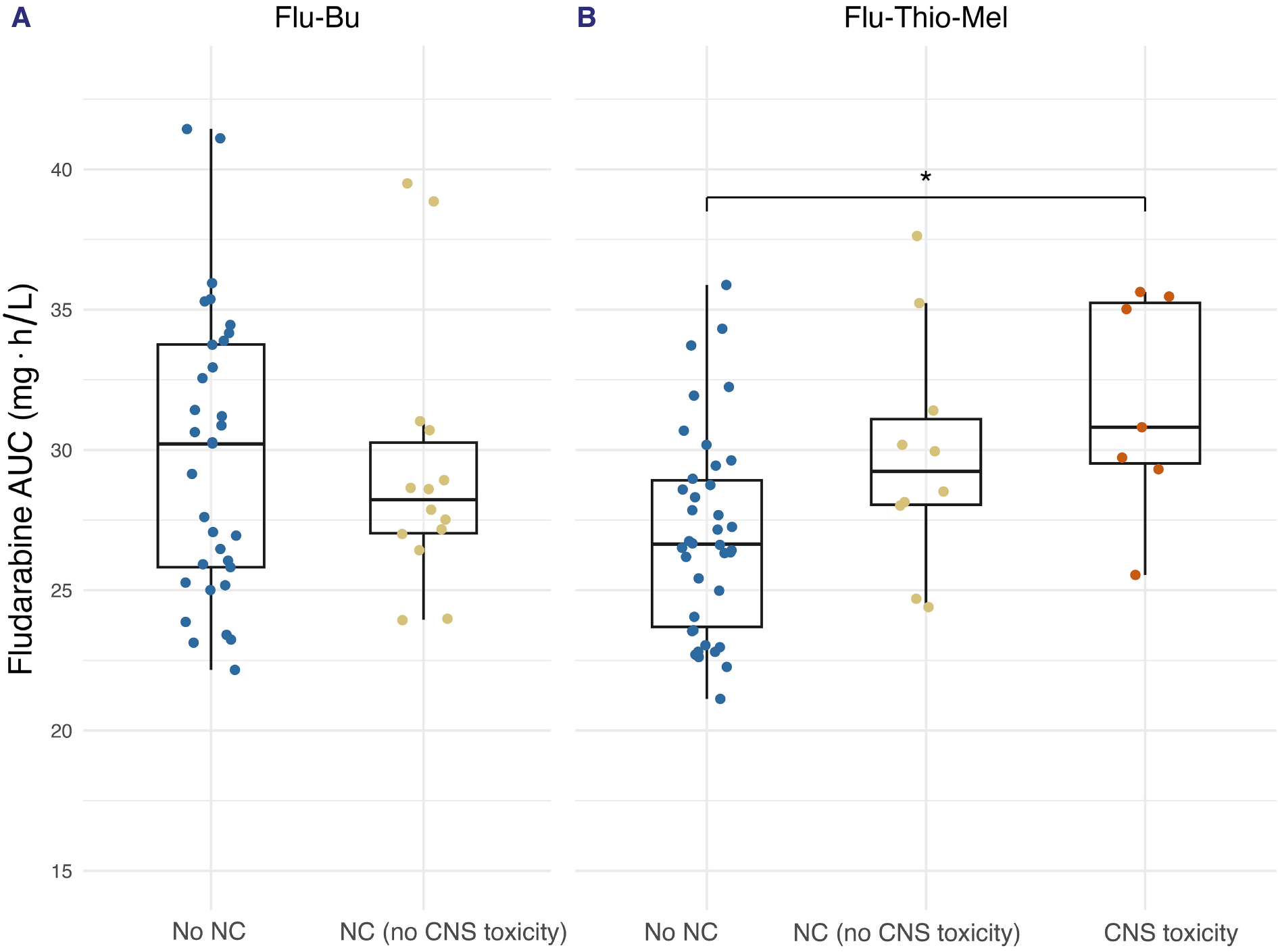


B



C




A
Flu-Bu
B
Flu-Thio-Mel
Fludarabine AUC (mg · h/L)
40
35
30
25
20
15
*
No NC
NC (no CNS toxicity)
No NC
NC (no CNS toxicity)
CNS toxicity

Online Supplements

Severe and fatal central nervous system toxicity after fludarabine, thiotepa and melphalan conditioning for allogeneic stem cell transplantation

Pleun Schonewille[1], Bram Kottink[2], Sharon Berendsen[3], Yousra van der Leest[1], Klaartje Nijssen[1], Lotte van der Wagen[1], Laura Daenen[1], Viola Kraak[1], Jan Willem Dankbaar[4], Arief Lalmohamed[2], Aurelia de Vries Schultink[2], Maaike Dirks[3], Jürgen Kuball[1,5], Moniek A. de Witte[1]

[1]Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands

[2]Department of Clinical Pharmacy, University Medical Center Utrecht, Utrecht, The Netherlands

[3]Department of Neurology, University Medical Center Utrecht, Utrecht, The Netherlands

[4]Department of Radiology, University Medical Center Utrecht, Utrecht, The Netherlands

[5]Center for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Corresponding author: Moniek A. de Witte; University Medical Center Utrecht; M.A.deWitte-7@umcutrecht.nl

Supplementary Methods

Renal function estimation

Renal function was assessed using a body surface area-normalized Cockcroft–Gault equation. The resulting eGFR was capped at a physiological maximum of 140 mL/min/1.73 m² and adjusted for age by assuming a decline in renal function of 0.86 mL/min/1.73 m² per year after the age of 30, as described previously.[1, 2] eGFR was reported in mL/min/1.73 m^2.

$$eGFR_{raw} = \frac{1.73 \times (140 - \text{Age}) \times \text{Weight} \times 0.85^{(1-Sex)}}{72 \times \text{Creatinine} \times \text{BSA}}$$

$$eGFR_{cap} = \begin{cases} 140, & \text{if Age} < 30 \\ 165.8 - 0.86 \times \text{Age}, & \text{if Age} \geq 30 \end{cases}$$

$$eGFR = min\left(eGFR_{raw}, eGFR_{cap}\right)$$

Age: Age at transplant in years, Weight: Weight at transplant in kg, Sex: Male=1, Female=0, Creatinine: Serum creatinine at transplant in mg/dL, BSA: Body surface area in m^2.

Fludarabine exposure modeling

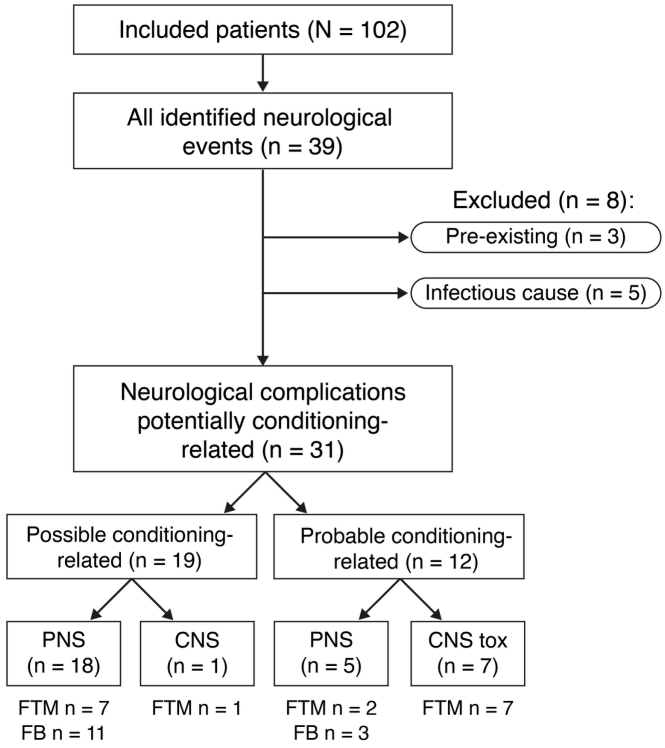
For the full Flu-Thio-Mel and Flu-Bu cohorts, fludarabine exposure was calculated based on patient characteristics and dosing records alone (a priori prediction). For a subset of the patients in the Flu-Thio-Mel cohort, a priori predicted AUCs were compared with sample-informed AUCs which incorporated available plasma concentration measurements, as a sensitivity analysis.

Plasma samples were collected as part of routine virological monitoring and stored at −80°C. Following identification of CNS toxicity, stored plasma samples from patients with CNS toxicity (n=7) and comparator patients (n=5) from the Flu-Thio-Mel cohort were retrieved for quantification of fludarabine plasma concentrations (as F-ara-A). Comparator patients were selected by age below the cohort median, eGFR > 90 mL/min/1.73 m^2, absence of post-transplant NC, and availability of a plasma sample obtained within 72 hours after the last fludarabine administration. F-ara-A concentrations were quantified using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS), based on a previously published method with minor modifications.[3] This method was developed in accordance with the EMA guideline for bioanalytical method validation.

For pharmacokinetic analysis, fludarabine phosphate doses were converted to F-ara-A equivalents by dividing the administered dose by 1.28.[1] Individual F-ara-A exposure was calculated as the cumulative area under the plasma concentration–time curve from the first dose until 192 hours

after treatment initiation ($AUC_{0\text{-}192h}$, mg*h/L). Individual empirical Bayes estimates were obtained using the previously developed three-compartment population pharmacokinetic model by Langenhorst *et al*. in NONMEM v7.5.1.[1] The individual parameters were used to calculate the patient-specific AUC values. Thiotepa and melphalan exposure were not modeled.

Supplementary Results

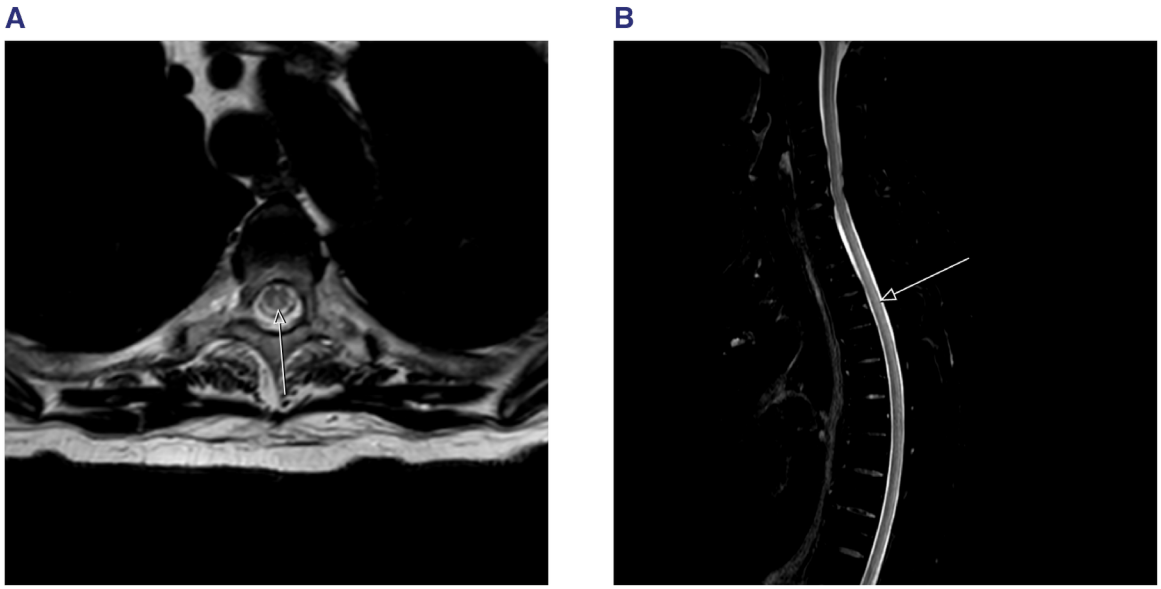


Supplementary Figure 1: Flow diagram of neurological complication identification and attribution to conditioning-related toxicity. Diagram shows the number of included patients, identified neurological events after allo-HSCT, excluded events, possible and probable conditioning-related neurological complications, and the affected anatomical locations. CNS: central nervous system (also including NC affecting both the CNS and PNS), FB: Fludarabine-Busulfan conditioning cohort, FTM: fludarabine-thiotepa-melphalan conditioning cohort, PNS: peripheral nervous system, tox: toxicity.

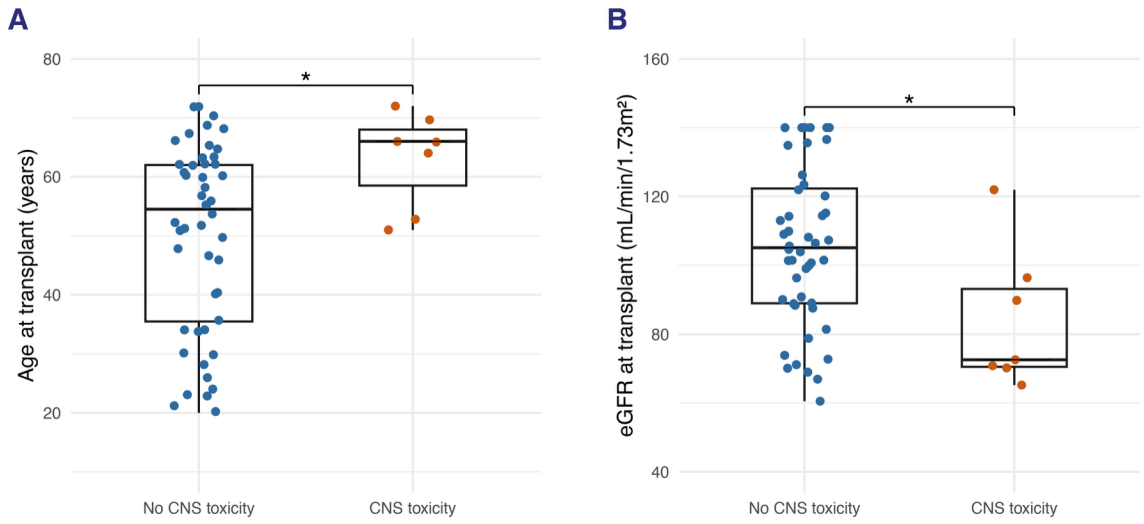
Supplementary Table: CSF microbiology analyses in central nervous system toxicity patients.

CNS toxicity case	Screened pathogens (all negative)
Leukoencephalopathy #1	Cytomegalovirus, enterovirus, Epstein-Barr virus, herpes simplex virus 1/2, human herpesvirus 6, human polyomavirus 2 (JC-virus), parechovirus, varicella zoster virus
Leukoencephalopathy #2	Cytomegalovirus, enterovirus, herpes simplex virus 1/2, human herpesvirus 6, human polyomavirus 2 (JC-virus), parechovirus, varicella zoster virus; bacterial/fungal culture
Leukoencephalopathy #3	[No CSF analysis]
Encephalomyelopathy #1	Cytomegalovirus, enterovirus, herpes simplex virus 1/2, human herpesvirus 6, parechovirus, varicella zoster viru; bacterial/fungal culture
Encephalomyelopathy #2	Enterovirus, herpes simplex virus-1/2, human polyomavirus 2 (JC-virus), parechovirus, varicella zoster virus; bacterial/fungal culture
Myeloneuropathy #1	Cytomegalovirus, enterovirus, herpes simplex virus 1/2, human herpesvirus 6, parechovirus, varicella zoster virus; bacterial/fungal culture
Myeloneuropathy #2	[No CSF analysis]

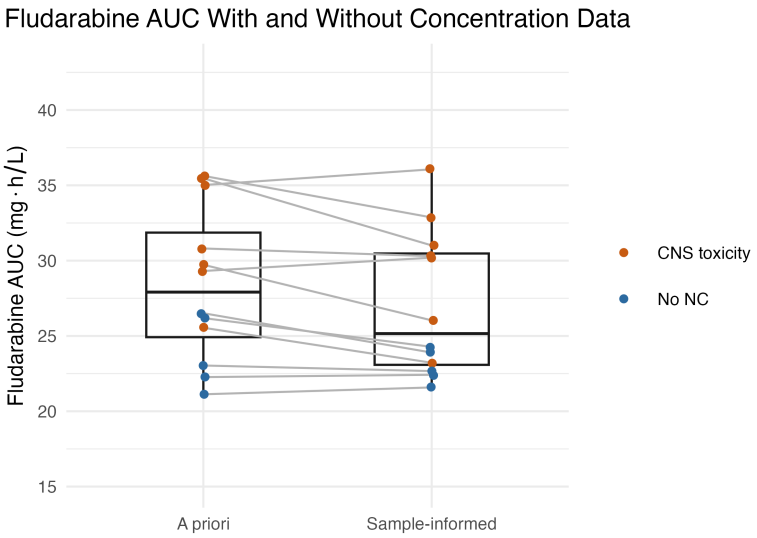
Table specifies which pathogens were screened for in the cerebrospinal fluid (CSF) (viruses: PCR, bacteria and fungi: culture) and ruled out, in patients with central nervous system (CNS) toxicity after fludarabine-thiotepa-melphalan conditioning for allo-HSCT.



Supplementary Figure 2: Representative MRI images of toxic myelopathy. Myelopathy was observed as hyperintense segments, indicated by arrows, within the dorsal column of the myelum on **A**) axial T2-weighted images and **B**) sagittal T2-weighted images with fat suppression. Enhancement after administration of gadolinium may occur. MRI images of both patients with encephalomyelopathy showed myelopathy, in the cervical (n=1) or thoracic (n=1) part of the myelum.



Supplementary Figure 3: Factors associated with central nervous system toxicity in patients after fludarabine, thiotepa and melphalan conditioning. In patients with central nervous system (CNS) toxicity post-transplant compared to those without CNS toxicity in the cohort conditioned with fludarabine, thiotepa and melphalan (Flu-Thio-Mel), **A**) the median age was higher (*P*=0.025), and **B**) the median estimated glomerular filtration rate (eGFR) was lower (*P*=0.024). Data points represent individuals**;** boxes represent the interquartile range with the median; whiskers represent the range of observed values. *: *P*<0.05, Wilcoxon rank-sum test.



Supplementary Figure 4: Fludarabine exposure calculated with and without incorporation of measured fludarabine concentrations. Fludarabine exposure (AUC) was calculated using the population pharmacokinetic model based on patient characteristics alone (a priori) and with incorporation of measured fludarabine concentrations (sample-informed) for the same 12 patients in the Flu-Thio-Mel cohort. Patients included all seven cases with central nervous system (CNS) toxicity and five comparator patients without neurological complications (NC). Data points represent individuals; grey horizontal lines connect paired predictions from the same individual; boxes represent the interquartile range with the median; whiskers represent the range of observed values.

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