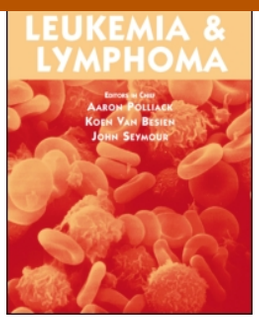

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ORIGINAL ARTICLE

The effect of asparaginase therapy on methotrexate toxicity and efficacy in children with acute lymphoblastic leukemia

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ABSTRACT

Asparaginase and methotrexate (MTX), both essential for pediatric acute lymphoblastic leukemia therapy, are often used concomitantly. Depending on the sequence, *in vitro*, asparaginase inhibits MTX-polyglutamate (MTXPG) formation, and side effects overlap. MTX toxicity and efficacy, reflected by intracellular erythrocyte MTXPG's, were compared between children treated with and without asparaginase during high dose MTX (HD-MTX) courses of the DCOG ALL-11 protocol (NL50250.078.14). Seventy-three patients, of whom 23 received asparaginase during the HD-MTX courses, were included. Grade 3–4 leukopenia and neutropenia occurred more often (59% and 86% vs. 30% and 62%). The number of infections, grade 3–4 hepatotoxicity, nephrotoxicity, and neurotoxicity did not differ. Patients with asparaginase had lower MTXPG levels, although to a lesser extent than *in vitro* studies. Although patients with asparaginase during HD-MTX courses showed more myelosuppression, this had no (serious) clinical consequences. Regarding the MTX efficacy, the schedule-related antagonism seen in *in vitro* seems less important *in vivo*.

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KEYWORDS
Asparaginase; methotrexate; acute lymphoblastic leukemia

Introduction

Asparaginase and methotrexate (MTX) are both essential for the treatment of pediatric acute lymphoblastic leukemia (ALL). These drugs are often used concomitantly, among other chemotherapeutics, to achieve better survival rates [1–8]. The sequence of administration, however, seems important as several *in vitro* studies have shown an antagonistic effect of native *Escherichia coli* asparaginase on MTX when asparaginase is administered prior to the MTX [9–12]. It has been shown that MTX efficacy, reflected by intracellular MTX polyglutamates (MTXPGs) [13–15], is decreased due to the asparagine depletion caused by asparaginase. The asparagine depletion inhibits folylpolyglutamyl synthetase (FPGS), the enzyme that forms the MTXPGs [10,12,16–18]. In contrast, if asparaginase is administered after MTX, there seems to be a synergistic effect *in vitro* [12]. Moreover, *in vivo*, MTX is administered prior to native *E. coli* asparaginase with the Capizzi regimen, increasing the dose guided by toxicity, which permits toleration of higher MTX doses and leads to successful remission rates [16].

Currently, most treatment protocols use PEGasparaginase, with therapeutic activity of at least two weeks, resulting in continuous asparagine depletion [19]. So treating patients with PEGasparaginase during MTX doses may influence the formation of MTXPGs, independently of the sequence of administration. Treatment protocols using these dosing schedules, however, are successful, suggesting that *in vivo* the effect of asparaginase on MTX efficacy seems less important [6,20,21].

Beside MTX efficacy, concomitant asparaginase and MTX therapy may alter toxicity profiles because the drugs have overlapping side effects, including neurotoxicity, hepatotoxicity, and myelosuppression. On the other hand, MTX toxicity can be decreased *in vitro* by drugs that prevent cells from entering the S-phase, which is the case when asparaginase depletes the extracellular asparagine pools [22].

In the current Dutch Childhood Oncology Group (DCOG) ALL-11 protocol, medium risk patients are being randomized either to a continuous or a discontinuous PEGasparaginase dosing schedule, which

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contains an asparaginase-free period of several months, to study the effect of the dosing schedule on the occurrence of hypersensitivity reactions. Patients who are treated according to the continuous dosing schedule are concomitantly treated with asparaginase and high dose MTX courses, enabling us to study the possible effects of asparaginase on MTX efficacy and toxicity. The aim of this study is to compare the MTX efficacy, reflected by intracellular erythrocyte MTXPG levels, and toxicity between patients who are treated with high dose MTX courses with and without concomitant PEGasparaginase treatment.

Methods

Patients and treatment

Pediatric patients with ALL, diagnosed between November 2014 and June 2017, and treated according to the medium or standard risk group of the DCOG ALL-11 protocol were included in this study. The patients were treated in the Sophia Children's Hospital, Rotterdam, The Netherlands; the Academic Medical Center, Amsterdam, The Netherlands; or the Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands. The study (CCMO register: NL50250.078.14) was approved by the local ethics committee and informed consent was signed by children >12 years old and/or the parents or guardians in accordance with the declaration of Helsinki.

The complete DCOG ALL-11 treatment protocol is described in Table 1. Both standard and medium risk patients treated according to the discontinuous asparaginase dosing group, received three doses of PEGasparaginase (1500 IU/m^2, IV, biweekly) during induction (course 1(A) and 1(B)), followed by an asparaginase-free interval of ~12 weeks (remaining course 1(B) and M). During the following intensification phase, standard risk patients received one more PEGasparaginase dose; medium risk patients another 14 doses. The medium risk patients who were treated according to the continuous dosing schedule, received the PEGasparaginase once every two weeks, also during the consecutive courses 1(B) and M. The PEGasparaginase doses were individualized after the third dose, based on asparaginase activity levels [23].

The asparaginase-free period for both standard and medium risk patients of the discontinuous dosing schedule started with course 1(B) containing 6-mercaptopurine, cytarabine, and cyclophosphamide, followed by course M with four high dose MTX courses (5000 mg/m^2/dose IV over 24 h) and 6-mercaptopurine (25 mg/m^2/day orally) (Table 1). MTX was administered biweekly, except if patients suffered from a (severe) infection, mucositis or hepatotoxicity (AST/ALT >10× upper limit of normal), or when the white blood count was $<1.5 \times 10^9$ or platelets were $<50 \times 10^9$. In that case, the course was postponed for at least one week. Folinic acid was administered after the MTX dose until the 48 h plasma MTX level was <0.4 μM or the 72 h plasma MTX level <0.25 μM. The patients with the continuous asparaginase dosing schedule also received PEGasparaginase during course 1(B) and M. The asparaginase doses were administered biweekly, even if the requirements of MTX administration were not fulfilled and MTX had to be postponed. This is also true for any delay in protocol 1(B), for example, if

Table 1. DCOG ALL-11 protocol for medium and standard risk patients.

Treatment phase	Therapy
Protocol 1(A)	
Prednisone	60 mg/m^2/day for 29 days followed by 9 days tapering
Vincristine	1.5 mg/m^2/dose at day 8, 15, 22 and 29
Daunorubicin	30 mg/m^2/dose at day 8, 15, 22 and 29 (not in case of Down syndrome)
PEGasparaginase	1500 IU/m^2 at day 12, 26
Intrathecal MTX, cytarabine, and prednisone	8–12 mg MTX, 20–30 mg cytarabine, 8–12 mg prednisone at day 15 and 33. Only intrathecal MTX at day 1.
Protocol 1(B)	
PEGasparaginase[a]	1500 IU/m^2 at day 40
Cyclophosphamide	1000 mg/m^2/dose at day 36 and 64
Cytarabine	75 mg/m^2/day at days 38–41, 45–48, 52–55, 59–62
6-Mercaptopurine	60 mg/m^2/day at days 36–63
Intrathecal MTX, cytarabine, and prednisone	8–12 mg MTX, 20–30 mg cytarabine, 8–12 mg prednisone at day 45 and 59
Protocol M	
6-Mercaptopurine	25 mg/m^2/day for 56 days
MTX	5000 mg/m^{2}over 24 h, at day 8, 22, 36 and 50
Intrathecal MTX, cytarabine, and prednisone	8–12 mg MTX, 20–30 mg cytarabine, 8–12 mg prednisone at day 8, 22, 36 and 50
PEGasparaginase	Only continuous group: individualized doses, biweekly

MTX: methotrexate.
[a]Medium risk patients in the continuous dosing schedule will continue PEGasparaginase treatment during course 1(B) and M, administered biweekly.

cyclophosphamide has to be postponed. Evidently, patients had to fulfill the requirements for asparaginase administration, which included the absence of hepatotoxicity (AST/ALT $>10\times$ and bilirubin $>3\times$ upper limit of normal), jaundice, clinical signs of pancreatitis, and cerebral thrombosis.

Toxicity

Methotrexate toxicity was prospectively studied using case report forms which were completed by the physician two weeks after each high dose MTX course. These toxicity forms included central neurotoxicity (ataxia, somnolence, a depressed level of consciousness, agitation, seizures, and posterior reversible encephalopathy syndrome), infections, mucositis, and diarrhea. In addition, complete blood count, liver enzymes (alanine transaminase (ALT) and aspartate transaminase (AST)), creatinine and albumin concentrations were measured prior to the next high dose MTX courses. Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03. In addition, total treatment delay due to toxicity, extra hospital admissions, and prolongation of hospital admissions for MTX administration were registered.

MTX polyglutamates

MTX polyglutamates were measured two to three weeks after each MTX course. Blood was drawn to measure intracellular MTXPG concentrations in the erythrocytes as described by Den Boer et al. [24] EDTA whole blood tubes were centrifuged at $2700 \times g$ for 10 min at room temperature. The red cell pellet was harvested and stored at $-80\,^{\circ}C$ until analysis. For the analysis, first stable-isotope-labeled internal standards were added, followed by incubation of the sample with 16% perchloric acid for protein precipitation. After centrifugation at $21{,}350 \times g$ for 7 min, MTXPG 1–5 concentrations were measured using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). MTXPG 1 is freely transportable out of the cells, so very variable [25]. Therefore, only MTXPG 2–5 were used for the analysis. Beside the intracellular MTXPG levels, 48 h plasma MTX levels were analyzed.

Statistical analysis

SPSS Statistics version 21.0 (IBM Corp, Armonk, New York, USA) and R Sigmaplot Version 3.4.1 (Systat Software Inc, London, UK) were used for the data analysis. Baseline characteristics were stated as mean and standard deviation (SD) for normally distributed data, and median and interquartile range (IQR) for skewed data. Student *t*-tests, Mann–Whitney U tests, χ^2-(trend)tests, Fisher exact tests, and corresponding *post-hoc* analyses were used to compare the baseline characteristics and maximal toxicity during protocol M. The MTXPG levels were longitudinally analyzed using marginal models to study the levels between patients with and without concomitant asparaginase treatment. The data were log transformed to obtain normally distributed data. We have corrected for the number of days between the MTX dose and sampling, the MTX dose number, age, sex, and whether there was an erythrocyte transfusion administered less than two weeks before the sample. In addition, we have studied whether asparaginase doses were administered directly after the MTX doses or one week after the MTX dose. The 48 h MTX plasma levels were also analyzed with marginal models. The data were log transformed to obtain normally distributed data. In the model, we have corrected for sex, age, the MTX dose number, albumin levels and whether patients had an increased creatinine level. Also in this analysis, it was included whether asparaginase doses were administered directly after the MTX doses or one week after the MTX dose.

Results

Baseline characteristics are described in Table 2. In total, 73 patients were included in the study. Twenty-three patients were concomitantly treated with asparaginase during the high dose MTX courses in protocol M. Of the group without asparaginase treatment during the MTX courses, 17/50 (34%) were treated according to the standard risk group; the other 33 patients according to the discontinuous dosing schedule of the medium risk group. Of the group with concomitant asparaginase treatment, one patient had to switch to the high-risk group halfway during protocol M due to high minimal residual disease.

There were no statistically significant differences in other baseline characteristics between the patients with and without asparaginase treatment during protocol M. Of note, some of the patients were standardly treated with intravenous immunoglobulins (IVIG) as part of another randomized study. The number of patients standardly treated with IVIG, however, did not differ between the groups.

Table 2. Patient characteristics.

	No asparaginase during high dose MTX courses $n = 50$	Asparaginase during high doses MTX courses $n = 23$	p Value
Sex (%)	–	–	.621
Male	27 (54%)	14 (61%)	–
Female	23 (46%)	9 (39%)	–
Age at diagnoses, years, median (IQR)	5.4 (3.0–9.2)	4.1 (3.4–5.5)	.319
Type of ALL (%)	–	–	.390
Pre-B cell ALL	12 (24%)	9 (39%)	–
Common B-cell ALL	31 (62%)	10 (44%)	–
Pro-B cell ALL	1 (2%)	4 (17%)	–
T-cell ALL	6 (12%)	0 (0%)	–
Risk group (%)	–	–	–
Standard risk	17 (34%)	–	–
Medium risk, discontinuous group	33 (66%)	–	–
Medium risk, continuous group	–	23 (100%)[a]	
IVIG therapy during protocol M (%)	–	–	.762
Yes	11 (22%)	4 (17%)	–
No	39 (78%)	19 (83%)	–
Average trough[b] asparaginase level during protocol M, IU/L, mean (SD)	–	249 (48)	–

MTX: methotrexate; IQR: interquartile range; ALL: acute lymphoblastic leukemia; IVIG: intravenous immunoglobulins; SD: standard deviation; MRD: minimal residual disease.
[a]One patient had to switch to high risk therapy due to high minimal residual disease.
[b]Trough asparaginase level: 14 ± 2 days after an asparaginase dose.

Table 3. Toxicity during protocol M.

	No asparaginase during high dose MTX courses $n = 50$		Asparaginase during high doses MTX courses $n = 22$[a]		p Value
Number of infections, median (IQR)	0 (0–1)		0 (0–1)		.347
Number of transfusions during protocol M, median (IQR)					
Erythrocytes	0 (0–0)		1 (0.75–2)		<.001
Thrombocytes	0 (0–0)		0 (0–0.25)		.033
	Maximal grade 1–2 n (%)	Maximal grade 3–4 n (%)	Maximal grade 1–2 n (%)	Maximal grade 3–4 n (%)	
Leukopenia	33 (66%)[d]	15 (30%)[d]	9 (41%)[d]	13 (59%)[d]	.022
Neutropenia	12 (24%)	31 (62%)[d]	3 (14%)	19 (86%)[d]	.032
Increased ALT/AST	16 (32%)[d]	1 (2%)	20 (91%)[d]	2 (9%)	<.001
Increased creatinine prior to MTX	4 (8%)	0	1 (5%)	0	.504
Increased creatinine 48 h after MTX	2 (4%)	0	3 (14%)	0	.163
Decreased albumine	0[d]	0	14 (100%)[c,d]	0	<.001
Neurotoxicity[b]	5 (10%)	2 (4%)	6 (27%)	0	.233
Mucositis	18 (36%)	16 (32%)	10 (46%)	5 (23%)	.580
Diarrhea	10 (20%)	1 (2%)	5 (23%)	1 (5%)	.572

MTX: methotrexate; IQR: interquartile range; ALT: alanine transaminase; AST: aspartate transaminase.
[a]In one patient, protocol M was not completed because he had to switch high risk therapy due to high minimal residual disease.
[b]Neurotoxicity included somnolence (grade 1–2), depressed consciousness (grade 1–2), agitation (grade 3–4), and seizures (grade 3–4).
[c]Albumine was standardly measured only in part of the patients ($n = 14$ and $n = 36$ for the groups without and with asparaginase, respectively).
[d]Statistically significant ($p < .05$) after *post-hoc* analysis.
Grading according to the common terminology criteria for adverse events version 4.03.

Toxicity

The maximum toxicity per group and the number of infections and transfusions during protocol M are described in Table 3. Patients with asparaginase treatment during the high dose MTX courses received significantly more erythrocyte and thrombocyte transfusions (median and IQR of 1 (0.75–2) and 0 (0–0.25), respectively) than patients without concomitant asparaginase treatment (median and IQR of 0 (0–0) and 0 (0–0), respectively). Patients with asparaginase treatment received a maximum of 5 erythrocyte and 4 thrombocyte transfusions; in the group without asparaginase, the maximum was 1 for both erythrocyte- and thrombocyte transfusions. In addition, the occurrence of grade 3–4 leukopenia and neutropenia was higher in the group of patients with concomitant asparaginase treatment (leukopenia 59% vs. 31%, and neutropenia 86% vs. 63%, respectively). However, the number of infections during the high dose MTX courses did not differ between patients with or without concomitant asparaginase treatment.

Table 4. Duration of protocol M.

	No asparaginase during high dose MTX courses $n = 49$[a]	Asparaginase during high doses MTX courses $n = 22$[a]	p Value
Duration of protocol M in days, mean ± SD	68 ± 7	80 ± 14	.001
Duration of hospital admissions for MTX administration in days, median (IQR)	–	–	–
Dose 1	2 (2–4)	2 (2–2)	.087
Dose 2	2 (2–3)	2 (2–3)	.610
Dose 3	2 (2–3)	2 (2–2)	.407
Dose 4	2 (2–4)	2 (2–3)	.099
Extra hospital admissions during protocol M (median, IQR)	0 (0–1)	0 (0–1)	.266
T48 MTX plasma level, μM (median, IQR)	0.39 (0.30–0.52)	0.39 (0.26–0.64)	.510[b]

MTX: methotrexate; SD: standard deviation; IQR: interquartile range.
[a]In two patients, protocol M was not completed. In one patient due to severe neurotoxicity, protocol M was postponed for several weeks; the other patient had to switch to high risk treatment during protocol M.
[b]The difference in T48 MTX plasma levels between the groups was analyzed using marginal models and corrected for sex, age and albumin levels.

Regarding hepatotoxicity, significantly more patients who were treated with asparaginase during the high dose MTX courses had grade 1–2 increased ALT and AST. On the other hand, grade 3–4 increased ALT and AST only occurred in one and two patients of the patients without and with concomitant asparaginase treatment, respectively.

All patients with asparaginase had grade 1–2 hypoalbuminemia, in contrast to patients without asparaginase, who all had normal albumin levels. This, however, had no clinical consequences.

The number of patients with increased creatinine, neurotoxicity, mucositis, and diarrhea did not significantly differ between the groups. Nephrotoxicity 48 h after one of the high dose MTX courses occurred in 5 patients (3 patients with and 2 patients without asparaginase), all grade 1–2.

The duration of protocol M, and prolonged and extra hospital admissions during this treatment phase are described in Table 4. Without any delay, protocol M would have a duration of 63 days. The mean duration of protocol M for patients without asparaginase administrations was 68 days (SD: 7 days); the mean duration for patients with asparaginase administrations was 80 days (SD: 14 days) ($p = .001$).

The hospital admission duration for MTX administration is usually 2 days, although, among other clinical reasons, admissions may be prolonged if plasma 48 h MTX plasma levels are >0.4 μM. There was no statistically significant difference in the hospital admission duration between the groups. Also, there was no statistically significant difference in the number of extra hospital admissions (median number and IQR of 1 (0–1) for both groups).

MTX polyglutamates

In total, 240 erythrocyte MTXPG samples were obtained. Longitudinally analyzed, MTXPG 2–5 were lower in patients treated with asparaginase during the high dose MTX courses. All MTXPG levels increased in the consecutive high dose MTX courses (Figure 1). The number of days between the MTX dose and sampling, sex, and age did not significantly alter the MTXPG levels. Also, the timing of the asparaginase dose with respect to the MTX dose did not have a significant influence on the MTXPGs. Figure 1 shows the median MTXPG levels two weeks (12–16 days) after the high dose MTX courses, corrected for the administration of erythrocyte transfusions. Comparing patients with and without concomitant asparaginase treatment, the median (IQR) MTXPG levels were 4.0 μM (2.1–7.6 μM) vs. 10.8 μM (6.6–19.8 μM) for MTXPG 2 ($p < .001$), 18.7 μM (9.9–27.2 μM) vs. 27.5 μM (18.5–37.4 μM) for MTXPG 3 ($p = .004$), 29.2 μM (13.0–42.1 μM) vs. 37.9 μM (26.6–58.3 μM) for MTXPG 4 ($p = .002$), and 19.4 μM (9.9–28.8 μM) versus 32.8 μM (20.3–48.1 μM) for MTXPG 5 ($p = .004$). Overall, the median (IQR) MTXPG 2–5 levels were 72.7 μM (37.7–110.4 μM) for patients with and 118.6 μM (74.8–16.1 μM) for patients without asparaginase treatment during protocol M ($p < .001$).

MTX plasma levels

The 48 h MTX plasma levels are shown in Table 4 and shown in Figure 2. The median 48 h MTX plasma level for patients without concomitant asparaginase treatment was 0.39 μM (IQR: 0.30–0.52 μM); for patients with concomitant asparaginase treatment, this was 0.39 μM (IQR: 0.26–0.64 μM). In the longitudinal analysis, we have corrected for sex, age, and albumin. Creatinine levels were not increased just prior to the MTX doses. During the analysis, outliers violated the normality assumption, even after log-transformation of the data. Therefore, we have performed the analysis also excluding the extreme outliers ($n = 5$). In both models, concomitant asparaginase treatment did not statistically significantly alter the 48 h plasma levels

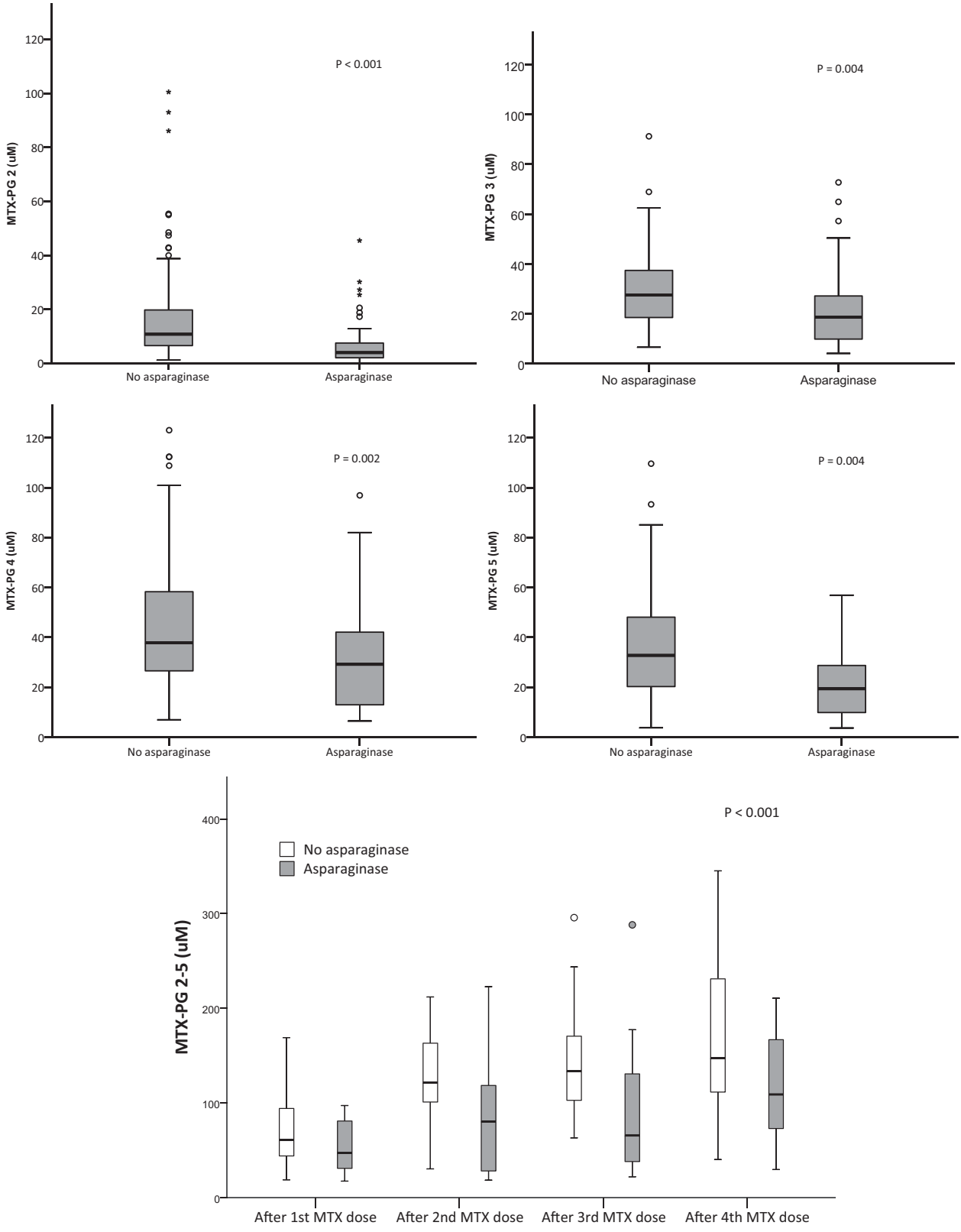

Figure 1. Erythrocyte MTXPG concentrations. This figure shows the concentrations of erythrocyte MTXPG 2, 3, 4, 5, and 2–5, measured two weeks (12–16 days) after the MTX dose, excluding measurements less than two weeks after an erythrocyte transfusion. The *p* values have been obtained from the longitudinal analysis. The MTXPG 2–5 levels are shown per MTX dose. The boxplot includes the 25th, 50th, and 75th percentile in the boxes, the outliers (○), extreme outliers (*), and the ranges (indicated by the whiskers).

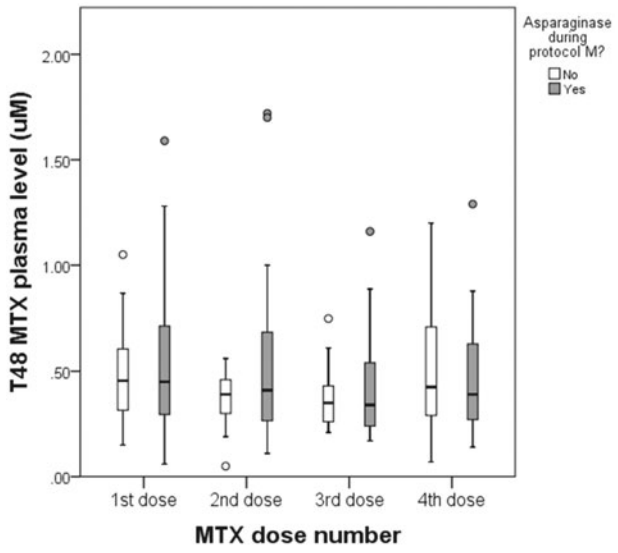

Figure 2. MTX plasma levels. This figure shows the 48 h MTX plasma levels after each high dose MTX for patients with and without concomitant asparaginase treatment. The *p* value has been obtained from the longitudinal analysis. The boxplot includes the 25th, 50th, and 75th percentile in the boxes, the outliers (○), and the ranges (indicated by the whiskers). The extreme outliers were excluded as described. The T48 MTX plasma levels did not differ between the patients who were treated with and without asparaginase (p=.510).

($p = .624$ with outliers and $p = .510$ without outliers). Also, the albumin levels did not significantly affect the 48 h MTX plasma levels.

Discussion

In this study, the toxicity and efficacy of high dose MTX were analyzed for patients with and without concomitant asparaginase therapy. Patients with asparaginase treatment had more often severe neutropenia and leukopenia, and they received more erythrocyte and thrombocyte transfusions. However, the most important consequence of myelosuppression, namely the occurrence of (severe) infections, did not differ between the groups. As a result of this myelosuppression, the high dose MTX courses had to be postponed more often in patients with asparaginase, resulting in a delay of protocol M. It could be questioned if this delay is clinically relevant. It could even have a positive effect as MTX therapy may be more effective when administered every three weeks instead of two: a possible rescue effect of folinic acid on leukemic cells may be diminished three weeks after the previous dose [26,27]. In addition to a difference in myelosuppression, all patients in the group with asparaginase had increased ALT and AST, although the far majority had grade 1–2 hepatotoxicity which had no clinical consequences.

Beside differences in myelosuppression and hepatotoxicity, we have found lower albumin levels in patients who were treated with asparaginase. MTX is a week acid and binds to serum albumin. Reiss et al. have shown that hypoalbuminemia is associated with decreased MTX clearance and an increased length of hospitalization [28]. In our patients, however, the 48 h MTX plasma levels and length of hospitalization did not differ in the patients with and without hypoalbuminemia. Unfortunately, in the study of Reiss et al., the severity of hypoalbuminemia was not reported. In our study, patients only had mild (grade 1–2) hypoalbuminemia, possibly explaining the lack of association between albumin levels, and MTX clearance, and hospitalization.

The occurrence of nephrotoxicity, neurotoxicity, and mucositis did not differ between the groups. The incidence of these side effects was in line with the incidences found by den Hoed et al., who have shown CTCAE grade 3–4 neurotoxicity in 3% and CTCAE grade 3–4 mucositis in 20% of the patients treated with high dose MTX [27]. The occurrence of nephrotoxicity has been reported in 2–10% of the patients after high dose MTX courses [29]. These toxicities have been correlated with MTX plasma levels [27,29]. In our study, asparaginase has no effect on these plasma levels, which may explain that the toxicity did not differ between the groups.

Beside toxicity during the high dose MTX courses, one should consider the toxicity during the intensification phase as well: patients in the continuous asparaginase dosing schedule will receive fewer asparaginase doses during the intensification and maintenance phase. These patients, probably, will tolerate a higher amount of 6-mercaptopurine and MTX, which is administered during these phases when they have completed their asparaginase doses, as reported by Merryman et al. [30] In addition, one could expect fewer infections and less hepatotoxicity during the intensification and maintenance phase in these patients.

Regarding MTX efficacy, we found that erythrocyte MTXPG levels were significantly lower in patients who were treated with asparaginase during the high dose MTX courses. The question is whether this is of clinical relevance. To the best of our knowledge, this is the first study comparing MTXPG levels in patients treated with and without asparaginase during high dose MTX. In earlier studies, Jolivet et al. and Sur et al. have concluded that asparaginase inhibits MTX polyglutamination *in vitro* by inhibition of FPGS due to asparagine

depletion [11,12]. However, this resulted in a decrease of mainly long chain MTXPG levels: MTXPG 4 levels were more than 80% lower, MTXPG 5 was not even measurable, but MTXPG 2 did not alter with asparaginase treatment. In our study, all MTXPG chains were lower, regardless of the chain length and, in contrast to the *in vitro* studies, all long chain MTXPGs were formed. Moreover, the overall decrease in levels is not as large as was found *in vitro* by Jolivet et al. This implies that the effect of asparaginase on MTX efficacy *in vivo* is smaller. However, it also has to be taken into account that erythroblasts, in which the erythrocyte MTX polyglutamination takes place, contain asparagine synthetase [31] and therefore, in contrast to leukemic blasts, do not depend on extracellular asparagine levels. This may influence the effect of asparaginase on MTX polyglutamination. In addition, the type and cytogenetic characteristics of the leukemic cells influence the degree of MTX polyglutamination. For example, the formation of MTXPGs is increased in hyperdiploid ALL and decreased in T-cell ALL [32,33]. On the other hand, also the asparaginase sensitivity varies, and leukemic cells which are less sensitive for asparaginase may encounter a smaller effect of asparagine depletion on MTX polyglutamination [34]. When the DCOG ALL-11 has been completed, survival analyses might provide differences between the asparaginase treatment arms, which then may be explained by the difference in MTXPG levels found in this study, although the number of relapses may be too low to draw these conclusions.

Though we found inhibition of MTX polyglutamylation, there was no effect of the timing of asparaginase administration on the MTXPG levels. In our study, asparaginase was either administrated directly or a week after the MTX. Several *in vitro* studies have shown that asparaginase inhibits MTX polyglutamylation and efficacy specifically when asparaginase has been administered prior to the MTX. Vice versa, asparaginase administration after MTX would have a synergistic effect [10–12,17]. In line with these findings, several treatment protocols prove to be successful when asparaginase is administered after MTX [2,6,35,36]. These protocols, however, used native *E. coli* asparaginase, which has shorter therapeutic activity than PEGasparaginase (three days versus two weeks). By administering PEGasparaginase biweekly, asparagine is continually depleted, which could explain our finding that the timing of asparaginase administration relative to the MTX doses had no effect on the formation of MTXPGs. However, in our study, patients were not treated with asparaginase directly prior to MTX. Therefore, we cannot draw conclusions about a possible effect of very high (top) asparaginase activity levels prior to MTX administration.

We conclude that the schedule-related antagonism seen in the early *in vitro* studies seems less important *in vivo*, especially when patients are treated with PEGasparaginase during the high dose MTX. In addition, the results of this study suggest that MTX toxicity caused by concomitant asparaginase therapy only slightly increases, and would be acceptable.

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