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Research Article

Effect of High Dose Methotrexate on Permeability Glycoprotein Serum Levels in Pediatric Patients with Acute Lymphocytic Leukemia

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ABSTRACT

Objectives To investigate the effect of high-dose methotrexate (HDMTX) on P-glycoprotein (P-gp) serum levels in pediatric patients with acute lymphocytic leukemia (ALL).

Methods This controlled parallel clinical study was conducted between April 2020 and February 2022. Thirty-three pediatric patients with a confirmed diagnosis of ALL were included in this study (ALL group). Patients were enrolled before receiving the HDMTX protocol and after completion of the induction phase. Age, body mass index (BMI), and sex ratio matched healthy pediatric subjects were also included in this study and were selected from a primary care center (control group). Blood samples were collected from the healthy control group at baseline, and patients with ALL at baseline and after MTX therapy, for biochemical analysis of P-glycoprotein. MTX concentration was also determined in ALL samples after treatment to correlate with P-gp serum level.

Results No statistically significant difference was observed in P-gp serum levels between the healthy pediatric subjects and patients with ALL before receiving MTX treatment. P-gp serum levels were significantly lower ($P < 0.05$) in pediatric patients with ALL after MTX treatment in all age segments as compared to its levels before MTX treatment (baseline data). Moreover, there was no statistically significant difference in P-gp serum levels between the healthy pediatric subjects and pediatric patients with ALL after MTX treatment in the first age segments (4–7 years old). However, P-gp serum levels were significantly lower in pediatric patients with ALL who received MTX in both the 8–10 and the 11–14 years old segments compared to the healthy group. In addition, there was no significant correlation between MTX concentration and P-gp serum level in patients with ALL after MTX treatment.

Conclusion High-dose methotrexate may exert a down-regulatory effect on P-gp serum level in pediatric patients with ALL. However, this needs further investigation. (**ClinicalTrials.gov Identifier**: NCT05021159, September 2021).

Keywords: Methotrexate, P-glycoprotein, Acute lymphocytic leukemia, Pediatrics.

1. INTRODUCTION

Acute lymphocytic leukemia (ALL) is a pediatric hematological malignancy of the highest prevalence, accounting for approximately one-third of all diagnosed cancers in the pediatric population.[1] High-dose methotrexate (HDMTX) (>500 mg/m^2) has been widely employed for several years as a critical component in ALL treatment protocols.[2] P-glycoprotein (P-gp), which is encoded by the ABCB1 gene, is one of the ABC superfamily efflux transporters with widespread expression in diverse body organs as well as in many blood cells.[3] Methotrexate (MTX) had been recognized as a specific substrate of P-gp[4]

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interpreting the resistance of cancerous tissues towards MTX treatment with a subsequent suboptimal response.[5] On the other hand, there are contradictory data regarding the effect of MTX on P-gp. In some pieces of literature, MTX was reported to down-regulate P-gp in tissue samples from treated individuals, while other authors reported inconsistent findings.[6,7]

Despite the availability of some studies that address the variations in tissue expression of P-gp in response to MTX treatment,[6] there is a scarcity of reports investigating P-gp serum levels after MTX treatment. In this context, our study aimed at investigating the effect of HDMTX on P-gp serum levels in pediatric patients with ALL as compared to its levels in healthy pediatric subjects.

2. MATERIALS AND METHODS

2.1. Study Design

The study was designed to be a controlled parallel study that was conducted between April 2020 and February 2022 at Tanta Cancer Center, Tanta, Egypt. During this study, a total of 40 ALL patients were screened for eligibility. Out of them, 5 patients were excluded (3 patients did not meet the inclusion criteria, and 2 patients declined to participate with their parents). The remaining 35 patients were included in the study. During the follow-up period, two patients dropped out, and only 33 patients completed the study. Forty healthy pediatric subjects with matched age, body mass index (BMI), and sex ratio were selected from a primary care center (control group), out of which seven declined to participate, as shown in the participants' flowchart in Figure 1. In both groups, participants were divided into three age segments which are, 4-7, 8-10, and 11-14 years old segments.

Patients with ALL (ALL group) received MTX (2–5 g/m^2) in the consolidation phase after completion of the induction phase. The study was approved by the National Research Ethics Committee of Tanta University (approval code: 34037/8/20) and by the Egyptian Ministry of Health and Population (approval code: 4-2021/12). The study protocol followed the ethical guidelines of the 1964 Declaration of Helsinki and its later amendments. The study was registered at ClinicalTrial.gov with ID: NCT05021159. Informed consents were given by the participants' representatives.

2.2. Patient inclusion & exclusion criteria

The study inclusion criteria involved pediatric patients aged between 2 and 18 years old with a confirmed diagnosis of ALL before receiving HDMTX and after their completion of the induction phase. Patients were selected to be candidates for treatment with HDMTX as the sole chemotherapeutic agent during the study period. The exclusion criteria included patients with severe renal impairment (eGFR <30 mL/min/1.73 m^2 at the time of screening), critically ill patients, and patients with other types of cancer. Following enrollment, demographic, clinical, and anthropometric data were collected, including age, gender, weight, and height, with the subsequent calculation of BMI. Patients' medical histories were taken to ensure their compliance with the study inclusion criteria.

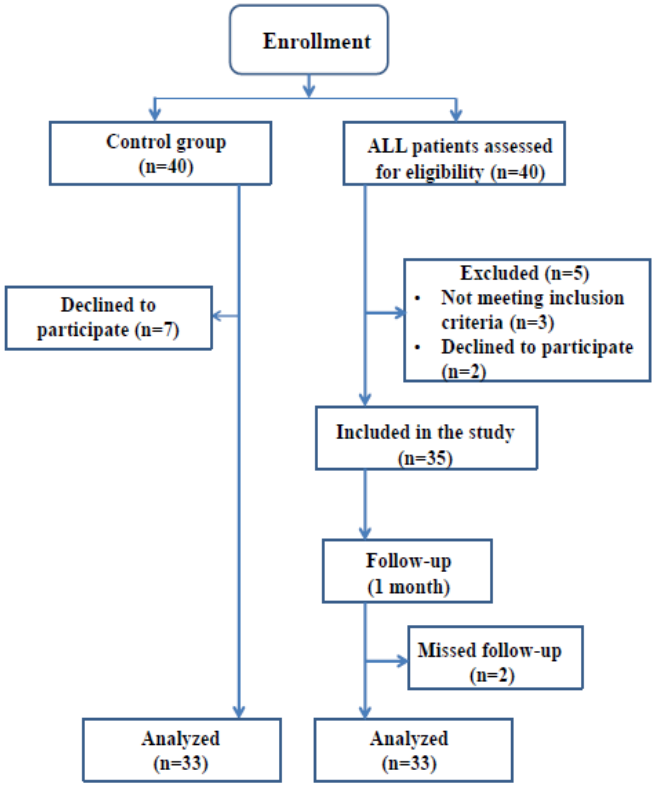

Figure 1: Flowchart of the study participants.

2.3. Biochemical investigations

Blood samples were withdrawn from each patient before MTX treatment, during the routine kidney function assessment, and also at 42 hours after MTX treatment. Blood samples were collected at the same time point for all individuals (fasting morning sample) by venipuncture into vacutainers, and then the blood samples were transferred into pre-rinsed plastic tubes. Blood samples were centrifuged at 4500 g for 10 min (Hettich Zentrifugen EBA 20), and the separated sera were kept at −80°C until analysis of the serum concentrations of both MTX and P-gp which were assayed by human enzyme-linked immunosorbent assay (ELISA) kits (SunRed Biotechnology, Shanghai, China, Catalog numbers: 201-12-1627 and 201-12-1727, respectively). All procedures and measurements were done according to the manufacturers' specifications using the supplied kits and the Biotek ELx800 UV-Vis Microplate Reader.

For the ALL group, P-gp serum level was measured at baseline and after MTX therapy to evaluate the impact of MTX on P-gp serum levels. For the control group, P-gp

serum level was measured at baseline. MTX concentrations were measured 42 hours after treatment to assess their correlation with P-gp serum levels in pediatric patients with ALL (ALL group).

2.4. Statistical analysis

Data were analyzed using the statistical package for the social sciences (IBM©SPSS® Statistics V26, 2019). The data was tested for normality using the Shapiro-Wilk test. Data were summarized using mean ± SD for quantitative variables and number/ratio for qualitative variables. The mean values of P-gp serum levels were compared between the control group and the ALL group before and after MTX treatment using an unpaired student *t*-test. The mean values of P-gp serum levels were compared in the ALL group before and after MTX treatment using the paired student *t*-test. For qualitative variables, a comparison between the two groups was done using Chi-Square ($\chi 2$) test. Comparison of the entire population of the healthy group, ALL groups before and after MTX treatment was done using ANOVA test followed by post hoc Tukey's test. Correlation analysis was performed using the Pearson coefficient of correlation to study the strength of a relationship between MTX concentration and P-gp serum level in ALL patients after receiving MTX treatment. P value <0.05 was considered statistically significant.

3. RESULTS

3.1. Demographic and anthropometric data of the study participants

There was non-significant variation between the two study groups regarding demographic and anthropometric data, including age, sex, weight, height, and BMI, as shown in Table 1. Detailed demographic and anthropometric data of all the study participants are included in Supplementary Table S1 together with the detailed statistics for each age segment.

Table 1: Baseline demographic and anthropometric data of the study participants.

Parameters	ALL group (n=33)	Control group (n=33)	P-value
Age (years)	8.69 ± 3.26	8.73 ± 3.24	0.96
Sex (male: female)	14:19	15:18	0.89
Weight (kg)	28.08 ± 8.73	28.24 ± 8.63	0.94
Height (m)	1.30 ± 0.17	1.28 ± 0.17	0.69
BMI (kg/m2)	16.16 ± 1.34	16.71 ± 1.44	0.12

Data are represented as mean ± SD and ratio. Data were analyzed using an Unpaired t-test, and a Chi-Square test was used for categorical data.

ALL group; pediatric patients with acute lymphocytic leukemia, Control group; healthy pediatric subjects.

3.2. Effect of disease state on P-gp serum levels in three different pediatric age segments

There was no statistically significant difference in P-gp serum levels between the healthy pediatric subjects (control group) and the patients with ALL (ALL group) before receiving MTX treatment (unpaired *t*-test). For the 4–7 year old age segment, P-gp serum level was (1.37 ± 0.02 ng/ml vs. 1.38 ± 0.02 ng/ml, p= 0.37); for the 8–10 year old segment, (1.25 ± 0.18 ng/ml vs. 1.22 ± 0.20 ng/ml, p= 0.52); and for the 11–14 year old segment, (1.39 ± 0.23 ng/ml vs. 1.39 ± 0.02 ng/ml, p= 0.14) as illustrated in Figure 2.

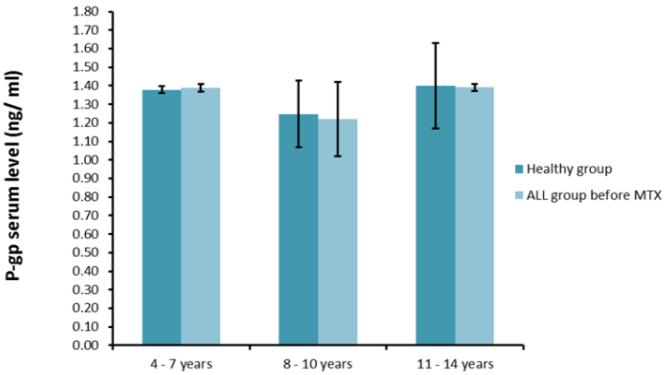

Figure 2: P-gp serum levels in three different age segments of healthy pediatric subjects and pediatric patients with ALL before receiving MTX.

3.3. Effect of MTX administration on P-gp serum levels in three different pediatric age segments

P-gp serum level was significantly lower in pediatric patients with ALL after MTX treatment in the three studied age segments compared to its level before MTX treatment (paired *t-test*). For the 4–7 year old segment; P-gp serum level was (1.41 ± 0.07 ng/ml vs. 1.39 ± 0.05 ng/ml, p= 0.018); for the 8–10 year old segment, (1.42 ± 0.05 ng/ml vs. 1.35 ± 0.13 ng/ml, p= 0.017); and for the 11–14 year old segment, (1.44 ± 0.01 ng/ml vs. 1.43 ± 0.03 ng/ml, p= 0.011) as illustrated in Figure 3.

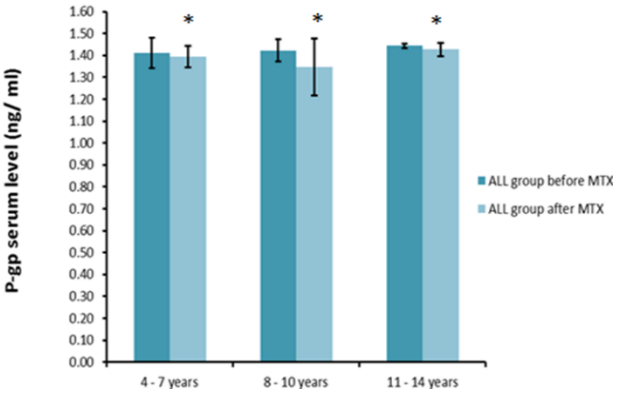

Figure 3: P-gp serum levels in three different age segments of pediatric patients with ALL before and after receiving MTX.* Significant difference (*P* <0.05).

3.4. Effect of disease state and MTX administration on P-gp serum levels in three different pediatric age segments

There was no statistically significant difference in P-gp serum levels between the healthy pediatric subjects and pediatric patients with ALL after MTX treatment in the first age segments (4–7 years old), (1.41 ± 0.07 ng/ml vs. 1.40 ± 0.05 ng/ml, p= 0.4). However, compared to the healthy group, P-gp serum levels were significantly lower in pediatric patients with ALL who received MTX in both the 8–10 years old and the 11–14 year old segments (1.42 ± 0.05 ng/ml vs. 1.32 ± 0.18 ng/ml, p= 0.016, and 1.44 ± 0.01 ng/ml vs. 1.42 ± 0.03 ng/ml, p= 5.04 x 10^{-6}, respectively), as illustrated in Figure 4.

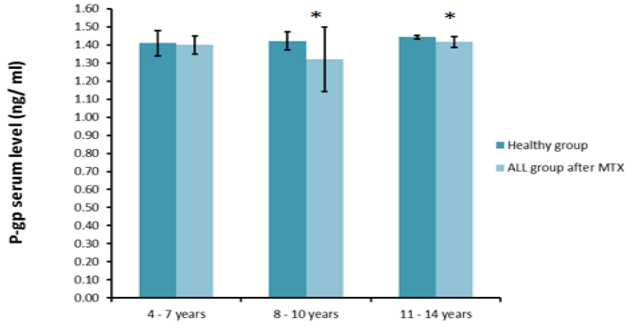

Figure 4: P-gp serum levels in three different age segments of healthy pediatric subjects and pediatric patients with ALL after receiving MTX

* Significant difference (P <0.05).

3.5. Comparison of P-gp serum levels between the healthy group and ALL groups (both before and after MTX treatment) for all participants in each group

The comparison of P-gp serum levels between the healthy group and the ALL group before and after MTX treatment using the whole population in each group showed that, there was no statistically significant difference in P-gp serum levels between the healthy pediatric subjects and pediatric patients with ALL after MTX treatment (P= 0.515). However, there were significant differences between the healthy group and the ALL group before MTX treatment (P= 0.002) and also between the ALL group before and after MTX treatment (P= 0.002) as shown in Figure 5.

3.6. Correlation analysis between MTX concentration and P-gp serum level in patients with ALL

No significant correlation was found between MTX concentration and P-gp serum level after MTX treatment in the three age segments of patients with ALL (r= 0.57, p= 0.11 for the 4–7 year old segment), (r= -0.62, p= 0.06 for the 8–10 year old segment), and (r= -0.49, p = 0.10 for the 11–14 year old segment), as shown in Figure 6 (A-C).

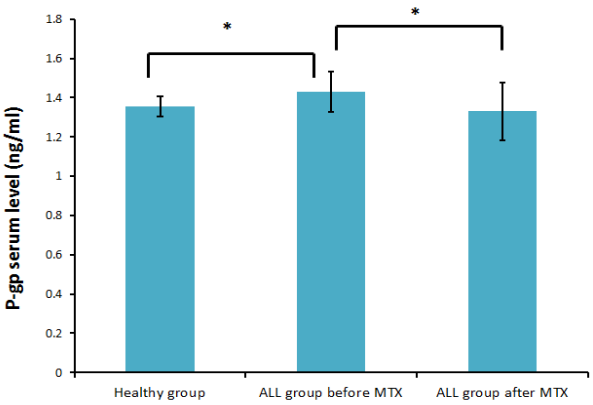

Figure 5: Mean P-gp serum levels of the whole group population of healthy pediatric subjects and pediatric patients with ALL before and after receiving MTX.

* Significant difference (P <0.05)

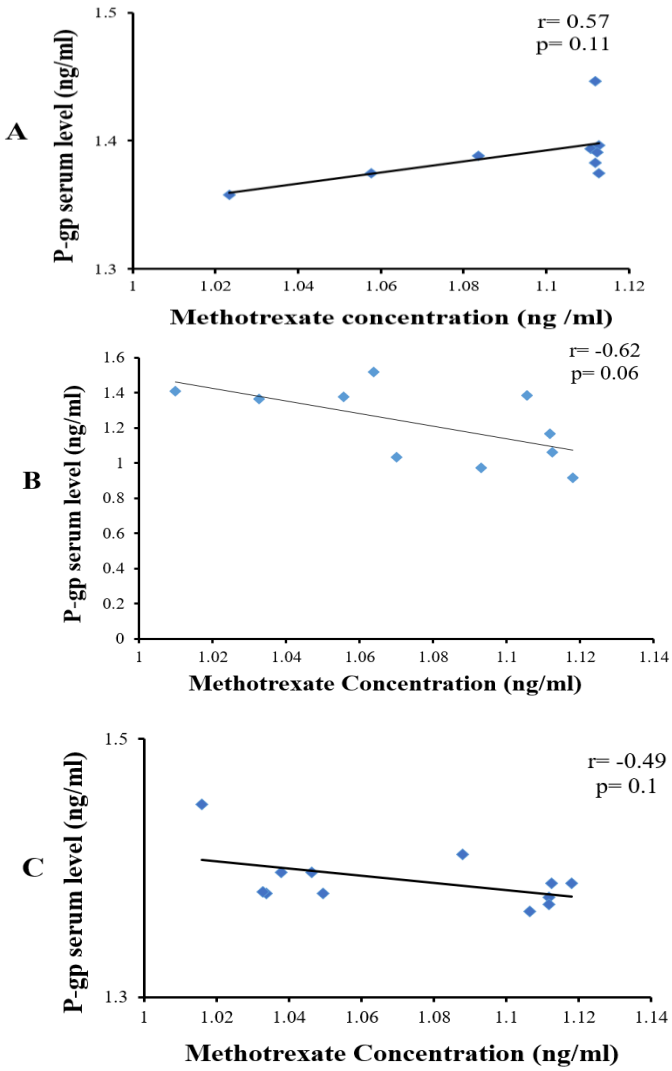

Figure 6: Correlations between MTX concentration and P-gp serum level after MTX treatment in the three studied ALL age segments (A) 4–7 years old, (B) 8–10 years old, and (C) 11–14 years old.

4. DISCUSSION

Acute lymphocytic leukemia is the most common childhood cancer, representing about one-third of all types of pediatric malignancies.[8] Successful treatment of children with ALL involves the administration of a drug regimen that is divided into several phases.[9] Methotrexate was reported to have a high value in the treatment of ALL, [10] especially when implicated in high-dose regimens.[10, 11] However, the clinical efficacy of MTX depends on intracellular drug retention and maintenance of therapeutic concentration.[12]

Continued administration of MTX is usually associated with the emergence of drug resistance, which may occur through different mechanisms. These mechanisms include MTX transporting proteins, dihydrofolate reductase (DHFR) level, and MTX polyglutamation.[13] Multidrug resistance, characterized by overexpression of drug transporters, has been shown to be one of the mechanisms involved in MTX resistance.[14]

The drug transporters that mediate MTX efflux remain somewhat controversial. Among others, P-gp had been recognized by some studies as a specific transporter of MTX.[4] However, other studies postulated that P-gp may confer resistance to MTX through indirect mechanisms.[15]

Although the variations in tissue expression and serum levels of P-gp have been shown to be important in determining the response/resistance to MTX, this has not widely been studied. Despite the availability of some studies addressing the variations of tissue expression of P-gp in response to treatment with MTX,[6] there is a scarcity of reports investigating the variations of P-gp serum levels in response to treatment with MTX.

To the best of our knowledge, this is the first clinical study that aimed at investigating the effect of the disease state and the treatment with HDMTX on P-gp serum levels in different age segments of pediatric patients with ALL as compared to healthy pediatric subjects.

Our results revealed that P-gp serum levels were not significantly changed between healthy pediatric subjects and pediatric patients with ALL before receiving MTX treatment. This aforementioned result indicates that leukemia (disease state) has no effect on P-gp serum levels. This result is not consistent with a previously reported finding indicating P-gp overexpression in bone marrow samples of 11 newly diagnosed pediatric patients (0–12 years old) with ALL.[16] These contradictory results could be explained on the basis of the variability of the sample studied. Despite we assessed the level of P-gp in serum samples, all the available studies were conducted in tissue samples. This contradictory result may be attributed to the notion that multi-drugs resistance 1 (MDR1) or P-gp gene mRNA expression levels do not necessarily correlate with P-gp level in different cell types.[17]

On investigating the effect of MTX administration on P-gp serum levels through comparing its level in the ALL group before and after receiving a single HDMTX, a statistically significant decrease was observed in P-gp serum levels in the three studied age segments after MTX treatment. This result indicates the connection between MTX administration and the decrease in P-gp serum levels. Moreover, there was no statistically significant difference in P-gp serum levels between the healthy pediatric subjects and pediatric patients with ALL after MTX treatment in the first age segments 4–7 years old, however, and as compared to the healthy group; P-gp serum levels were significantly lower in pediatric patients with ALL who received MTX in the other two age segments. Our former result comes in consonance with previously reported studies revealing that MTX down-regulates P-gp expression [6] and MTX can produce a dose-response reduction in P-gp expression in rheumatoid arthritis peripheral blood mononuclear cells.[18] This down-regulatory effect of MXT on P-gp seems highly favorable since a significantly lower serum P-gp level was observed in patients who responded to MTX therapy compared to patients who showed treatment failure by MTX.[19] However, our result seems in contradiction with the findings of other studies reporting the up-regulatory effect of MTX on P-gp. The absence of strong evidence that proves the correlation between the gene expression of P-gp and its protein levels may explain this contradiction.

No significant correlation was observed between MTX blood concentrations and P-gp serum levels in the three age segments of ALL patients after MTX treatment. This could be explained on the basis that MTX serum level depends on both expression and serum levels of several transporters other than P-gp which are involved in MTX flux. The limited time between MTX administration and sample collection (42 hours) may be an important reason for the lack of correlation. In addition, the relatively small sample size could attribute to the absence of a significant correlation between MTX blood concentrations and P-gp serum levels. Moreover, there is a reported inter-patient variability regarding the efficacy and toxicity of MTX. This variability may be related to genetic polymorphisms in the genes encoding MTX transporters and their substantial influence on the kinetics and the response to HDMTX therapy in pediatric patients with ALL.[20]

Despite of absence of similar studies that allow the comparison to our data, our findings provide new insight into the regulatory effect of MTX on P-gp serum levels. However, this study has some limitations which included the relatively small sample size which is not representative of all pediatric populations. In addition, the lack of assessment of other transporters involved in MTX efflux represents another limitation. Moreover, in our study, we did not address the relation between P-gp serum levels and patients' responses. In this context, we recommend future large-scale studies with the assessments of other transporters involved in MTX resistance.

5. CONCLUSION

The data obtained from our study revealed that MTX may have a negative effect on p-gp serum levels in spite of controversial previous results; further large-scale studies are still required to confirm our preliminary results. Furthermore, the effect of MTX on other transporters warrants further investigation.

AUTHORS' CONTRIBUTIONS

Osama M. Ibrahim and Tarek M. Mostafa reviewed the literature and constructed the study design. Maha M. Khalaf contributed to the conceptualization and eligibility evaluation. Yasmine M. Elmorsi contributed to the investigation and samples analysis. Yasmine M. Elmorsi and Tarek M. Mostafa performed the statistical and data analysis. All authors wrote, reviewed, and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest.

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LIST OF ABBREVIATIONS

- **ALL:** acute lymphocytic leukemia
- **P-gp**: permeability glycoprotein
- **MTX**: methotrexate
- **HDMTX:** high dose methotrexate
- **ELISA**: Enzyme-linked immunosorbent assay

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