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CHAPTER 3

Methotrexate resistance in relation to treatment outcome in childhood acute lymphoblastic leukemia

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ABSTRACT

Background

Methotrexate (MTX) eradicates leukemic cells by disrupting *de novo* nucleotide biosynthesis and DNA replication, resulting in cell death. Since its introduction in 1947, MTX-containing chemotherapeutic regimens have proven instrumental in achieving curative effects in acute lymphoblastic leukemia (ALL). However, drug resistance phenomena pose major obstacles to efficacious ALL chemotherapy. Moreover, clinically relevant molecular mechanisms underlying chemoresistance remain largely obscure. Several alterations in MTX metabolism, leading to impaired accumulation of this cytotoxic agent in tumor cells, have been classified as determinants of MTX resistance. However, the relation between MTX resistance and long term clinical outcome of ALL has not been shown previously.

Methods

We have collected clinical data for 235 childhood ALL patients, for whom samples taken at the time of diagnosis were also broadly characterized with respect to MTX resistance. This included measurement of concentrations of MTX polyglutamates in leukemic cells, mRNA expression of enzymes involved in MTX metabolism (FPGS, FPGH, RFC, DHFR and TS), MTX sensitivity as determined by the TS inhibition assay and FPGS activity.

Results

Herein we demonstrate that higher accumulation of long-chain polyglutamates of MTX is strongly associated with better overall (10-year OS: 90.6% vs 64.1%, P=0.008) and event-free survival (10-year EFS: 81.2% vs 57.6%, P=0.029) of ALL patients. In addition, we assessed both the association of several MTX resistance-related parameters determined *in vitro* with treatment outcome as well as clinical characteristics of pediatric ALL patients treated with MTX-containing combination chemotherapy. High MTX sensitivity was associated with DNA hyperdiploid ALL (P<0.001), which was linked with increased MTX accumulation (P=0.03) and elevated RFC expression (P=0.049) in this subset of ALL patients. *TEL-AML1* fusion was associated with increased MTX resistance (P=0.023). Moreover, a low accumulation of MTX polyglutamates was observed in *MLL*-rearranged and *TEL-AML1* rearranged ALL (P<0.05).

Conclusions

These findings emphasize the central role of MTX in ALL treatment thereby expanding our understanding of the molecular basis of clinical differences in treatment response between ALL individuals. In particular, the identification of patients that are potentially resistant to MTX at diagnosis may allow for tailoring novel treatment strategies to individual leukemia patients.

Keywords: acute lymphoblastic leukemia, antifolate, methotrexate, drug resistance, hyperdiploid, FPGS, DHFR, RFC, TS

INTRODUCTION

The treatment outcome of pediatric acute lymphoblastic leukemia (ALL) has greatly improved over the past 7 decades with the current regimens resulting in a 5-year event-free survival (EFS) of around 80%.[1–3] This impressive improvement has been largely attributed to novel prognostic factors, including cytogenetic abnormalities such as *TEL-AML1* and *E2A-PBX1* gene fusions associated with good prognosis as well as *MLL* gene rearrangements that confer unfavourable prognosis.[4,5] Yet, the high cure rates achieved with current treatment protocols are still paralleled by approximately 20% risk of relapse, which is consequently associated with poor prognosis.[2,3] The emergence of relapse is largely attributable to drug resistance phenomena of leukemic cells. Thus, further advances in understanding of the molecular basis underlying these drug resistance phenomena as well as accurate prediction of chemotherapy resistance prior to drug treatment may pave the way to overcoming of chemoresistance.

Historically, one of the backbones of contemporary ALL treatment is the folate antimetabolite – methotrexate (MTX).[6] Folates are essential enzyme cofactors involved in one-carbon metabolism, which includes several cellular biosynthetic pathways including thymidylate and *de novo* purine biosynthesis, amino acid metabolism and mitochondrial protein synthesis.[7] Antifolates potently inhibit several folate-dependent enzymes engaged in nucleotide biosynthesis, which leads to cessation of DNA replication eventually resulting in cell death.[6] High dose (HD)-MTX is used as a part of central nervous system (CNS)-directed therapy (intrathecal MTX), and MTX is an essential component of the maintenance treatment.[1] MTX is predominantly taken up into cells via the reduced folate carrier (RFC/SLC19A1) and in the case of HD-MTX treatment also by passive diffusion across cellular membranes, at least to some extent.[8,9] Upon entry into the cytoplasm, MTX undergoes polyglutamylation – a unique metabolic conversion catalyzed by folylpolyglutamate synthetase (FPGS).[9] This polyglutamylation, which is based on the sequential addition of multiple glutamate residues to the γ-carboxyl group of both folates and MTX ensures efficient intracellular retention as well as sustains and enhances target enzyme inhibition.[10–12] The main targets of polyglutamylated MTX are dihydrofolate reductase (DHFR - also inhibited by MTX monoglutamates), thymidylate synthase (TS) and several enzymes involved in purine synthesis.[9] On the other hand, a lysosomal glycoprotein - folylpolyglutamate hydrolase (FPGH) can counteract polyglutamylation, thereby increasing the efflux of MTX by the efflux transporters of the ATP-binding cassette superfamily, including for example ABCC1 and ABCG2.[9,13,14] Overall, the intracellular accumulation of MTX polyglutamates in leukemic cells proved to be an important determinant of the anti-leukemic activity of MTX in childhood ALL patients *in vivo*.[15–17] At the same time, high concentration of long-chain but not total MTX polyglutamates was associated with inhibition of *de novo* purine synthesis.[15] Consequently, a spectrum of alterations in MTX metabolism resulting in its decreased cellular accumulation, have been identified to induce MTX resistance and compromise its curative effect. MTX resistance has been attributed to inactivating mutations or down-regulation affecting the *RFC* gene, as well as increased levels of DHFR and TS enzymes together with mutations that decrease their affinity for antifolates.[8,9,18–21] In addition, different polymorphisms in *RFC*, *TS* and *DHFR* were previously reported, several of which were related to increased risk of relapse.[22–24] The cytotoxic effect elicited by MTX is also largely influenced by FPGS activity. Consequently, loss of FPGS function is a well-established mechanism of resistance to

MTX and other polyglutamylation dependent antifolates in leukemic cells.[17,25–28] Differential MTX sensitivity was shown to be associated with several cytogenetic abnormalities. Precursor B-cell ALL displaying *TEL-AML1* or *E2A-PBX1* gene fusions were characterized by decreased levels of MTX polyglutamates as compared to precursor B-cell ALL with normal karyotype,[16] while patients with hyperdiploid karyotype were highly sensitive to MTX.[29,30] Next, to its own cytotoxic effect, MTX is also important in the metabolism of other chemotherapeutics, such as mercaptopurine, used routinely in ALL treatment. MTX was shown to promote the conversion of mercaptopurine to one of its active metabolites – thioguanine nucleotides[31,32] – of which high concentration in leukemic cells was associated with increased EFS in leukemia patients.[33] Therefore, it is imperative to characterise the extent of resistance to this important chemotherapeutic, as well as the mechanisms underlying this phenomenon.

The aim of the current study was therefore to determine which parameters of MTX resistance are related to the long-term clinical outcome in childhood ALL patients treated with combination chemotherapy. Towards this goal, we have determined a range of *in vitro* parameters associated with MTX resistance in a large cohort of pediatric ALL patients and subsequently assessed their relation with treatment outcome as well as with clinical characteristics.

MATERIALS AND METHODS

ALL patients

We analysed a total of 235 newly diagnosed (see Supplemental Table S1 for patient characteristics), untreated pediatric ALL patients treated on Dutch Childhood Oncology Group (DCOG – N=125) protocols ALL6 – ALL9[34,35], German Co-operative ALL study group (COALL – N=93) protocols 92-97[2,36] and Nordic Society of Paediatric Haematology and Oncology (NOPHO – N=17) protocols ALL-92 – ALL-2000.[37] All patients (or parents or legal guardians of patients) have provided a written informed consent.

Immunophenotyping and DNA index flow cytometry were performed at reference laboratories of the participating groups; patient characteristics were collected by the study centers.

For all patients, leukemic cells freshly obtained from the bone marrow or peripheral blood of ALL patients at diagnosis were isolated within 48h of sampling as described previously.[38] When necessary, contaminating normal cells were removed by monoclonal antibodies linked to magnetic beads as described previously.[39] All samples contained > 80% leukemic blasts with the majority of samples reaching blast percentages around 90% (only 11 out of 235 patient samples contained <85% blasts), as determined by cytospin preparations stained with May-Grunwald-Giemsa (Merck, Darmstadt, Germany).

This study was approved by the Local Ethics Committee VUmc. Date of approval: December 5, 2000 (file number TJFS/bz2568a).

In situ Thymidylate Synthase inhibition assay (TSIA)

The TSIA was used to measure *in vitro* efficacy of MTX to inhibit TS, which is known to determine cytotoxicity in MTX exposed cells. Inhibition of TS was determined in whole cells by measuring the TS-catalysed conversion of ^{3}H-dUMP to dTMP and $^{3}H_2O$, as described previously.[40] Two conditions were used: a continuous 21 h incubation or a short 3 h exposure, followed by an 18 h drug-free

period. Data are expressed as the concentration of MTX necessary to inhibit 50% of the TS activity (TSI_{50}) compared to the controls incubated without MTX (in triplicate).[40,41]

MTX accumulation and polyglutamylation

Ten million leukemic cells were incubated for 24 h with 1 µM [3', 5', 7-^{3}H]-MTX (Moravek Biochemicals; final specific activity 2 Ci/mmol) in 5ml culture medium, as described previously.[17] After washing, samples were counted for radioactivity, cell number and viability. The remaining suspension was centrifuged and the cell pellet was used for measurement of polyglutamates using high performance liquid chromatography on an anion exchange column as described previously.[17,41] The data are expressed as pmol MTX-Glu_n/10^9 cells. Total MTX polyglutamates included MTX associated with 1 up to 6 glutamate residues, while long-chain MTX polyglutamates corresponded to MTX associated with 4 up to 6 glutamate residues.

RNA extraction, reverse transcription and quantitative PCR assay

Freshly isolated cells were washed twice in RPMI (Gibco) containing 2% fetal calf serum, followed by lysis in 1ml RNAzol. Next, the samples were frozen in liquid nitrogen and stored at -80°C until further processing. RNA extraction was performed according to manufacturer's instructions with previously described adjustments.[42] Competitive template PCR was performed as described previously.[42]

FPGS activity assay

The FPGS activity assay was performed as described previously.[17] Briefly, the reaction was carried out in crude cell extracts employing a 2 h incubation with a final concentration of 250 µM MTX and 4 mM [^{3}H]-L-glutamic acid at 37°C, which was next terminated with ice-cold glutamic acid. The resulting MTX-[^{3}H]-Glu_2 was separated from unreacted [^{3}H]-L-glutamic acid by reverse phase column chromatography and quantified as described previously.[17]

Statistical analysis

All statistical analyses were performed using the IBM SPSS Statistics 20 software. The Mann-Whitney U test was used to compare the levels of MTX resistance-related variables between different subgroups of ALL patients, while Spearman's Rho test and linear regression were applied to assess correlations. Kaplan-Meyer analysis and Cox regression were used in the analysis of EFS as well as overall survival (OS) of the patients. The cut off values for survival analysis were selected based on literature (clinical factors).[2,34–37] For MTX-related variables, if the distribution was bimodal, we chose the cut-off value midway between the two modes of the distributions, otherwise we used the median as a cut-off. Kaplan-Meyer analysis and Cox regression were used for testing associations between MTX resistance-related variables and event-free (defined as time from complete remission to an event) as well as overall survival of the patients. Events considered in event-free survival were relapse and death. MTX resistance-related variables which showed significant impact on survival in the univariate analysis ($p<0.05$ at two-sided testing) were added to a multivariate Cox regression model with identified prognostic clinical factors in order to assess their added prognostic value. As different protocols were used with different patients, additional analyses were performed in which we corrected for the protocol used by including protocol as an additional independent variable in the Cox regression model.

RESULTS

The association of *ex vivo* MTX resistance and the clinical outcome

To assess the relevance of MTX resistance in pediatric ALL we determined several *ex vivo* parameters characterizing MTX metabolism in leukemic blasts of ALL patient specimens. This primarily included the measurement of the cellular concentration of total (1-6 glutamate residues) as well as long-chain (4-6 glutamate residues) MTX polyglutamates and the extent of TS inhibition (TSIA). For the TSIA parameter we used both a continuous (TSIA cont.) and a short-term (TSIA short) exposure to MTX, followed by a drug free period. TSIA cont. represents the overall ability of cells to accumulate MTX, while TSIA short enables the efflux of MTX during the drug free period and hence rather points to the polyglutamylation capacity of the cells. This approach reliably reflects MTX sensitivity of leukemic blasts.[40] In addition, FPGS activity and mRNA expression of enzymes and proteins involved in MTX metabolism and transport were determined including *DHFR*, *TS*, *FPGS*, *FPGH* and *RFC*. The above-mentioned parameters were determined in a cohort of 235 samples derived from pediatric ALL patients at diagnosis. The patients under study were enrolled in several treatment protocols. The proportion of patients assigned to specific protocols along with their clinical characteristics is listed in Additional file 1 (Supplemental Table S1). Due to logistic challenges and leukemic blast number limitations, none of the MTX-related parameters could be measured in all the patients in our cohort. Therefore the number of cases included in the statistical analysis varied considerably for the different parameters and the number of patients used for each measurement as listed in the various tables.

The prognostic value of MTX resistance associated parameters was assessed with respect to long-term OS as well as EFS of childhood ALL patients. We identified three MTX related variables that were significantly associated with OS in Kaplan-Meyer analysis (Figure 1). Higher levels of both the total concentration of cellular MTX as well as long-chain MTX polyglutamates were predictive of a better outcome. A 10-years OS reached 89.7% in patients with high concentrations of total MTX polyglutamates and 90.6% in case of long-chain MTX polyglutamates, compared to 64% in patients with low levels of these MTX metabolites (P=0.018 for total concentration and P=0.008 for long-chain MTX polyglutamates, respectively). Consistently, higher FPGS activity was also predictive of a better OS (P=0.039). For EFS, only the accumulation of long-chain MTX polyglutamates was a significant predictor of a better outcome (Figure 2; P=0.029). The total concentration of MTX showed a similar trend, although it did not reach significance (Figure 2, P=0.055). The univariate Cox regression model further confirmed the concentration of both long chain (EFS: HR=2.61, P=0.035, OS: HR=4.44, P=0.015) and total MTX polyglutamates (EFS: HR=2.29, P=0.062, OS: HR=3.8, P=0.028) as the strongest prognostic variables (Table 1 and Supplemental Table S2). Due to the large heterogeneity of treatment protocols applied in therapy of the patients under study, we performed our analyses both with and without the correction for the treatment protocol. In this analysis, the concentration of the long chain MTX polyglutamates was still significantly associated with OS (HR=3.82, P=0.03), but not with EFS (HR=2.23, P=0.084); whereas the levels of total MTX polyglutamates were not significant for both the EFS and OS (EFS: HR=1.73, P=0.232, OS: HR=2.57, P=0.125). Intriguingly, low *RFC* mRNA expression was a significant predictor of a longer EFS when corrected for the treatment protocol (HR=0.15, P=0.009). Trends observed for the concentration of both the total as well as long-chain MTX polyglutamates in the whole patient cohort were also

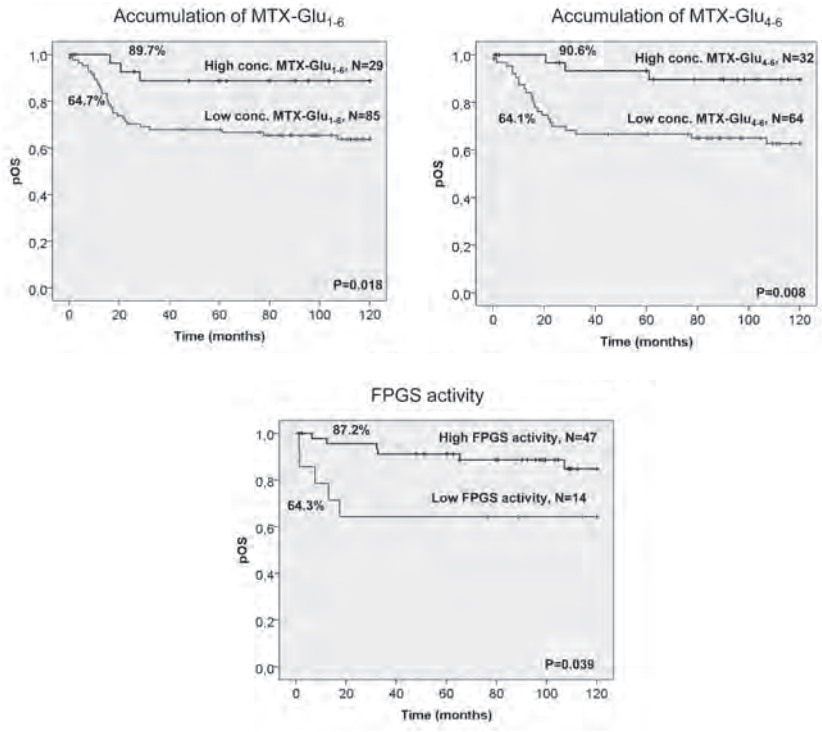

Figure 1. Kaplan-Meyer analysis of the accumulation of MTX polyglutamates and FPGS activity in relation to overall survival (P logrank) of total ALL patients. Each survival curve is labelled with the OS rates at 10 years and the number of patients included in each subgroup. The cut-off values used were: 1935 pmol/10^9 cells for total accumulation of MTX polyglutamates, 1000 pmol/10^9 cells for long chain MTX polyglutamates and 0.35 pmol MTX-Glu_2 formed/h/10^6 cells for FPGS activity.

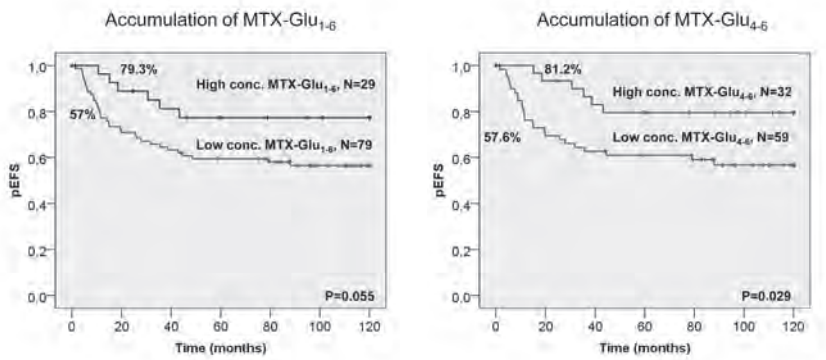

Figure 2. Kaplan-Meyer analysis of the accumulation of MTX polyglutamates in relation to event-free survival (P logrank). Each survival curve is labelled with the EFS rates at 10 years and the number of patients included in each subgroup. The cut-off values used were: 1935 pmol/10^9 cells for total accumulation of MTX polyglutamates and 1000 pmol/10^9 cells for long chain MTX polyglutamates.

Table 1. Results of univariate Cox regression model of MTX resistance related variables in relation to event-free survival.

	Not corrected for protocols			Corrected for protocols		
	N	**Hazard Ratio (95%CI)**	**p-value**	**N**	**Hazard Ratio (95%CI)**	**p-value**
TSIA short (cut-off 1.44 µM)						
Low	79	1.00		73	1.00	
High	23	1. 39 (0.67-2.89)	0.374	22	1.34 (0.60-3.01)	0.477
TSIA contin. (cut-off 0.035 µM)						
Low	25	1.00		22	1.00	
High	75	1.19 (0.54-2.60)	0.671	70	1.30 (0.53-3.18)	0.567
MTX-Glu_{1-6} accumulation (cut-off 1935 pmol/10^9 cells)						
High	29	1.00		29	1.00	
Low	79	2.29 (0.96-5.45)	0.062	72	1.73 (0.70-4.27)	0.232
MTX-Glu_{4-6} accumulation (cut-off 1000 pmol/10^9 cells)						
High	32	1.00		32	1.00	
Low	59	2.61 (1.07-6.36)	0.035*	56	2.23 (0.90-5.57)	0.084
DHFR mRNA (cut-off 3.74)						
High	27	1.00		27	1.00	
Low	18	1.50(0.50-4.47)	0.466	18	1.16 (0.37-3.67)	0.796
TS mRNA (cut-off 10)						
Low	34	1.00		33	1.00	
High	12	0.77 (0.21-2.81)	0.696	12	0.78 (0.21-3.22)	0.781
FPGS mRNA (cut-off 4.13)						
High	19	1.00		19	1.00	
Low	23	1.17 (0.36-3.83)	0.798	22	0.58 (0.16-2.18)	0.421
FPGH mRNA (cut-off 3.17)						
High	16	1.00		15	1.00	
Low	18	1.18 (0.32-4.40)	0.805	18	0.80 (0.19-3.33)	0.764
RFC mRNA (cut-off 0.86)						
High	19	1.00		19	1.00	
Low	25	0.70 (0.23-2.19)	0.544	24	0.36 (0.10-1.32)	0.123
FPGS activity (cut-off 0.35)						
High	48	1.00		48	1.00	
Low	12	2.32 (0.79-6.80)	0.125	11	2.61 (0.88-7.76)	0.084

TSIA continuous (TSIAcont.) and short-term exposure (TSIA short) are expressed as the concentration of MTX (in µM) necessary to inhibit 50% of the TS activity (TSI_{50}) compared to the controls incubated without MTX (in triplicate); the concentration of MTX polyglutamates (MTX-Glu_{1-6} and MTX-Glu_{4-6}) is expressed as pmol MTX-Glu_n/10^9 cells; mRNA expression of folate enzymes is expressed as ratio normalized to β-actin; FPGS activity is expressed as pmol MTX-Glu_2 formed/h/10^6 cells. The asterisk * denotes significant associations.

apparent when precursor B-cell ALL patients were analyzed separately (data not shown), while the numbers of T-cell ALL patients were too low for further analysis.

Next, the independent prognostic values of the concentration of total as well as long chain MTX polyglutamates were examined in the multivariate Cox regression model, in combination with clinical factors significantly associated with the EFS (white blood cell count - WBC, age and CNS

involvement – see Supplemental Tables S3 and S4) and OS (WBC, age, CNS involvement, lineage and DNA index). Neither of the parameters tested had independent prognostic value in the multivariate analysis of EFS (Supplemental Table S5), both with (HR=1.65, P=0.292 for the total MTX polyglutamates, HR=2.16, P=0.112 for the long chain MTX polyglutamates) and without the correction for treatment protocol (HR=1.9, P=0.170 for the total MTX polyglutamates, HR=2.33, P=0.073 for the long chain MTX polyglutamates). *RFC* mRNA expression showed a significant association with OS in the multivariate analysis both with (HR=0.18, P=0.025) and without (HR=0.13, P=0.026) the correction for the treatment protocol. As in the univariate analysis, low *RFC* expression was predictive of a better OS which is the opposite of what was expected. Neither the accumulation of the total nor the long chain MTX polyglutamates showed significant association with the OS (Supplemental Table S6).

Ex vivo MTX resistance in pediatric ALL patient specimens

To substantiate the associations found in the clinical outcome analysis, we performed more in detail characterization of MTX resistance in the patient cohort under study. Since, precursor B-cell ALL and T-cell ALL have a different MTX response, we first compared the distribution of the MTX-resistance parameters between these two subtypes of ALL (Table 2). Indeed, our results support previously reported findings in a patient group partially overlapping with the currently analysed cohort.[17,40,42] Accordingly, precursor B-cell ALL patient specimens showed higher MTX sensitivity than T-cell ALL samples, as determined by lower median TSI_{50} values for the short-term exposure (3.9-fold difference in the median, P<0.001), as well as higher accumulation of both total and long-chain MTX polyglutamates (1.9-fold and 3.3-fold respectively, P<0.001). These differences were paralleled by higher mRNA expression (2.6-fold, P=0.008) and enzyme activity (5.6-fold, P<0.001) of FPGS in precursor B-cell ALL as well as elevated levels of *DHFR* and *TS* mRNA in T-cell ALL (3.2-fold and 3.9-fold respectively, both P<0.001).

Table 2. MTX resistance-related variables in precursor B- and T-cell ALL.

	Precursor B-cell ALL		T-cell ALL		
	N	**Median (Range)**	**N**	**Median (Range)**	**P-value**
TSIA cont.	85	0.058 (0.01-0.76)	30	0.062 (0.01-0.22)	0.595
TSIA short	87	0.37 (0.16-12.97)	30	1.44 (0.16-40.0)	<0.001
MTX-Glu_{1-6}	97	1210 (120-4838)	31	630 (314-1943)	<0.001
MTX-Glu_{4-6}	83	856 (80-3190)	25	260 (0-843)	<0.001
DHFR mRNA	37	3.86 (0.27-55.06)	17	12.42 (1.48-34.11)	<0.001
TS mRNA	37	2.64 (0.24-23.04)	18	10.26 (1.64-25.04)	<0.001
FPGS mRNA	32	6.26 (1.12-39.64)	20	2.39 (0.52-17.20)	0.008
FPGH mRNA	27	2.12 (0.16-18.00)	16	4.83 (0.18-27.65)	0.196
RFC mRNA	35	0.89 (0.24-6.90)	20	0.79 (0.23-4.79)	0.668
FPGS activity	57	1.01 (0.03-11.33)	16	0.18 (0.02-1.57)	<0.001

P-value is determined by the Mann-Whitney U test; TSIA continuous (TSIAcont.) and short-term exposure (TSIA short) are expressed as the concentration of MTX (in μM) necessary to inhibit 50% of the TS activity (TSI_{50}) compared to the controls incubated without MTX (in triplicate); the concentration of MTX polyglutamates (MTX-Glu_{1-6} and MTX-Glu_{4-6}) are expressed as pmolMTX-Glu_n/10^9 cells; mRNA expression of folate enzymes is expressed as ratio normalized to β-actin; FPGS activity is expressed as pmol MTX-Glu_2 formed/h/10^6 cells.

Next, in order to determine which variables strongly influence MTX resistance in ALL patients, we examined the correlation between cellular levels of MTX polyglutamates, activities and mRNA expression levels of MTX metabolic enzymes with *in vitro* MTX sensitivity as determined by the TSIA. In line with previous findings,[40] both the TSIA results based on continuous and the short-term MTX exposure correlated with concentrations of total (TSIA continuous: R=-0.432 and TSIA short-term: R=-0.524, P<0.001) and long chain MTX polyglutamates (Table 3, R=-0.399, P=0.001 and R=-0.568, P<0.001) as well as with the *TS* mRNA expression (TSIA continuous: R=0.466, P=0.033 and TSIA short-term: R=0.693, P=0.001). Similar correlations were obtained when precursor B-cell patients were examined separately. Weaker, and non-significant correlations were found in T-cell ALL, suggesting that there might be a substantial difference between the factors determining MTX resistance in precursor B-cell and T-cell leukemia. To formally test whether the strength of association between TSIA and MTX polyglutamates or *TS* mRNA expression differed between B-cell and T-cell leukemia, we used a linear regression model that included an interaction between the level of MTX polyglutamates and the lineage. No significant difference in association between the concentration of MTX polyglutamylates and both the continuous and short-term TSIA was found between the precursor B-cell and T-cell ALL (data not shown).

Intriguingly, the accumulation of both total and long-chain MTX polyglutamates correlated with FPGS activity but not with its mRNA levels (Table 4). This association was observed for the whole cohort, as well as for precursor B-cell ALL alone. Consequently, *FPGS* mRNA expression did not correlate with FPGS activity (P=0.125 for the whole cohort, P=0.75 for precursor B-cell ALL and P=0.544 for T-cell ALL).

Table 3. Correlation of the TSIA assay (continuous and short exposure) with accumulation of MTX polyglutamates and TS mRNA expression.

	Precursor B-cell ALL		T-cell ALL		Total ALL	
	TSIA cont.	**TSIA short**	**TSIA cont.**	**TSIA short**	**TSIA cont.**	**TSIA short**
MTX-Glu$_{1-6}$						
correlation coefficient	-0.547*	-0.495*	-0.094	-0.128	-0.432*	-0.524*
P-value	<0.001	<0.001	0.685	0.612	<0.001	<0.001
number of patients	64	58	21	18	85	76
MTX-Glu$_{4-6}$						
correlation coefficient	-0.494*	-0.503*	-0.258	0.046	-0.399*	-0.568*
P-value	<0.001	<0.001	0.336	0.875	0.001	<0.001
number of patients	54	50	16	14	70	64
TS mRNA						
correlation coefficient	0.591	0.678*	0.139	0.483	0.466*	0.693*
P-value	0.056	0.045	0.701	0.187	0.033	0.001
number of patients	11	9	10	9	21	18

P-value was estimated using Spearman's Rho test; TSIA continuous (TSIAcont.) and short-term exposure (TSIA short) are expressed as the concentration of MTX (in μM) necessary to inhibit 50% of the TS activity (TSI_{50}) compared to the controls incubated without MTX (in triplicate); the concentration of MTX polyglutamates (MTX-Glu_{1-6} and MTX-Glu_{4-6}) is expressed as pmol MTX-Glu_n/10^9 cells; TS mRNA level is expressed as ratio normalized to β-actin. The asterisk * denotes significant associations.

Table 4. Correlation of cellular MTX polyglutamates levels with mRNA expression of FPGS and FPGH, as well as FPGS activity.

	Precursor B-cell ALL		T-cell ALL		Total ALL	
	MTX-Glu_{1-6}	MTX-Glu_{4-6}	MTX-Glu_{1-6}	MTX-Glu_{4-6}	MTX-Glu_{1-6}	MTX-Glu_{4-6}
FPGS mRNA						
correlation coefficient	-0.168	-0.082	0.345	0.002	0.264	0.280
P-value	0.456	0.724	0.176	0.994	0.104	0.104
number of patients	22	21	17	14	39	35
FPGH mRNA						
correlation coefficient	0.117	0.195	0.366	0.537	0.101	0.045
P-value	0.622	0.424	0.219	0.110	0.576	0.817
number of patients	20	19	13	10	33	29
FPGS activity						
correlation coefficient	0.429*	0.419*	0.265	0.05	0.522*	0.533*
P-value	0.005	0.01	0.431	0.898	<0.001	<0.001
number of patients	41	37	11	9	52	46

P-value was estimated using Spearman's Rho test; the concentration of MTX polyglutamates (MTX-Glu_{1-6} and MTX-Glu_{4-6}) is expressed as pmol MTX-Glu_n/10^9 cells; mRNA expression of folate enzymes is expressed as ratio normalized to β-actin; FPGS activity is expressed as pmol MTX-Glu_2 formed/h/10^6 cells. The asterisk * denotes significant associations.

Ex vivo MTX resistance in association with chromosomal abnormalities

Next, we examined the possible association of MTX resistance with the hyperdiploid karyotype, as well as 3 other recurrent cytogenetic abnormalities seen in ALL: *MLL*-rearrangements, *TEL-AML1* and *E2A-PBX1*. We found that blasts of hyperdiploid ALL patients were significantly more sensitive to MTX than blasts from non-hyperdiploid ALL patients (Figure 3), both in the continuous and the short-term TSIA (P<0.001). Consistent with previous reports,[30,43] the leukemic blasts of these patients accumulated significantly higher levels of total MTX and in the same time displayed elevated *RFC* mRNA levels, confirming that increased uptake of MTX contributes to MTX hypersensitivity observed in this group.

Similarly, the analysed cytogenetic subtypes of ALL showed several associations with MTX resistance-related parameters (Table 5). In line with findings from others,[16] we established that *TEL-AML1* fusion results in lower accumulation of both total and long-chain MTX polyglutamates (2.2-fold, P=0.013 and 2.8-fold, P=0.007, respectively). In addition, these patients showed higher TSI_{50} values for both continuous and short-term exposure (3.3-fold, P=0.023 and 2.9-fold P=0.06, respectively). Interestingly, ALL patients with *MLL* rearrangements also displayed lower accumulation of total and long-chain MTX polyglutamates (3.6-fold, P=0.005 and 4.3-fold, P=0.012, respectively), however this was not paralleled by elevated TSI_{50} values. No significant correlations were observed for patients harbouring the *E2A-PBX1* nor *BCR-ABL* gene fusions, which can partially be explained by the small patient numbers in both groups.

DISCUSSION

In the current study, which used multiple parameters associated with MTX sensitivity, we demonstrate that the cellular level of MTX-induced cytotoxicity is an important determinant of

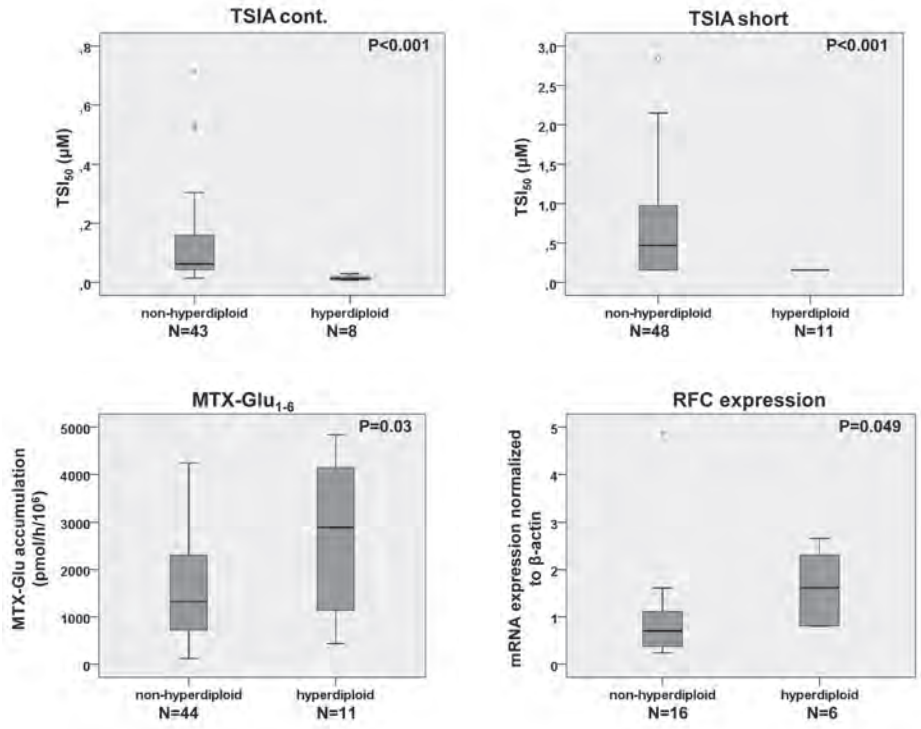

Figure 3. MTX resistance related variables that are significantly different in precursor B-cell ALL patients with hyperdiploid karyotype compared to non-hyperdiploid precursor B-cell ALL patients. The box plots depict means with standard deviation (P Mann-Whitney U test).

long-term treatment outcome in childhood ALL. We showed that accumulation of long-chain MTX polyglutamates was the strongest MTX resistance-associated variable, as reflected by its significant association with both OS and EFS in the analysed patient cohort. Our data support and further extend previous studies documenting better antileukemic effect of MTX as well as favourable 5-year event-free survival (EFS) in childhood ALL patients, whose leukemic cells accumulated higher levels of MTX polyglutamates.[15,29,44] On the other hand, a study of Children's Oncology Group (COG) suggested that the association between the concentration of MTX polyglutamates and EFS is abrogated by higher dose MTX therapy.[45] However, this study involved a different mode of MTX administration and cut-off levels for high/low polyglutamate accumulation, which might have influenced the different outcome of the analysis. When analysed in multivariate Cox regression with correction for established clinical factors (including WBC, age, CNS involvement, lineage and DNA index) as covariates, the cellular level of long-chain MTX polyglutamates was not an independent prognostic factor. This indicates that despite the important role in ALL treatment response, MTX polyglutamates may not have additional predictive value to the already existing models. Surprisingly, low levels of *RFC* were predictive of a better overall survival in both univariate and multivariate analysis, contradicting previous reports.[9,46] The relation between *RFC* mRNA expression and overall survival was the strongest when corrected for the treatment protocol, suggesting that it might be linked to high doses of MTX used in some treatment protocols. When

Table 5. MTX sensitivity and accumulation of MTX polyglutamates in relation to cytogenetic abnormalities.

		TSIA cont.	TSIA short	MTX-Glu_{1-6}	MTX-Glu_{4-6}
Normal cytogenetics	*N*	16	23	18	17
	Median	0.05	0.28	2102	1514
	(range)	(0.01–0.71)	(0.16–2.84)	(436–4240)	(209–3190)
MLL-rearranged	*N*	8	6	4	3
	Median	0.06	0.21	591	350
	(range)	(0.03–0.16)	(0.16–1.33)	(360–1016)	(280–443)
	P value	0.610	0.896	0.005*	0.012*
BCR-ABL	*N*	4	2	4	2
	Median	0.04	–	2088	1630
	(range)	(0.03–0.28)		(360–3721)	(1212–2047)
	P value	0.750	0.240	0.712	0.749
TEL-AML1	*N*	10	9	8	7
	Median	0.16	0.82	945	550
	(range)	(0.06–0.37)	(0.54–11.21)	(290–2476)	(380–1152)
	P value	0.023*	0.06	0.013*	0.007*
E2A-PBX1	*N*	3	3	1	0
	Median	0.14	0.77	–	–
	(range)	(0.05–0.29)	(0.57–1.12)		
	P value	0.254	0.157	–	–

First, the Kruskal-Wallis analysis of variance was used to compare all the 4 groups and variables with the P-value above 0.1 were further examined in detail. The Mann-Whitney U test was used to compare MTX related variables between each subgroup of ALL patients carrying certain cytogenetic abnormality and patients displaying normal karyotype as a reference group; TSIA continuous (TSIAcont.) and short-term exposure (TSIA short) are expressed as the concentration of MTX (in μM) necessary to inhibit 50% of the TS activity (TSI_{50}) compared to the controls incubated without MTX (in triplicate); the concentration of MTX polyglutamates (MTX-Glu_{1-6} and MTX-Glu_{4-6}) is expressed as pmol MTX-Glu_n/10^9 cells. The asterisk * denotes significant associations.

examined within each treatment protocol separately, this association was only observed in patients treated at DCOG ALL8 protocol, on which patients depending on the risk group were administered MTX at 2 g/m^2 or 5 g/m^2.[34] In contrast, on COALL protocol ALL-97, for which this relation was absent, MTX was administered at 1 g/m^2.[2,36] This discrepancy may be explained by the limited number of patients treated with 1 g/m^2 in this cohort (6 patients for COALL-97 and 16 for DCOG ALL8). Decreased *RFC* expression as well as mutations in the *RFC* gene, resulting in less efficient uptake of antifolates by tumour cells, have been associated with MTX resistance.[9,46] On the other hand, some of the mutations found in the *RFC* gene were previously reported to increase the affinity of RFC to folates, resulting in increased intracellular accumulation and competition of folate with MTX.[47] It is therefore possible that extremely high *RFC* expression results in high concentration of folates in the cells, leading to decreased efficacy of MTX and worse treatment outcome. Unfortunately, the numbers of patients treated on the other protocols for which *RFC* mRNA expression data were available were too low to allow this analysis.

Moreover, we show that the lineage as well as a number of chromosomal abnormalities is associated with distinct levels of MTX sensitivity. In agreement with previous analysis in a partially overlapping patient cohort as well as in other studies,[17,40,42] we observed here elevated accumulation

of MTX polyglutamates together with decreased expression of *DHFR* and *TS* mRNA and consequent higher MTX sensitivity in precursor B-cell ALL, as compared to T-cell ALL. The difference in the accumulation of the long-chain MTX polyglutamates between these two distinct subtypes of ALL exceeded that obtained for the total MTX polyglutamates. This observation, together with the FPGS activity being significantly increased in the precursor B-cell ALL, suggests that differences in MTX response between the precursor B-cell and T-cell leukemia are highly dependent on FPGS activity and consequent MTX polyglutamylation. Indeed, a previous study comparing lineage-based differences in MTX sensitivity in leukemia cell lines, also pointed to the involvement of FPGS activity and DHFR levels.[48] The importance of the cellular accumulation of MTX polyglutamates was further corroborated by its association with the TSIA, which was previously shown to be a good determinant of MTX sensitivity, as reflected by the correlation between the TSIA results and MTX cytotoxicity.[40] This association was found in both the entire patient cohort as well as in precursor B-cell ALL alone but not in T-cell ALL when analyzed separately. The linear regression analysis did not show that the association of cellular MTX polyglutamates levels with the TSIA is actually different between precursor B-cell and T-cell leukemia. The apparent differences between the correlations may be due to a large uncertainty in the estimates for the correlations of the T-cell ALL, which may have been caused by the small sample size for this subgroup. Taken together, our data indicate that despite the differences in concentration of MTX polyglutamates and MTX sensitivity profiles between precursor B-cell and T-cell ALL, the accumulation of MTX polyglutamates is likely an indicator of MTX resistance as determined by the TSIA in both of these ALL subtypes.

Intriguingly, in parallel to differences in both FPGS mRNA levels and activity observed between precursor B-cell and T-cell ALL, we found no correlation between *FPGS* mRNA level and enzyme activity or the concentration of MTX polyglutamates. This suggests that the level of *FPGS* mRNA might not directly translate to its enzymatic activity. A strong correlation was previously reported between *FPGS* mRNA expression and activity in leukemic cell lines.[48] In fact, our previous finding of the high propensity of human *FPGS* gene in ALL specimens to undergo impaired splicing, is consistent with the fact that transcript levels may not correlate at all with FPGS catalytic activity.[28,49] Moreover, decreased rates of *FPGS* mRNA translation affecting its activity were previously detected in murine leukemic cell lines selected with another polyglutamylation-dependent antifolate - edatrexate.[50] In addition, we have reported that aberrant *FPGS* splicing is a potential contributing factor to the loss of FPGS function, as various *FPGS* mRNA splicing alterations were detected in MTX resistant leukemic cell lines devoid of FPGS activity, as well as in adult ALL patient samples.[28]

MTX sensitivity in the patient cohort under study differed between several genetic subtypes of precursor B-cell ALL. This included the hyperdiploid patients, displaying high MTX sensitivity associated with increased *RFC* expression, as well as *TEL-AML1* fusion and *MLL*-rearrangements displaying an increased MTX resistance. Elevated *RFC* expression might be caused by an additional copy of *RFC* gene-carrying chromosome 21, which is often observed in hyperdiploid ALL. Our results support previous findings showing that hyperdiploid patients accumulate higher levels of MTX polyglutamates associated with elevated *RFC* expression.[29,30] Consequently, this subset of patients responded favorably to MTX-containing chemotherapy as compared to patients with a non-hyperdiploid karyotype.[44] Although the remarkable sensitivity of hyperdiploid ALL cannot be entirely explained by high MTX sensitivity, the current results as well as observations

of others[29,30,44] suggest, that it is an important biological feature contributing to sensitivity of this ALL subtype. Precursor B-cell ALL with *TEL-AML1* fusion as well as with rearranged *MLL* gene were associated with lower accumulation of MTX polyglutamates, which in the case of *TEL-AML1* was accompanied by increased MTX resistance as reflected by the TSIA. Precursor B-cell ALL carrying either *TEL-AML1* or *E2A-PBX1* gene fusions were previously shown to accumulate decreased levels of MTX polyglutamates as compared to precursor B-cell ALL which are devoid of these cytogenetic aberrations.[16] This was associated with diminished expression of RFC in *E2A-PBX1* ALL and elevated expression of ABCG2 - an MTX efflux transporter - in ALL patients with *TEL-AML1* fusion.[16] Decreased RFC activity resulting from transcriptional silencing as well as mutations and allele loss was previously reported as the cause of antifolate resistance in several tumor types, including leukemia, breast cancer and osteosarcoma.[19–21] Similar overexpression of ABC transporters is an established mechanism of antifolate resistance.[9,13,51] Moreover, another study suggests that both *TEL-AML1* and *E2A-PBX1* gene fusions downregulate FPGS expression by interacting with its promoter region.[52,53] Interestingly, *TEL-AML1* and *E2A-PBX1* are generally associated with a relatively good prognosis, as opposed to patients harbouring rearranged *MLL* gene,[4] which can be explained by the differences in sensitivity to other chemotherapeutics between these ALL subtypes.[5] The numbers of ALL patients displaying these cytogenetic abnormalities for which MTX sensitivity-associated variables were measured was very limited in this study. Hence, we were not able to evaluate the role of particular trisomies (i.e. trisomy 21) across the patients with hyperdiploid karyotype or the analyzed cytogenetic abnormalities. Therefore, these associations should be further addressed in large future studies. However, the current data suggest that *TEL-AML1*, *E2A-PBX1* and *MLL*-rearranged ALL may benefit from courses with high-dose MTX, partly overcoming the polyglutamylation and transport defects.[16,18]

One of the major limitations of the current study was the low numbers of patients for which particular parameters were recorded. This especially influenced analyses in various ALL groups, such as cytogenetic subtypes, where the patient numbers were extremely low. Moreover, patients included in this study were treated on diverse protocols with distinct MTX administration doses. This issue however, was addressed in the statistical analysis by including the treatment protocol as a covariate. Finally, the lack of MTX related toxicity data limited our insight into the associations of particular MTX resistance-related parameters and the clinical outcome of ALL patients. These issues should be carefully considered in future studies.

Taken together, our study clearly shows that the low cellular level of long-chain polyglutamates of MTX is an important predictor of MTX resistance and is associated with dismal therapeutic outcome. Its additional prognostic value warrants further investigation in larger studies using more up-to-date treatment regimens. As MTX remains one of the mainstays of contemporary ALL treatments, expanding our understanding of its contribution to the treatment outcome is of supreme therapeutic value. In particular, the identification of patients that are potentially resistant to MTX at start of the treatment may allow for tailoring novel treatment strategies to individual leukemia patients in the context of combination chemotherapy.

COMPETING INTERESTS

The authors report no potential conflicts of interest.

AUTHORS' CONTRIBUTIONS

JC was the principal investigator and takes primary responsibility for the paper; GE, KS and ES provided patient samples and clinical data; AW, NLW, PMV and BD performed the research; JC, GJLK, GJ, GJP and YGA designed the research; AW wrote the paper; JC, GLK, GJ, GJP, GE, KS, ES, NLW, PMV, BD, YGA and RP edited the paper.

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SUPPLEMENT

Supplemental Table S1. Clinical characteristics of pediatric ALL patients included in this study.

Treatment protocol	TOTAL	COALL92	COALL97	DCOG ALL7	DCOG ALL8	DCOG ALL9	NOPHO ALL92	Other
Number of patients	235	67	26	10	55	42	17	18
CR	227	66	25	10	53	40	17	16
Relapse	65	18	5	4	19	6	4	9
Median follow up (months)	107.9	89.6	103.7	140.5	108.7	108.1	183.9	139.1
Event free survival at 10 years (%)	67.2	67.8	66.7	60	64.2	80.6	75	43.8
Overal survival at 10 years (%)	72.5	72.9	73.3	80	67.3	82.1	93.8	44.4

COALL 92 and 97 – studies of the German Co-operative ALL group; DCOG ALL 7-9 – studies of the Dutch Childhood Oncology Group; NOPHO ALL 92 – a study of the Nordic Society of Paediatric Haematology and Oncology; other – low number of patients included in several other treatment protocols.

Supplemental Table S2. Results of univariate Cox regression model of MTX resistance related variables in relation to overall survival.

	Not corrected for protocols			Corrected for protocols		
	N	**Hazard Ratio (95%CI)**	**p-value**	**N**	**Hazard Ratio (95%CI)**	**p-value**
TSIA short (cut-off 1.44 µM)						
Low	82	1.00		76	1.00	
High	27	1.685 (0.842-3.369)	0.140	26	1.655 (0.772-3.549)	0.195
TSIA cont. (cut-off 0.035 µM)						
Low	26	1.00		23	1.00	
High	79	1.281 (0.556-2.952)	0.561	74	1.175 (0.457-3.025)	0.738
MTX-Glu$_{1-6}$ (cut-off 1935 pmol/10^9 cells)						
High	29	1.00		29	1.00	
Low	85	3.796 (1.158-12.442)	0.028	77	2.571 (0.769-8.600)	0.125
MTX-Glu$_{4-6}$ (cut-off 1000 pmol/10^9 cells)						
High	32	1.00		32	1.00	
Low	64	4.438 (1.332-14.788)	0.015	61	3.819 (1.135-12.850)	0.03
DHFR mRNA (cut-off 3.74)						
High	27	1.00		27	1.00	
Low	19	2.888 (0.843-9.886)	0.091	19	2.399 (0.654-8.804)	0.187
TS mRNA (cut-off 10)						
High	13	1.00		13	1.00	
Low	34	1.051 (0.279-3.962)	0.942	33	1.236 (0.317-4.822)	0.761
FPGS mRNA (cut-off 4.13)						
High	19	1.00		19	1.00	
Low	24	2.143 (0.553-8.302)	0.270	23	1.190 (0.271-5.217)	0.818
FPGH mRNA (cut-off 3.17)						
High	15	1.00		14	1.00	
Low	20	2.670 (0.538-13.242)	0.229	20	2.072 (0.373-11.501)	0.405
RFC mRNA (cut-off 0.86)						
High	21	1.00		21	1.00	
Low	24	0.323 (0.83-1.250)	0.102	23	0.147 (0.034-0.625)	0.009
FPGS activity (cut-off 0.35)						
High	47	1.00		47	1.00	
Low	14	3.260 (0.993-10.704)	0.051	13	3.196 (0.966-10.579)	0.057

TSIA continuous (TSIAcont.) and short-term exposure (TSIA short) are expressed as the concentration of MTX necessary to inhibit 50% of the TS activity (TSI_{50}) compared to the controls incubated without MTX (in triplicate); the concentration of MTX polyglutamates ($MTX\text{-}Glu_{1-6}$ and $MTX\text{-}Glu_{4-6}$) is expressed as pmol $MTX\text{-}Glu_n/10^9$ cells; mRNA expression of folate enzymes is expressed as a ratio normalized to β-actin; FPGS activity is expressed as pmol $MTX\text{-}Glu_2$ formed/h/10^6 cells.

Supplemental Table S3. Treatment outcome (EFS) in the whole cohort according to presenting features (not corrected for the treatment protocol).

	Number of patients	10-year event free survival (%)	Hazard Ratio (95% CI)	p-value
Age*				
1-9	127	70.9	1.00	
≥10	52	67.3	1.23 (0.692-2,185)	0.481
<1	24	45.8	2.56 (1.358-4.825)	0.004
Sex				
Female	84	73.8	1.00	
Male	120	62.5	1.486 (0.892-2.475)	0.128
White blood cell count (x 10⁹/l)*				
<50	118	76.3	1.00	
≥50	83	55.4	2.265 (1.386-3.703)	0.001
Immunophenotype				
Precursor B-cell ALL	168	69	1.00	
T-cell ALL	35	57.1	1.522 (0.857-2.705)	0.152
CNS involvement				
no	166	66.9	1.00	
yes	12	50	2.09 (0.899-4.859)	0.087
DNA index				
<1.16	111	67.6	1.00	
≥1.16	19	89.5	0.305 (0.073-1.266)	0.102

* - significantly different survival in the Kaplan-Meyer analysis; CNS – central nervous system

Supplemental Table S4. Treatment outcome (EFS) in the whole cohort according to presenting features (corrected for the treatment protocol).

	Number of patients	Hazard Ratio (95% CI)	p-value
Age*			
1-9	121	1.00	
≥10	49	1.286 (0.704 -2.347)	0.413
<1	17	2.751 (1.245-6.081)	0.012
Sex			
Female	78	1.00	
Male	110	1.752 (1.0 -3.068)	0.05
White blood cell count (x 10⁹/l)*			
<50	116	1.00	
≥50	69	2.182 (1.272 -3.743)	0.005
Immunophenotype			
Precursor B-cell ALL	153	1.00	
T-cell ALL	34	1.695 (0.932-3.081)	0.084
CNS involvement			
no	156	1.00	
yes	8	1.948 (0.581 -6.536)	0.280
DNA index			
<1.16	106	1.00	
≥1.16	19	0.331 (0.077 -1.43)	0.139

* - significantly different survival in the Kaplan-Meyer analysis; CNS – central nervous system

Supplemental Table S5. Results of multivariate Cox regression model of MTX resistance related variables in relation to event-free survival.

	Not corrected for protocols			Corrected for protocols		
	N	**Hazard Ratio (95%CI)**	**p-value**	**N**	**Hazard Ratio (95%CI)**	**p-value**
MTX-Glu$_{1-6}$ accumulation (cut-off 1935 pmol/10^9 cells)						
High	26	1.00		29	1.00	
Low	68	1.897 (0.760-4.738)	0.170	71	1.648 (0.651-4.173)	0.292
MTX-Glu$_{4-6}$ accumulation (cut-off 1000 pmol/10^9 cells)						
High	29	1.00		32	1.00	
Low	52	2.329 (0.924-5.872)	0.073	55	2.158 (0.835-5.579)	0.112

The concentration of MTX polyglutamates (MTX-Glu$_{1-6}$ and MTX-Glu$_{4-6}$) is expressed as pmol MTX-Glu$_n$/10^9 cells.

Supplemental Table S6. Results of multivariate Cox regression model of MTX resistance related variables in relation to overall survival.

	Not corrected for protocols			Corrected for protocols		
	N	**Hazard Ratio (95%CI)**	**p-value**	**N**	**Hazard Ratio (95%CI)**	**p-value**
MTX-Glu$_{1-6}$ accumulation (cut-off 1935 pmol/10^9 cells)						
High	18	1.00		26	1.00	
Low	38	0.945 (0.219-4.074)	0.940	65	1.808 (0.480-6.809)	0.382
MTX-Glu$_{4-6}$ accumulation (cut-off 1000 pmol/10^9 cells)						
High	19	1.00		29	1.00	
Low	31	1.726 (0.399-7.463)	0.465	52	3.069 (0.804-11.715)	0.101
RFC mRNA (cut-off 0.86)						
High	12	1.00		19	1.00	
Low	16	0.126 (0.020-0.780)	0.026	19	0.176 (0.039-0.806)	0.025

The concentration of MTX polyglutamates (MTX-Glu$_{1-6}$ and MTX-Glu$_{4-6}$) is expressed as pmol MTX-Glu$_n$/10^9 cells; RFC mRNA level is expressed as a ratio normalized to β-actin.