Polymorphisms of the SLCO1B1 gene predict methotrexate-related toxicity in childhood acute lymphoblastic leukemia

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Abstract

Background: Methotrexate is an important component of the therapy for childhood acute lymphoblastic leukemia. Treatment with high-dose methotrexate often causes toxicity, recommending a dose reduction and/or cessation of treatment. Polymorphisms in genes involved in the methotrexate metabolism have been associated with toxicity with controversial results. The discrepancies could be due to differences in treatment protocols among studies, small or non-homogeneous populations or the use of different toxicity criteria. The aim of the present study was to analyze the possible correlation of polymorphisms of genes involved in the methotrexate metabolism with the toxicity during therapy with the well established LAL/SHOP protocol.

Procedure: We analyzed 10 polymorphisms in 7 genes (*MTHFR*, *TS*, *SHMT1*, *RFC1*, *ABCB1*, *ABCG2* and *SLCO1B1*) from the methotrexate metabolism in 115 Spanish pediatric B-ALL patients, using methotrexate plasma concentration as an objective and quantifiable marker of toxicity.

Results: We confirmed the suitability of methotrexate plasma levels as a toxicity marker. We found a statistically significant association between methotrexate plasma concentration and the *SLCO1B1* rs11045879 CC genotype (p=0.030). The rs4149081 AA genotype, in the same gene, could also be an indicator for high MTX plasma concentrations. We did not find any significant association in the other genetic polymorphisms analyzed.

Conclusions: Identification of the rs4149081 and rs11045879 *SLCO1B1* polymorphisms in children with ALL could be a useful tool for monitoring patients at risk of low methotrexate clearance in order to avoid MTX-related toxicity.

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer, accounting for 30% of all pediatric malignancies (1-2). During the last 20 years, survival rates for ALL have improved dramatically due to advances in chemotherapy for childhood ALL, with expected cure rates higher than 80% (3).

An important component of ALL therapy is methotrexate (MTX). MTX enters the cell via active transport mediated by the reduced folate carrier (RFC1) (4-5). Then, MTX acts by inhibiting two enzymes. MTX inhibits dihydrofolate reductase (DHFR), which converts folates (DHF) to their active form tetrahydrofolate (THF), affecting other important enzymes, such as methylenetetrahydrofolate reductase (MTHFR) and serine hydromethyl transferase (SHMT1). On the other hand, the polyglutamated forms of MTX (MTXPG) inhibit thymidylate synthase (TS) directly. As a result, nucleic acid synthesis is inhibited, favoring cell death (6). Finally, different transporters act pumping MTX out of the organism. These include ABC transporters, such as the multidrug resistance protein (ABCB1) and the breast cancer resistance protein (ABCG2) (7), and organic anion transporters, such as SLCO1B1 (8-9) (Figure 1).

Despite its clinical success, treatment with high-dose MTX often causes toxicity, requiring a dose reduction or cessation of treatment. Therefore, for appropriate use of MTX, it would be useful to identify a predictor of the adverse effects of MTX (10). In this context, polymorphisms of the methotrexate transporters, methotrexate targets and folate-metabolizing enzymes that could influence the effectiveness and toxic effects of methotrexate in pediatric ALL have been described (11). For instance, a polymorphism in *RFC1* (G80A), resulting in a less efficient transporter, has been associated with

gastrointestinal (12) and hepatic toxicity (13). Polymorphisms in the *MTHFR* (C677T and A1298C) and *SHMT1* (C1420T) genes, that affect the enzymatic activity, have been associated with hepatic toxicity (14-16). *TS* 28-bp tandem repeat and 6bp deletion have been linked to differences in *TS* expression and with treatment outcome (17). The *ABCB1* C3435T and *ABCG2* C421A polymorphisms result in less active transporters and have been associated with nervous system toxicity and infections (18-19). And more recently, the *SLCO1B1* rs4149081 and rs11045879 polymorphisms have been associated with MTX clearance (8).

However, the associations of polymorphisms and toxicity found by several groups are not always confirmed. For example, *MTHFR* C677T polymorphism has been associated with toxicity in some populations (13; 15) but other authors did not find any association (12; 14). This lack of replication could be due to differences in treatment protocols among studies, small or non-homogeneous populations or the use of different toxicity criteria. Consequently, studies with patients treated homogeneously and the use of an objectively quantifiable marker of toxicity are needed. In this context, MTX plasma levels at the terminal phase could be an objective marker to analyze MTX-associated toxicity (10).

In the present study, we have retrospectively analyzed the polymorphisms in genes involved in the MTX metabolism (*MTHFR*, *TS*, *SHMT1*, *RFC1*, *ABCB1*, *ABCG2* and *SLCO1B1*) and their association with toxicity during therapy with the well established LAL/SEHOP protocol in a Spanish pediatric B-ALL population (115 patients) using MTX plasma concentration as a toxicity marker.

Methods

Patients

This is a retrospective study based on the systematic appraisal of chart data regarding treatment protocols. The patients included in this study were 115 children all diagnosed with B-ALL from 2000 to 2010 at the Pediatric Oncology Units of 3 Spanish reference hospitals (Hospital Cruces; Hospital Donostia; and Hospital La Paz). Informed consent was obtained from all patients or their parents before sample collection.

Treatment and toxicity evaluation

All patients were homogeneously treated with the LAL-SHOP 99 and 2005 protocols, which included the same consolidation therapy for all the risk groups: three doses of methotrexate (each dose consisted of 3 g/m^2 or 5 g/m^2 of MTX), 6-mercaptopurine (30 mg/m^2/day for 6 weeks), four doses of cytarabine (1 g/m^2) and four doses of triple intrathecal therapy. MTX was given in 24 h infusion with folinic acid rescue. Monitoring of MTX concentration in plasma was carried out every day until the concentration was below 0.2 μM.

Toxicity data were collected objectively, blinded to genotypes, from the patients' medical files. Toxicity was graded according to the Spanish Society of Pediatric Hematology and Oncology (SHOP) standards, adapted from the WHO criteria (grades 0-4). The highest grade of toxicity observed for each patient during the consolidation therapy period was recorded. Data collected included: hepatic toxicity (AST/ALT), hyperbilirubinemia, vomiting, diarrhea, mucositis and renal toxicity (creatinine) and MTX concentrations 72 h and 96 h after infusion. MTX levels were considered high if the concentration was over 0.2 μM.

Genes and polymorphisms selection and genotype analyses

We selected 10 polymorphisms in 7 genes within the MTX pathway already studied in association with MTX response by other authors with controversial results and/or with a demonstrated functional effect:

In *RFC1 gene*, G80A polymorphism changes an aminoacid (His to Arg) in the protein and reduces its activity. In *MTHFR*, the SNPs C677T and A1298C change an Ala to Val and Glu to Ala, respectively, and reduce the enzymatic activity. In *TS*, in 5' region, 28-bp tandem repeat (3R allele) increases gene expression and in 3' region, 6-bp deletion (allele -) reduced gene expression. In *SHMT1*, C1420T changes Leu to Phe and decreases enzymatic activity. In *SLCO1B1*, rs4149081 and rs11045879 are intronic SNPs with unknown function. In *ABCB1*, C3435T is a synonymous change that is related with decreased mRNA expression. Finally, in *ABCG2*, C421A changes a Gln to Lys that reduces the transporter activity.

Genomic DNA was extracted with the phenol-chloroform method as previously described (20) from peripheral blood or bone marrow slides.

The methods for genotyping were PCR, PCR-RFLP and PCR allele-specific (Table I). Each PCR was performed with 50 ng DNA. DNA fragments were visualized on a 3% agarose gel with ethidium bromide. An 8% acrylamide gel was used for analyzing *TS* 6-bp deletion.

Statistical analysis

The association between MTX plasma levels, and toxicity parameters was evaluated by the $\chi 2$ or Fisher's exact test. To assess the strength of the association we calculated the odds ratio (OR) ant its 95% confidence intervals followed by the area under the curve (AUC) from a receiver operating characteristic (ROC) approach. This last estimate gives an indication of the probability of discriminating between MTX plasma concentration knowing the reported toxicity. The association between MTX plasma levels, and genetic polymorphisms was evaluated by the $\chi 2$ or Fisher's exact test as well as the Hardy-Weinberg equilibrium. The effect sizes of the associations were estimated by the OR's from univariate logistic regressions and multivariate logistic regressions to account for the possible confounding effect of sex and age. In all cases the significance level was set at 5%. The p-values obtained in the analyses (univariate and multivariate) of the 10 tested polymorphisms were corrected for multiple comparisons by using the Benjamini & Hochberg (21) false discovery rate (FDR). Analyses were performed by using Stata v11 and R v2.11 software. Linkage disequilibrium (LD) between the SNPs was analyzed using Haploview 4.2 by calculating pairwise D' and r^2.

Results

Patients' baseline characteristics

In this study, we have analyzed 115 B-ALL patients, whose characteristics are reported in Table II. Clinical data about therapy-related toxicity were available for 102 patients. Among all patients who developed toxicity (n=52, 51%), the prevalence of different types of toxicity were as follows: hepatic (n=30, 29.4%), vomits (n=22, 21.6%), mucositis (n=11, 10.8%), renal (n=9, 8.8%), diarrhea (n=7, 6.9%), and hyperbilirubinemia (n=5, 4.9%). Clinical data about MTX plasma concentration were available for 111 patients 72 h after infusion and for 108 patients after 96 h. 35 patients (31.5%) had high MTX plasma levels (>2 μM) 72 h after MTX infusion and 25 (23.1%) continued with high MTX levels after 96 h.

Methotrexate clearance and toxicity

In order to confirm the suitability of MTX plasma levels as a toxicity marker, we analyzed the association between different toxicity parameters and plasma concentration of MTX 72 h (Table III) and 96 h after MTX treatment For analyses, toxicity grades were used to dichotomize toxicities as "present" versus "absent," with grade 2 to 4 considered as present as defined in Table III.

When we analyzed the toxicity in patients with high MTX plasma levels *versus* toxicity of those with low MTX plasma levels, there was a significantly higher frequency of patients with global toxicity in the group of individuals with high MTX concentration (72 h, p=0.004). The frequency of renal toxicity, by itself, was significantly increased in patients with a high MTX concentration at 72 h (p=0.005). Similar results were obtained at 96h. The frequency of vomiting in the total population was also significantly higher

in the group of patients with a high MTX concentration, but only at 72 h after infusion (p=0.020).

Our results show that MTX plasma concentration is a good toxicity marker in our population. Indeed it has a strong association with the parameter "global toxicity", here we considered grouped any kind of toxicity. With these results, we decided to use it as marker of toxicity in the following analyses, as it is an objectively quantifiable variable.

Polymorphisms in association with toxicity

In order to investigate if genetic variation may influence the clearance of MTX, we tested the association between 10 polymorphisms in 7 genes and MTX plasma concentration 72 h after intravenous infusion, as a toxicity index.

The 10 polymorphisms were genotyped with an average rate of success of 99.57%. For each polymorphism, we considered two genotypic groups, one of risk of higher toxicity and other of normal expected toxicity, according to the function and previous reports (Table IV). All the genotypes analyzed were in Hardy-Weinberg equilibrium.

In the gene MTHFR, individuals with the genotype 1298CC had lower frequency of toxicity than expected, without reaching the significance level. When we analyzed the correlation of polymorphisms in SHMT1, TS, ABCB1, ABCG2 and RFC1 genes with MTX plasma concentration at 72 or 96h, we did not find any significant association.

We found a statistically significant association between MTX plasma concentration and the *SLCO1B1* rs11045879 CC genotype. All the patients with the CC genotype had high

MTX plasma concentrations 72 h after MTX infusion (p= 0.030) (Table IV). In the multivariate analysis, SLCO1B1 rs11045879 remained associated with MTX plasma levels (p=0.008) (Table IV). When we corrected for multiple comparisons, we obtained a p-value near the significance level (p= 0.08). In the rs4149081 polymorphism, of the same gene, *SLCO1B1*, the AA genotype was always associated with high MTX plasma concentrations at 72 h, although this association did not reach statistical significance (p= 0.097; p=0.057). As shown in Figure 2, in the group of patients with the rs11045879 TT/TC and rs4149081 GG/GA genotypes only a third of them had high MTX concentrations in plasma, while all the individuals with the rs11045879 GG and rs4149081 AA had high MTX plasma levels. It is worth to note that all the individuals homozygous for the risk allele rs4149081 AA had also the rs11045879 GG risk genotype. This was expected due to the high degree of linkage disequilibrium between both SNPs (r^2=0.807), which are located in the same linkage block. None of the other polymorphisms analyzed were in high linkage disequilibrium (pairwise D' was lower than 0.70).

Discussion

In the present study, we evaluated the correlation of polymorphisms in several genes involved in the methotrexate pathway with toxicity in a group of 115 children diagnosed with B-ALL and treated according to the standardized LAL/SHOP protocol. Among the strengths of our retrospective study were a good sample size, a homogeneous diagnostic, a standardized treatment protocol followed by all patients, and objective and well recorded toxicity data.

MTX is the backbone of pediatric ALL therapy, but it is also a drug known for its high toxicity. For drugs such as MTX, with a very narrow therapeutic index, every effort should be made to minimize interpatient variability in drug exposure in order to maximize the benefit while keeping the risk of serious adverse effects at an acceptable level.

Several studies have investigated the relationship between genetic variation and MTX-related toxicity. However, very often, these data are not comparable since each study uses different toxicity parameters. Even, for some parameters, such as vomits, the determination of toxicity may vary among clinicians.

We have used the MTX plasma levels as a good and objective MTX-related toxicity marker. We observed that MTX plasma levels were strongly linked to the development of global toxicity. In fact, 70.6% of the patients with high MTX plasma levels 72h after drug infusion developed toxicity, while only 39.7% of the patients with low MTX plasma levels showed toxicity events (OR= 3.64; p= 0.004). Other authors also used this parameter in their studies (8; 10; 12; 16).

One of the advantages of using MTX plasma concentration as toxicity marker is that it can be directly associated with methotrexate, and, if needed, treatment adjustments could be performed in the future in a specific way. Another advantage of using this parameter is its availability in the patients' files and the fact that we can avoid the lack of homogeneity in the toxicity data collection, as it is an objectively quantifiable data.

In recent years, the relationship between genetic polymorphisms and MTX toxicity has been a controversial topic in childhood ALL. In our study, we selected polymorphisms of several genes from the MTX metabolism with functional effect and whose implication in ALL toxicity had been previously suggested by other authors and still are under discussion: *MTHFR*, *SHMT1*, *TS*, *ABCB1*, *ABCG2*, *RFC1*, and *SLCO1B1*.

With our results, we conclude that the polymorphisms analyzed in *MTHFR*, *TS*, *SHMT1*, *ABCB1*, *ABCG2* and *RFC1* are not useful markers for toxicity in pediatric ALL. Previous associations found in the literature could be due to the analysis of small or non-homogeneous samples, the use of non objective toxicity markers or differences in the protocol of treatment. For instance, the *MTHFR* 677T allele encodes a protein with decreased enzymatic activity, so people with the CT genotype exhibit only 60% of the MTHFR activity and those with the TT genotype demonstrate only 30% (22-23). We did not find an association between *MTHFR* C677T polymorphism and MTX plasma levels. In agreement with our results, most of the studies performed did not find significant associations between the 677T allele and toxicity (12; 14; 24-27). The associations observed by other authors can be explained if we take into account that they studied small samples (10, 13, 15-16) or patients were treated with lower

methotrexate doses (15mg/m^2) (28). We can consider the possibility that the functional differences in the activity of the enzyme due to this polymorphism can only modulate toxicity with low methotrexate doses and are not useful at the high doses used in most treatment protocols for acute lymphoblastic leukemia.

In our study, an interesting result was that all the patients with the rs11045879 CC genotype had high MTX plasma concentrations (p=0.008 at 72 h following MTX infusion). When we corrected for multiple comparisons, the statistical significance was lost, which was expected due to the frequency of the risk genotype, although we must emphasize that the p-value (p= 0.08) is near the significance level. Also, the rs4149081 AA genotype was always associated with high MTX plasma concentrations, although this association did not reach statistical significance (p=0.057). In addition, the 3 individuals with the rs11045879 CC genotype and the 2 patients with the rs4149081 AA genotype developed toxicity during the consolidation therapy. Both SNPs, rs4149081 and rs1104579, are in linkage disequilibrium. Consequently, the fact that both SNPs are associated with toxicity suggests the implication of these SNPs or other SNPs in the same linkage block in MTX clearance and an important role for SLCO1B1 in MTX toxicity. We are aware that one of the limitations of this study is the fact that the number of patients with the minor alleles is not very high and further studies with larger populations would be necessary. However, it must be remarked that patients included in this study were recruited from three hospitals over ten years and considering the frequency of those minor alleles it will not be easy to increase the frequency of homozygotes significantly.

In a recent work, using a genome-wide approach, the polymorphisms rs4149081 and rs11045879 of SLCO1B1 gene have been strongly associated with MTX clearance (8), being the first time that this transporter was proposed as a candidate gene in clinical pharmacogenetic studies of MTX. Now, our findings confirm the association found by Treviño and colleagues between *SLCO1B1* and MTX plasma levels, suggesting that *SLCO1B1* polymorphisms may influence methotrexate-related toxicity in pediatric ALL.

The relationship between SLCO1B1 and MTX clearance can be understood if we consider its function. SLCO1B1 is localized at the sinusoidal membrane of hepatocytes, and its transcript has been detected in enterocytes. SLCO1B1 mediates uptake of substrates from sinusoidal blood, resulting in their excretion, likely via biliary excretion (8). Moreover, SLCO1B1 has been shown to transport methotrexate *in vitro* (29) and, by using a transgenic mouse model, SLCO1B1 has also shown to be an important transporter for MTX in vivo, with a rate-limiting role for plasma elimination (30). This supports a putative role of genetic polymorphisms in SLCO1B1 on plasma pharmacokinetics of MTX in ALL patients.

The function of these two SNPs, which are located in an intronic region, is still unknown. It must be remarked that both SNPs are in strong LD with a non-synonymous SNP (rs4149056), that had previously been shown to impair statin transport and was associated with higher plasma levels of statins (31). However, in the study by Treviño, when genotypes at rs4149056 and at rs11045879 were allowed to compete, only the rs11045879 SNP remained in the model (8). In this study we have selected in each gene the SNPs that had previously been more significantly associated with toxicity in other works. Nevertheless, once confirmed the association with SLCO1B1 gene, it would be

of great interest to analyze other SNPs, such as rs4149056, whose association with MTX toxicity has been described to be weaker than that of rs4149081 and rs11045879.

In summary, identifying the rs4149081 and rs11045879 SLCO1B1 polymorphisms in children with ALL could be a useful tool for monitoring patients at risk of low methotrexate clearance in order to avoid MTX-related toxicity.

In this context, it would be also of great interest to study in depth the possible functional effects of these polymorphisms and the implication of other polymorphisms, such as CNVs in SLCO1B1 and other related genes (e.g. SLCO1A2 and SLCO1B3) in MTX toxicity.

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Conflict of Interest Statement

The authors reported no potential conflicts of interest.

Authorship

AGO was the principal investigator and takes primary responsibility for the paper. MAP, PGM and AN recruited the patients and collected the clinical data. ELL and IMG performed the laboratory work for this study. JB participated in the statistical analysis, AGO and AN coordinated the research. ELL and AGO wrote the paper. All other authors have read, revised, and accepted the manusctript.

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Figure 1. Methotrexate pathway. Genes are marked in italics. Open circles show genes selected in the present study.

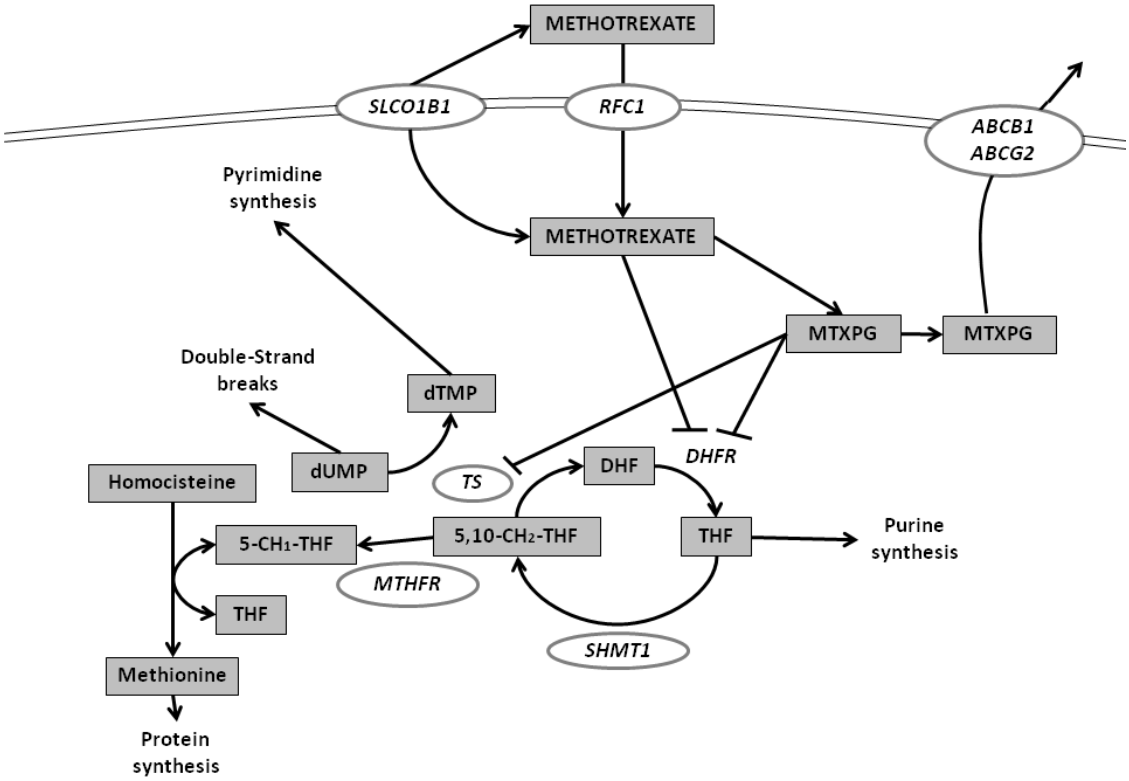

Figure 2. Frequency of ALL patients with high and normal MTX plasma concentration at 72 (A, C) and 96 hours (B, D) after MTX infusion according to genotypes of the SLCO1B1 polymorphisms rs4149081 and rs11045879.

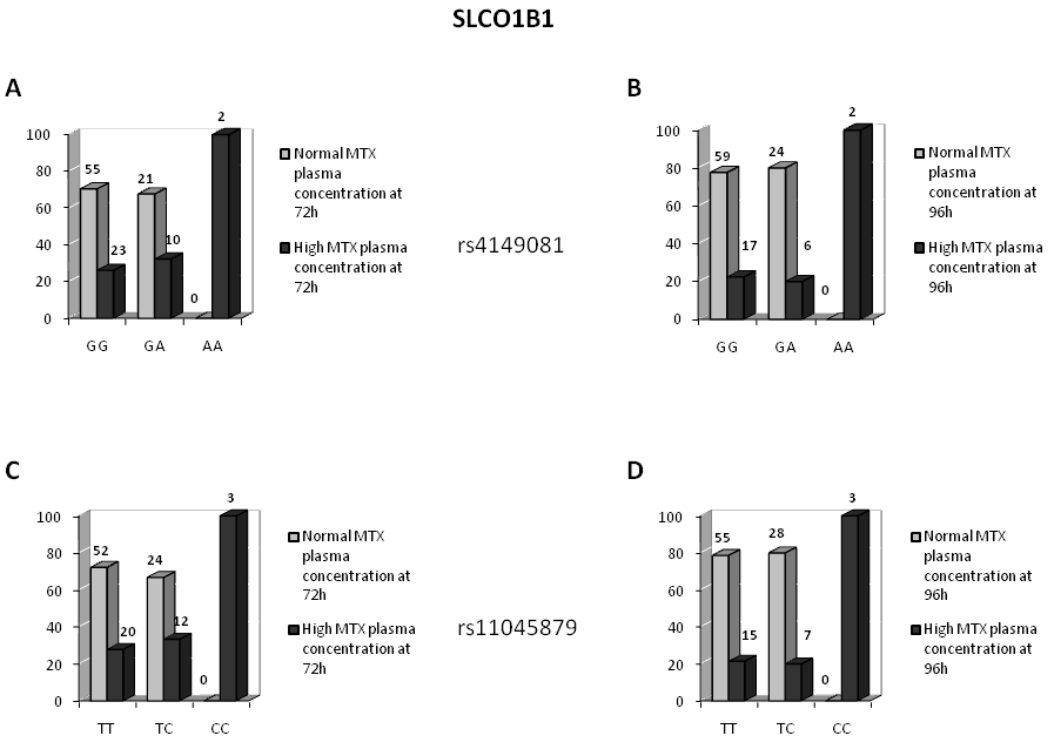

Table I: Polymorphisms selected within the MTX pathway and genotyping method used to carry out the analysis.

Polymorphism	Method	Primers	Restriction enzyme
RFC1 G80A (1, 3)	PCR-RFLP (7)	F: CTGCAGACCATCTTCCAAGG R: AGGAGGTAGGGGGTGATGAA	HhaI (Takara) 0.5U, 37ºC on
MTHFR C677T (1, 3)	PCR-RFLP (7)	F: GGAAGGTGCAAGATCAGAGC R: CTCACCTGGATGGGAAAGAT	Hinf I (Takara) 0.5U, 37ºC on
MTHFR A1298C (1, 3)	PCR-RFLP (7)	F: GTAAAACGACGGCCAGGGAGGAGCTGACCAGTGCAG R: GAAACAGCTATGACGCTGCGGTCAGGCCAGGGGCAG	Fnu4HI (NEB) 0.5U, 37ºC on
TS 2R/3R (2, 4)	PCR	F: CTCCGTTCTGTGCCACACC R: GGAGGATGTGTTGGATCTGC	-
TS 6bp-del (1, 5)	PCR	F: GGAGCTGAGTAACACCATCG R: CAGAATGAACAAAGCGTGGA	-
SHMT1 C1420T (1, 5)	PCR allele specific	F1: GTTGAGAGCTTCGCCTCTT R1: GTCAACAGTTCCCCTTTGGA F2: GCCACCCTGAAAGAGTTCAA R2: GCCAGGCAGAGGGAAGAG	-
SLCO1B1 rs4149081 (1, 6)	PCR allele specific	F1: GTGATTCAAGGATAATAACCAACTTG R1: GCCCCAGCTAGTCATTCTGT F2: CTGACTTTGCATGCAGTATGG R2: CCATTTTCTATTATCTCTGATTTTTGAT	-
SLCO1B1 rs11045879 (1, 6)	PCR allele specific	F1: TGTTTCTTTGATGATATATATGAAGATGC R1: GAAATTGTCTTTGTTTGCAATATGAC F2: TTAATCACATGCATTTAAATTTCCTC R2: ATCCAGGGTTAATATAACAGAATCAAA	-
ABCB1 C3435T (1, 3)	PCR-RFLP (5)	F: TTCAAAGTGTGCTGGTCCTG R: GCATGTATGTTGGCCTCCTT	MboI (Takara) 0.5U, 37ºC on
ABCG2 C421A (1, 5)	PCR allele specific	F1: CTCTGACGGTGAGAGAAAACTAAC R1: TGCTGATCATGATGCTTTCA F2: CATGGTCTTAGAAAAGACTCATTATCA R2: CGAAGAGCTGCTGAGAAGTT	-

on: Over night; (1)Master mix: 0.2 mM dNTPs, 1.5 mM MgCl2 (except for SLCO1B1, 2mM MgCl2), 1X ImmoBuffer, 10 pmoles of each primer and 0.5 U Immolase enzyme (BIOLINE); (2) Master mix: GC-RICH PCR System (Roche Applied Science), according to the manufacturer's instructions; (3) PCR protocol: 95ºC for 7 min, (95ºC for 30 s, 60ºC for 30 s, 72ºC for 30 s) 35 cycles and 72ºC for 10 min; (4) PCR protocol: 95ºC for 3 min, (95ºC for 30 s, 58ºC for 30 s, 72ºC for 45 s) 35 cycles and 72ºC for 7 min; (5) PCR protocol: 95ºC for 7 min, (95ºC for 30 s, 58ºC for 30 s, 72ºC for 30 s) 35 cycles and 72ºC for 10 min; (6) PCR protocol: 95ºC for 7 min, (95ºC for 30 s, 60ºC for 30 s, 72ºC for 30 s) 30 cycles and 72ºC for 10 min; (7) 15 µl of amplified DNA were used in each digestion.

Table II. Characteristics of the population of our study.

No. of patients, n	115
Mean age at diagnosis ± SE, years	5.49 ± 0.33
Sex, n (%)	
Female	53 (46.1)
Male	62 (53.9)
Risk group, n (%)	
Standard	44 (38.3)
High	52 (45.2)
Very high	19 (16.5)
Treatment protocol, n (%)	
LAL-SHOP 99	45 (39.1)
LAL-SHOP 2005	70 (60.9)
MTX dose in consolidation, n (%)	
$3g/m^2$	51 (44.3)
$5g/m^2$	64 (55.7)
Toxicity during consolidation therapy, n (%)	
Global toxicity	52 (51.0)
Hepatic	30 (29.4)
Vomits	22 (21.6)
Diarrhea	7 (6.9)
Mucositis	11 (10.8)
Hyperbilirrubinemia	5 (4.9)
Renal	9 (8.8)
MTX concentration in plasma, n (%)	
Higher than 0.2uM at 72h	35 (31.5)
Higher than 0.2uM at 96h	25 (23.1)

SE: standard error.

Table III. MTX clearance and toxicity.

Toxicity	Status	MTX concentration in plasma at 72h <0.2 µM n (%)	>0.2 µM n (%)	OR (95% CI)	p	AUC Roc (95%CI)
Global toxicity	No toxicity	41 (60.3)	10 (29.4)			
	Toxicity	27 (39.7)	24 (70.6)	3.64 (1.51-8.82)	0.004*	0.65 (0.56-0.75)
Hepatic	Grade 0-1	51 (75.0)	21 (61.8)			
	Grade 2-4	17 (25.0)	13 (38.2)	1.86 (0.77-4.49)	0.169	0.57 (0.47-0.66)
Vomits	Grade 0-1	58 (85.3)	22 (64.7)			
	Grade 2-4	10 (14.7)	12 (35.3)	3.16 (1.20-8.36)	0.020*	0.60 (0.51-0.70)
Diarrhea	Grade 0-1	65 (95.6)	30 (88.2)			
	Grade 2-4	3 (4.4)	4 (11.8)	2.89 (0.61-13.72)	0.182	0.54 (0.48-0.60)
Mucositis	Grade 0-1	60 (88.2)	31 (91.2)			
	Grade 2-4	8 (11.8)	3 (8.8)	0.73 (0.18-2.93)	0.653	0.49 (0.42-0.55)
Hyperbilirubinemia	No toxicity	66 (97.1)	31 (91.2)			
	Toxicity	2 (2.9)	3 (8.8)	3.19 (0.51-20.10)	0.216	0.53 (0.48-0.58)
Renal	No toxicity	67 (98.5)	26 (76.5)			
	Toxicity	1 (1.5)	8 (23.5)	20.62 (2.46-173.06)	0.005*	0.61 (0.54-0.68)

*p<0.05; Grade 0-1 is considered as no toxicity and grade 2-4 is considered as toxicity.

Table IV. Genetic polymorphisms and methotrexate clearance.

Gene	Polymorphism	Chromosome position	Location	Genotype	MTX concentration in plasma at 72h		Univariate analysis			Multivariate analysis		
					< 0.2 µM n (%)	>0.2 µM n (%)	OR (95% CI)	p	p (corrected)	OR (95% CI)	p	p (corrected)
MTHFR	C677T	1:11778965	exon 4	CC/CT	58 (65.9)	30 (34.1)	1.00			1.00		
				TT	18 (78.3)	5 (21.7)	0.54 (0.18-1.59)	0.244	0.415	0.53 (0.17-1.64)	0.256	0.427
MTHFR	A1298C	1:11777063	exon 7	AA/AC	65 (65.7)	34 (34.3)	1.00			1.00		
				CC	10 (90.9)	1 (9.1)	0.19 (0.02-1.56)	0.060	0.300	0.21 (0.03-1.69)	0.078	0.260
SHMT1	C1420T	17:18172821	exon 12	CC	40 (71.4)	16 (28.6)	1.00			1.00		
				CT/TT	36 (65.5)	19 (34.5)	1.32 (0.59-2.95)	0.498	0.622	1.15 (0.49-2.67)	0.748	0.749
TS	28bp	18:647646	5’ region	2R3R/3R3R	62 (70.5)	26 (29.5)	1.00			1.00		
				2R2R	12 (57.1)	9 (42.9)	1.79 (0.67-4.76)	0.249	0.415	1.31 (0.47-3.69)	0.608	0.749
TS	6bp-del	18:663444	3’ region	++	34 (72.3)	13 (27.7)	1.00			1.00		
				+-/--	42 (65.6)	22 (34.4)	1.37 (0.60-3.11)	0.450	0.622	1.67 (0.70-4.00)	0.245	0.427
ABCB1	C3435T	7:86976581	exon 26	CC/CT	60 (72.3)	23 (27.7)	1.00			1.00		
				TT	16 (57.1)	12 (42.9)	1.96 (0.80-4.76)	0.142	0.355	2.19 (0.86-5.61)	0.104	0.260
ABCG2	C421A	4:89271347	exon 5	CC	64 (69.6)	28 (30.4)	1.00			1.00		
				CA/AA	12 (63.2)	7 (36.8)	1.33 (0.47-3.74)	0.588	0.636	1.19 (0.41-3.46)	0.749	0.749
RFC1	G80A	21:45782222	exon 2	AA	20 (64.5)	11 (35.5)	1.00			1.00		
				AG/GG	54 (69.2)	24 (30.8)	0.81 (0.34-1.95)	0.636	0.636	0.84 (0.33-2.11)	0.709	0.749
SLCO1B1	rs4149081	12:21269288	intron 14	GG/GA	76 (69.7)	33 (30.3)	1.00			1.00		
				AA	0 (0)	2 (100)	N.E.	0.097	0.323	N.E.	0.057	0.260
SLCO1B1	rs11045879	12:21273886	intron 14	TT/TC	76 (70.4)	32 (29.6)	1.00			1.00		
				CC	0 (0)	3 (100)	N.E.	0.030*	0.300	N.E.	0.008*	0.080

N.E. Not Estimable; *p<0.05; Genotypes MTHFR 677 TT, MTHFR 1298 CC, SHMT1 CC, TS 2R2R, TS +-/-- ABCB1 TT, ABCG2 CA/AA, RFC1 AA, SLCO1B1 AA and SLCO1B1 CC were considered of higher risk of toxicity. Genotypes MTHFR 677 CC/CT, MTHFR 1208 AA/AC, SHMT1 CT/TT, TS 2R3R/3R3R, TS ++, ABCB1 CC/CT, ABCG2 CC, RFC1 AG/GG, SLCO1B1 GG/GA and SLCO1B1 TT/TC were considered of low risk of toxicity.