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Thiopurine methyltransferase in acute lymphoblastic leukaemia: biochemical and molecular biological aspects☆

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Abstract

Thiopurine S-methyltransferase (TPMT) is a cytosolic enzyme, catalysing S-methylation of aromatic and heterocyclic sulphhydryl compounds. TPMT activities and genotypes have been determined in patients with acute lymphoblastic leukaemia (ALL) and in control children. Median red blood cell (RBC) TPMT activity in ALL patients at diagnosis was significantly lower than in controls (median 11.5 pmol/10^7 RBC*hr; range 1.7–30.7; $n = 191$ *vs.* 14.6 pmol/10^7 RBC*hr; range 1.6–50.7; $n = 140$). This reduction of TPMT activity in ALL patients was not due to differences in the frequency of mutations in the *TPMT* gene. In concordance with other authors, we found a higher TPMT activity during maintenance treatment with 6-mercaptopurine (6MP) than at diagnosis and in controls. However, we observed that TPMT activity was already significantly increased after the induction therapy, before the patients received 6MP (median 17.5; range 3.9–40.3 pmol/10^7 RBC*hr; $n = 139$). *In vitro* experiments indicate that the early increase of TPMT activity during treatment may be explained by the use of antifolates, e.g., methotrexate and trimethoprim.

Keywords: Thiopurine methyltransferase; Acute lymphoblastic leukaemia; Childhood leukaemia; 6-mercaptopurine; Antifolates

1. Introduction

6-mercaptopurine (6MP) is a hypoxanthine analogue. Metabolic activation occurs by its conversion to thioinosine monophosphate (thio-IMP). This reaction is catalysed by hypoxanthine-guanine phosphoribosyltransferase in the presence of phosphoribosyl pyrophosphate (PRPP) as a cosubstrate. Thio-IMP can be converted to thioguanine nucleotides (TGNs) which are incorporated into DNA and RNA to exert cytotoxicity. A second cytotoxic pathway is the methylation of thio-IMP to methylthio-IMP by thiopurine-S-methyltransferase (TPMT), a cytosolic enzyme catalysing S-methylation of aromatic and heterocyclic sulphhydryl compounds. Methylthio-IMP has been shown to inhibit purine *de novo* synthesis [1]. By contrast, 6MP can be methylated to methyl-6MP. This reaction is part of the catabolic pathway, since methyl-6MP cannot be converted to active compounds.

TPMT exhibits autosomal codominant polymorphism, with 89–94% of the population having a high activity [2,3]. One out of 300 individuals has a very low or non-detectable TPMT activity and 6–11% of the population show an intermediate activity. The gene

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encoding TPMT is localised on chromosome 6 [4]. Polymerase chain reaction (PCR)-, single-strand conformational polymorphism (SSCP)-, denaturing high-performance liquid chromatography (DHPLC) and hybridisation probe assays have been developed to detect TPMT mutations [5–10]. Mutations in the *TPMT* gene often lead to an enzyme which is more susceptible to proteolysis resulting in a faster degradation of the enzyme [11]. Many studies have been conducted in genotyping individuals. *TPMT*3A* mutations are the most prevalent ones, with an allele frequency of 4.0%. In addition, *TPMT*3C* alleles have been found in some cases (1.4%). Other mutations occur rarely. In 93.7% of the cases, wild-type alleles were found [12]. Similar findings have been described in an ALL population by McLeod and colleagues [13].

Knowledge of TPMT activity is of special importance when patients are treated with 6-thiopurines e.g., 6MP, 6-thioguanine or azathioprine. Lennard and colleagues [14] have shown that when TPMT activity is very high, and, consequently, the RBC-TGN-concentration is relatively low, treatment failure occurs more often. They observed no differences in TPMT activity between children in a control population and children suffering from ALL at diagnosis [15]. TPMT activity significantly increased during maintenance treatment of patients with ALL compared with the activity at diagnosis. After termination of treatment, TPMT activity decreased to control values [15,16].

McLeod and colleagues [16] have shown that the TPMT activity in RBC at diagnosis correlates well with the TPMT activity in lymphoblasts at diagnosis. Furthermore, a good correlation of TPMT activity in RBC and lymphocytes in control subjects has been shown [17]. TPMT assays are mainly performed in RBC due to the low number of white blood cells (WBC) during treatment of ALL.

In this study, we monitored the dynamics of RBC-TPMT activity at diagnosis, and during and after therapy in patients with ALL. Monitoring TPMT activity may be of benefit to improve thiopurine therapy. Additional experiments were performed to investigate the biochemical mechanism causing increase in TPMT activity during drug therapy compared with TPMT activity at diagnosis. A group of patients and controls were also genotyped to look for genotype/phenotype correlations.

2. Materials and methods

2.1. Chemicals

6MP, 6-methyl mercaptopurine (6MeMP), S-adenosyl methionine (SAM), allopurinol, dithiotreitol (DTT), methotrexate (MTX), vincristine, dexamethasone, L-asparaginase and trimethoprim were obtained from Sigma Chemicals (St Louis, MO, USA). Perchloric acid (PCA) was purchased from Fluka (Buchs, Switzerland). Methanol and acetonitrile were obtained from Labscan (Dublin, Ireland). Glutamax-1, gentamycin and deoxynucleotide triphosphates (dNTPs) were purchased from Invitrogen (UK). Sodium pyruvate was obtained from BDH Chemicals Ltd (UK). Potassium dihydrogenphosphate, dipotassium hydrogenphosphate, dimethylsulphoxide and L-methionine were obtained from Merck (Darmstadt, Germany). Fetal bovine serum was obtained from Integro B.V. (Dieren, the Netherlands). Isolation of genomic DNA was performed with the Puregene Genomic DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA). Primers were all purchased from Invitrogen Corporation (Carlsbad, CA, USA). Restriction enzymes used were purchased from New England Biolabs (Beverly, MA, USA), except for *Mse I*, which was obtained from Invitrogen Corporation (Carlsbad, CA, USA). Primers and restriction enzymes used are listed in Table 1.

2.2. Patients' and control material

2.2.1. Patients

Patients' blood samples were obtained from children with ALL included in the DCOG, formerly the Dutch Childhood Leukaemia Study Group (DCLSG)-ALL-9 protocol after written informed consent of the patients and/or their parents or guardians (Table 1). This protocol excluded patients with mature B-ALL. Patients were stratified into two treatment regimens: non-high-risk (NHR) and high-risk (HR). Criteria for HR were: WBC count $>50*10^9$/l, T-cell leukaemia, broadened mediastinum, initial central nervous system (CNS) or testicular involvement, presence of a t(9;22) or a *bcr-abl*-rearrangement, presence of a t(4;11) or a 11q23 aberration with a *mll*-rearrangement. Patients who did not meet any of these criteria at diagnosis were treated according to the NHR protocol except for two changes [18]. In the ALL-9, protocol triple intrathecal therapy is administered. And 4 intravenous (i.v.) Paronal® (L-asparaginase) infusions are given instead of the 14 Crasnitin® (L-asparaginase) infusions according to ALL-6. Maintenance treatment consisted of alternating cycles of 2 weeks vincristine and dexamethasone, and 5 weeks of oral 6MP (50 mg/m^2 daily) and oral methotrexate (30 mg/m^2 weekly) until week 109.

TPMT activity of children with NHR-ALL was determined at diagnosis (week 0), after induction treatment (week 6), after the prophylactic treatment of the CNS with 3 weekly medium-dose MTX infusions, i.e., at the start of maintenance therapy (week 11), during maintenance therapy (weeks 25, 53, 81 and 109) and one year after cessation of treatment.

Table 1
Treatment phases and measure points during treatment of acute lymphoblastic leukaemia (ALL) patients according to the Dutch childhood leukaemia study group (DCLSG)-ALL-9-protocol

	NHR			HR		
	Treatment period (weeks)	Cytostatics	Sampling week	Treatment period (weeks)	Cytostatics	Sampling week
Induction treatment	0–6	Dexamethasone (6 mg/m^2, p.o.) Vincristine (2 mg/m^2, i.v.) L-Asparaginase (6000 IU/m^2, i.v.) Intrathecal therapy (MTX, diadreson-F. aquosum and cytosine-arabinoside)	0 6 	0–6	Dexamethasone (6 mg/m^2, p.o.) Vincristine (2 mg/m^2, i.v.) Daunorubicin (25 mg/m^2, p.o.) L-Asparaginase (6000 IU/m^2, i.v.) Intrathecal therapy (MTX, diadreson-F. aquosum and cytosine-arabinoside)	0 6
Prophylactic CNS treatment	7–10	MD-MTX (2 g/m^2, p.i.) Intrathecal therapy	11	7–14	HD-MTX (3 g/m^2, p.i.) Intrathecal therapy 6MP (50 mg/m^2, p.o.)	15
Intensification treatment I				15–24	6MP (50 mg/m^2, p.o.) Dexamethasone (6 mg/m^2, p.o.) Vincristine (2 mg/m^2, i.v.). Daunorubicin (25 mg/m^2, i.v.) L-Asparaginase (6000 IU/m^2, i.v.) Intrathecal therapy	
Intensification treatment II				25–31	6 courses of: cytosine cytosine-arabinoside (80 mg/m^2, i.v.) Cyclophosphamide (80 mg/m^2, i.v.)	32
Maintenance treatment	11–109	Alternating: 2 weeks: Intrathecal therapy Dexamethasone (6 mg/m^2, p.o.) Vincristine (2 mg/m^2, i.v.) and 5 weeks: 6MP (50 mg/m^2, p.o.) MTX (30 mg/m^2, p.o.)	25 53 81 109	32–109	Alternating: 2 weeks: Intrathecal therapy Dexamethasone (6 mg/m^2, p.o.) Vincristine (2 mg/m^2, i.v.) and 5 weeks: 6MP (50 mg/m^2, p.o.) MTX (30 mg/m^2, i.v.)	53 81 109

NHR, non-high-risk; HR, high-risk. CNS, central nervous system; p.i., per infusion; p.o., orally; i.v, intravenously; MTX, methotrexate. Intrathecal therapy during maintenance treatment is given until week 60 (NHR group) or (HR group) of the DCLSG-ALL-9-protocol.

The induction therapy of HR patients (weeks 0–6) was similar to that of NHR patients with the addition of 4 doxorubicin injections in the first 4 weeks and 4 weekly intrathecal triple injections in case of CNS disease at diagnosis. This was followed by a period of 8 weeks of prophylactic CNS treatment (weeks 6–14) with oral 6MP (50 mg/m^2) and 4 high-dose MTX (3 g/m^2) infusions. Intensification treatment I (weeks 15–23) consisted of daily oral 6MP (50 mg/m^2), weekly i.v. L-asparaginase, every 3 weeks oral dexamethasone (7 days) and vincristine and daunorubicin. In weeks 24–32 six intensification-courses with cyclophosphamide and cytosine-arabinoside Ara-C were given. During maintenance treatment (weeks, 33–109), MTX was administered i.v.

TPMT activity of children with HR-ALL was determined at week 0, 6, 15, 32 and during maintenance therapy (weeks 53, 81 and 109) and one year after cessation of treatment.

During maintenance treatment, blood was sampled after a 5-weeks period of 6MP and MTX administration. During the entire treatment period until 3 months after cessation of ALL treatment, patients also received oral low dose cotrimoxazole, a mixture of sulphomethoxazole and trimethoprim (15 and 3 mg/kg, respectively, 3 days weekly), to prevent *Pneumocystis carinii* pneumonia.

2.2.2. Controls

As control material, heparinised blood was used from control paediatric patients, after their written informed consent and/or after consent of their parents or guardians. These patients were non-critically ill children from the outpatient clinic of our department of Pediatrics at the University Medical Center, Nijmegen. They were not suffering from either haematological, immunological or nephrological diseases, and were also not treated with thiopurines or other antimetabolites, e.g., antifolates.

2.3. Sample isolation

Within 24 h after blood sampling, RBC and WBC were isolated by density gradient centrifugation, as described earlier in Ref. [19]. The mononuclear fraction (pMNC) was used for mutation analysis and the RBC were lysed for TPMT activity measurements, following the procedure as described in Ref. [20]. RBC and WBC were counted with an automated cell counter (Sysmex F520, Goffin, Meyvis, Tiel, the Netherlands). RBC were washed three times with phosphate-buffered saline and then lysed in three volumes of ice cold water. Subsequently, the lysate was centrifuged at 14000*g* for 10 min at 4 °C to remove cellular debris. The supernatant was kept at −80 °C until analysis, as were the pMNC.

2.4. In vitro experiments

The effects of various drugs, used during induction treatment of ALL, on TPMT activity were studied in Molt F4-cells, a human T-lymphoblastic cell line. Conditions of cell culture were as described by Stet and colleagues in Ref. [21] with some modifications. The cells were cultured in Roswell Park Memorial Institute (RPMI)1640 medium with Glutamax-1 supplemented with 10% fetal calf serum, 2 mM sodium pyruvate and gentamicin (50 μg/ml). Twenty four hours prior to the start of the experiments, logarithmically growing cells were seeded in a concentration of 0.3×10^6 cells per ml. Drugs were added to the medium in clinically relevant concentrations: for vincristine 0.0012, 0.012, 0.120 μM, for dexamethasone 0.0025, 0.025, 0.25 μM, for L-asparaginase 0.001, 0.01, 0.1 IU/ml, for trimethoprim 0.24, 2.4, 24 μM, and for MTX 0.005, 0.01 and 0.1 μM. Drugs were added as single doses in 1/100 volume. Blanks, i.e., no drugs added, were included (indexed as 100% in each experiment).

Furthermore, we investigated the effect of methionine, a precursor for SAM, on the TPMT increase observed after incubation with trimethoprim. Cells were incubated with or without the addition of trimethoprim (2.4 μM), together with or without the addition of 1.5 mM methionine. After 24 h of incubation, cells were harvested and counted in a Coulter Counter (model Z1, Coulter Electronics, Luton, UK). Cell viability was determined by the tryphan blue exclusion test, and TPMT activity was measured (see below). Experiments were performed in triplicate.

2.5. Determination of the TPMT activity

Control and patients' TPMT activities were measured as described by Keizer-Garritsen and colleagues in Ref. [20] and expressed as pmol 6MeMP formed per 10^7 RBC per hour of incubation. In Molt F4-cells, TPMT activity was expressed as pmol 6MeMP formed per 10^6 viable Molt F4-cells per hour of incubation.

2.6. Screening on TPMT mutations

Each assay was done using 75 ng genomic DNA. The standard PCR protocol consisted of a denaturation step (95 °C for 5 min) followed by 30 cycles of denaturation (94 °C for 1 min), annealing (specific annealing temperature for 2 min) and extension (72 °C for 1 min). The final extension was performed at 72 °C for 7 min.

*TPMT*2*, *TPMT*3A*, *TPMT*3B* and *TPMT*3C* were screened according to the method described by Yates and colleagues [5] with slight modifications. To detect the *TPMT*2* mutation, we used an allele-specific PCR. The PCR reaction was carried out in 50 μl of a solution containing buffer (20 mM Tris–HCl, 50 mM

Table 2
Primers and restriction enzymes used for the detection of mutations in the thiopurine S-methyltransferase (TPMT) gene

Mutation	Primer	Sequence $5' \rightarrow 3'$	PCR fragment-length	Restriction enzyme	Restriction fragment length
*TPMT*2*	P2FW	gta tga ttt tat gca ggt ttg	254 bp		
	P2FM	gta tga ttt tat gca ggt ttc			
	P2R	taa ata gga acc atc gga cac			
*TPMT*3B*	P3F	agg ctc cta aaa cca tga ggg	317 bp	*Mwo I*	77, 240 bp
	P3R	gta tac taa aaa att aag aca gc			
*TPMT*3C*	P3CF	cag gct tta gca taa ttt tca att cct c	293 bp	*Acc I*	86, 207 bp
	P3CR	tgt tgg gat tac agg tgt gag cca c			
*TPMT*3D*	P2FW	gta tga ttt tat gca ggt ttg	254 bp	*Apo I*	49, 74, 131 bp
	P2FM	gta tga ttt tat gca ggt ttc			
	P2R	taa ata gga acc atc gga cac			
*TPMT*4*	P4FW	ttt aac atg tta ctc ttt ctt gtt tca a	209 bp		
	P4FM	ttt aac atg tta ctc ttt ctt gtt tca g			
	P3CR	tgt tgg gat tac agg tgt gag cca c			
*TPMT*5*	P5F	cta ctc gga tac tga ggt ac	233 bp	*Mse I*	110, 123 bp
	P5R	ctt gta tcc caa gtt cac tg			
*TPMT*6*	P6FW	cct ggg aaa gaa gtt tca ga	188 bp		
	P6FM	cct ggg aaa gaa gtt tca gt			
	P6R	gac agt caa ttc ccc aac tt			
*TPMT*7*	P7FW	ggt gat gct ttt gaa gaa cga cat	149 bp		
	P7FM	ggt gat gct ttt gaa gaa cga cag			
	P3CR	tgt tgg gat tac agg tgt gag cca c			

bp, base pairs; PCR, polymerase chain reaction.

KCl; pH 8.5), 4 mM $MgCl_2$, 1 mM dNTPs, 0.5 μM of primers P2FW and P2R for the wild-type allele or primers P2FM and P2R for the mutant allele and 1 U Taq polymerase (Life Technologies, Scotland). Annealing temperature during amplification was 57 °C. Wild-type or mutant DNA fragments were selectively amplified resulting in fragments lengths of 254 base pairs (Table 2). *TPMT*3A*, containing the *TPMT*3B* mutation (G460A) and the *TPMT*3C* mutation (A719G) on one allele, is detected by a restriction enzyme analysis. The PCR assay to detect *TPMT*3B* was carried out in 50 μl of a solution containing buffer G (Invitrogen; 60 mM Tris–HCl, 15 mM ammonium sulphate, 2.5 mM $MgCl_2$, pH 9.0), 1 mM dNTPs, 0.4 μM of each primer P3F and P3R, and 1 U Taq polymerase. The annealing temperature was 53 °C. 10 μl of PCR product were used for digestion with 1 U *Mwo I*. For optimal digestion, the samples were incubated overnight at 60 °C. The PCR assay to detect *TPMT*3C* was carried out in 50 μl of a solution containing buffer 1 (Invitrogen; 60 mM Tris–HCl, 15 mM ammonium sulphate, 1.5 mM $MgCl_2$, pH 9.5), 1 mM dNTPs, 0.4 μM of each primer P3CF and P3CR, and 1 U Taq polymerase. The annealing temperature was 57 °C. 10 μl of PCR product were used for digestion with 1 U *Acc I*. For optimal digestion, the samples were incubated overnight at 37 °C.

We also developed a PCR assay for *TPMT*3D*, *TPMT*5*, *TPMT*6* and *TPMT*7*. To detect *TPMT*3D* fragments, containing a third mutation (G292T) on a *TPMT*3A* allele, the *TPMT*2* PCR products (with primers P2FM and P2R) were used. Digestion of the PCR products with 1 U *Apo* I for 2 h at 50 °C provided three fragments with lengths of 131, 74 and 49 bp for wild-type alleles. PCR products containing the G292T mutation will be cut in two fragments (131 and 123 bp). *TPMT*4*, a G → A splice site mutation of intron 9, could be detected using an allele-specific PCR. The PCR reaction mixture contained buffer A (Invitrogen; 60 mM Tris–HCl, 15 mM ammonium sulphate, 1.5 mM $MgCl_2$; pH 8.5), 1 mM dNTPs, 0.4 μM of primers P4FW and P3CR for the wild-type allele or primers P4FM and P3CR for the mutant allele, and 1 U Taq polymerase. The annealing temperature was 57 °C. Wild-type or mutant DNA fragments were selectively amplified resulting in fragment lengths of 209 base pairs. *TPMT*5* (T146C mutation) could be detected using restriction enzyme analysis. The PCR reaction mixture contained buffer A, 1 mM dNTPs, 0.4 μM of each primer P5F and P5R, and 1 U Taq polymerase. Annealing temperature was 55 °C. Digestion of the PCR product with 1 U *Mse I* for 2 h at 37 °C resulted in 2 bands of 110 and 123 bp for the wild-type allele; mutant alleles will not be cut. To detect *TPMT*6*, an A539T mutation, we used an allele-specific PCR. The PCR reaction mixture contained buffer D (Invitrogen; 60 mM Tris–HCl, 15 mM ammonium sulphate, 3.5 mM $MgCl_2$; pH 8.5), 1 mM dNTPs, 0.4 μM of each primer P6FW and P6R for the wild-type allele or P6FM and P6R for the mutant allele, and 1 U Taq polymerase. Annealing temperature was 55 °C. Amplification resulted in a fragment of 188 bp. *TPMT*7*, a T681G mutation, was detected by selective amplification as well. The PCR reaction mixture contained buffer A, 1 mM dNTPs, 0.4 μM of each primer P7FW and P3CR

for the wild-type allele or P7FM and P3CR for the mutant allele, and 1 U Taq polymerase. Annealing temperature was 55 °C. Amplification resulted in a PCR fragment of 149 bp. All PCR fragments were separated on a 3% agarose gel. We decided not to include screening for mutation *TPMT*8* as this is not a common mutation and the costs of the restriction enzyme needed (*Mae II*) are high.

2.7. Statistics

Ages in the control population and patients with ALL at diagnosis were compared using the Students' *t*-test. Differences in TPMT activities in these groups, and both genders in the leukaemic patient group were calculated using the Wilcoxon *W* test. The relationship of TPMT activity with age was established with Spearman's correlation analysis. Intrapatient differences of RBC TPMT activity at diagnosis, during ALL treatment and one year after cessation of treatment were calculated by means of the Wilcoxon Signed Ranks Test. The gender data in controls and the ALL population were compared using the χ^2 test, whereas the difference in prevalence of mutations in both groups was calculated using the Fisher's Exact Test. TPMT activities in the control cells and the cells incubated with drugs were compared using the non-parametric Wilcoxon *W* test.

3. Results

3.1. TPMT activity in a control population and patients diagnosed with ALL

TPMT activities in control children were compared with those in patients with ALL at diagnosis and during treatment. With respect to gender, no significant differences were observed in controls ($n = 122$; 51 females and 71 males) and in patients with ALL at diagnosis ($n = 173$; 57 females and 116 males; $\chi^2 = 2.712$; $P = 0.100$). Mean age in the control group was 8.6 ± 5.4 years, whereas the mean age in the ALL group at diagnosis was 5.9 ± 4.1 years ($P < 0.001$). The mean ages in both groups were different, but in an earlier study we observed no differences in control TPMT activities concerning both gender or age [20]. In the patients monitored in this study, TPMT activity was not related with age (Fig. 1) nor gender. Median TPMT activity in male patients was 12.4 (range 1.7–30.7), in females 12.8 (range 5.8–30.4) pmol/10^7 RBC*hr ($P = 0.841$). Control RBC TPMT activity showed an overall median activity of 14.6 (range 1.6–50.7) pmol/10^7 RBC*hr ($n = 140$). The median TPMT activity in patients with ALL at diagnosis was significantly lower than control values (11.5; range 1.7–30.7) pmol/10^7 RBC*hr; ($n = 191$; $P < 0.001$).

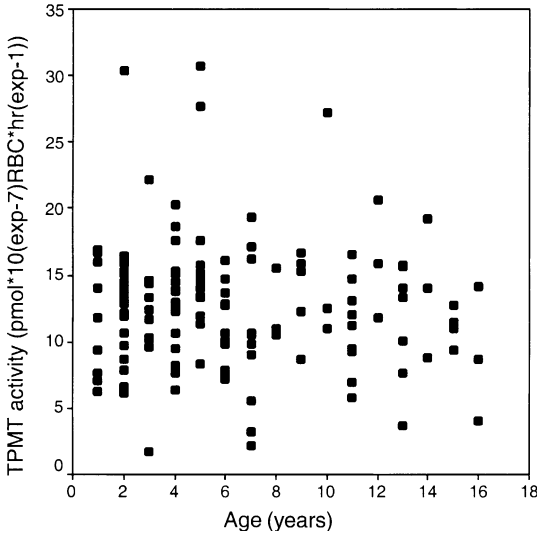

Fig. 1. Scatter diagram of thiopurine S-methyltransferase (TPMT) activity at diagnosis and age in patients with acute lymphoblastic leukaemia (ALL). Spearman's correlation coefficient was −0.070; $P = 0.419$. TPMT activity is expressed in pmol/10^7 RBC*hr; age in years.

3.2. TPMT mutations

In Fig. 2, the genotype/phenotype correlations are shown for the controls and children with ALL. Forty three controls and 109 children with ALL have been genotyped. We found one child in each group to be homozygous for *TPMT*3A*. The TPMT activities of these children were 1.6 and 1.8 pmol/10^7 RBC*hr. Two of the controls showed a heterozygous genotype (one TPMT*1/*2 and one TPMT*1/*3A), whereas twelve patients with ALL carried one mutant allele. Nine of these patients showed a TPMT*1/*3A and three a TPMT*1/*3C genotype. All children possessing one or two mutant alleles had a RBC-TPMT

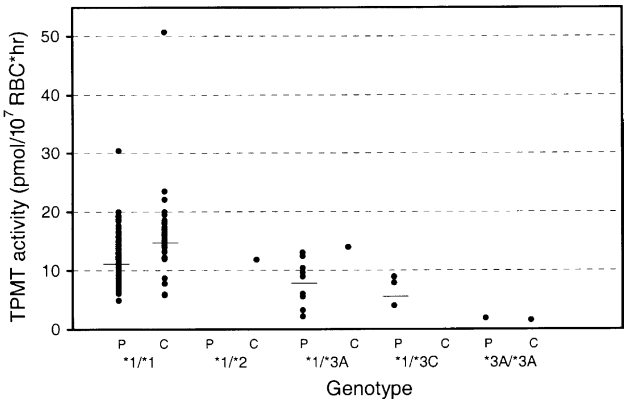

Fig. 2. Correlation of *TPMT* genotype and phenotype in 109 children with ALL at diagnosis and 43 controls. TPMT activity is expressed as pmol/10^7 RBC*hr. "P" indicates patients with ALL, "C" controls. Horizontal lines represent the median activities.

Table 3
Allele frequencies found in controls and children with ALL

Alleles	Control population (*n*, %)	ALL population (*n*, %)
*1	82 (95.3)	204 (93.6)
*2	1 (1.2)	0
*3A	3 (3.5)	11 (5.0)
*3C	0	3 (1.4)
Total	86	218

activity below 15 pmol/10^7 RBC*hr. In ALL patients at diagnosis, the median TPMT activity was significantly higher in the wild-type (TPMT*1/*1) compared with the heterozygotes: 12.0 (range: 3.2–30.4; $n = 95$) *vs.* 7.5 (range: 1.7–13.0; $n = 14$) pmol/10^7 RBC*hr, respectively ($P = 0.002$). The frequency of mutant alleles as shown in Table 3, was not significantly different in controls compared with the ALL population ($P = 0.254$). The median TPMT activity of the wild-type controls (15.1; range 5.8–50.7 pmol/10^7RBC*hr; $n = 40$) is higher compared with the TPMT activity of the wild-type of patients with ALL at diagnosis (12.0; range 3.2–30.4 pmol/10^7 RBC*hr; $n = 95$; $P = 0.005$).

3.3. TPMT activity in patients during ALL treatment

TPMT activities during the various treatment phases in both the NHR (Fig. 3(a)) and HR groups (Fig. 3(b)) are shown. In the total group (NHR + HR), TPMT activity significantly increased compared with the value at diagnosis during the first six weeks of treatment to a median TPMT activity of 17.5 (range: 3.9–40.3) pmol/10^7 RBC*hr ($n = 111$; $P < 0.001$), which is 176% (range: 30–854%) compared with the level at diagnosis in the same patients.

TPMT activity was even significantly higher at this treatment stage than control values ($P < 0.001$). During maintenance treatment, TPMT activity was still significantly increased, compared with that at diagnosis and control values, and remained stable during this whole treatment period (Fig. 3). At the end of treatment (week 109), values were still significantly higher compared with diagnosis ($P < 0.001$). One year after cessation of treatment, the TPMT activity had normalised to control patient values (12.8; range: 8.2–23.7) pmol/10^7 RBC*hr; $n = 17$; ($P = 0.672$).

Interestingly, a transient, but statistically significant, TPMT decrease was observed in HR patients at week 32 of ALL treatment ($P = 0.043$) compared with TPMT activity at week 15.

3.4. Influence of RBC transfusions on TPMT activity

In a subgroup of 29 homozygous wild-type patients, treated in our paediatric department, the influence of

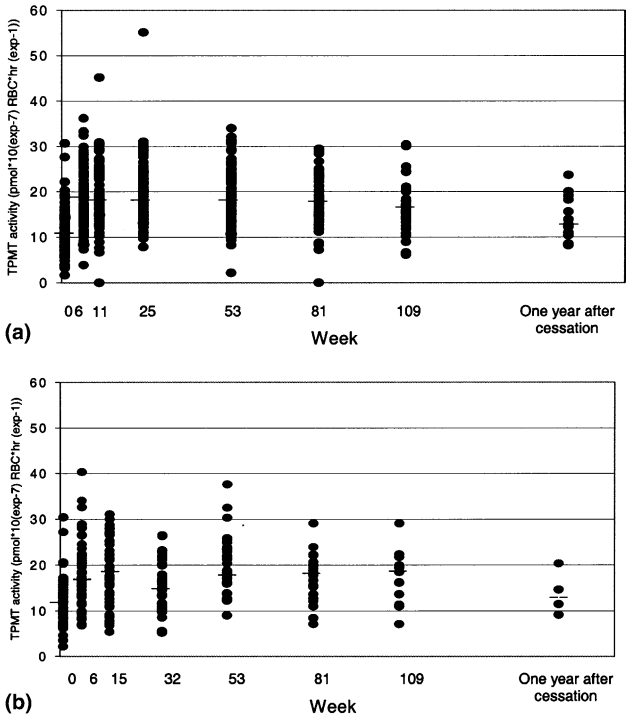

Fig. 3. TPMT activity during ALL treatment; (a) non-high risk (NHR) patients, (b) high-risk (HR) patients. Activity is expressed as pmol/10^7 RBC*hr. Horizontal lines represent the median TPMT activity, the dots represent the individual values. The category axis indicates the week of sampling. Number of patients are given as follows: time-point (*n*). Concerning the NHR patients: week 0 (125), week 6 (88), week 11 (94), week 25 (105), week 53 (87), week 81 (51), week 109 (35), one year after cessation of treatment (13). Concerning the HR patients: week 0 (66), week 6 (51), week 15 (51), week 32 (41), week 53 (34), week 81 (25), week 109 (15), one year after cessation of treatment (4).

RBC transfusions on the median TPMT activity at diagnosis was studied in more detail. Thirteen of 29 patients were transfused within one week prior to diagnosis, blood sampling and initiation of therapy. The median TPMT activity of these transfused patients was not higher than in the patients who did not get a RBC transfusion at this stage: activities were 10.5 (range 3.2–15.9) *vs.* 11.1 (range 1.7–19.8) pmol/10^7 RBC*hr, respectively ($P = 0.878$). Thus, for TPMT analyses in a group, RBC tranfusions do not play a significant role.

3.5. Influence of drugs on TPMT activity

We studied the effects of drugs on Molt F4-cells *in vitro* during six weeks of treatment. The effects on TPMT activity of different concentrations of these drugs are shown in Fig. 4. TPMT activity in the untreated cells was 18.1 ± 2.2 pmol/10^6 Molt-F4 cells*hr. Following an incubation of the cells with 2.4 μM trimethoprim, a significant increase to 148% was observed (actual TPMT activity: 26.8 ± 4.7 pmol/10^6 Molt-F4 cells*hr: $P = 0.004$; $n = 6$). The increase could be partly

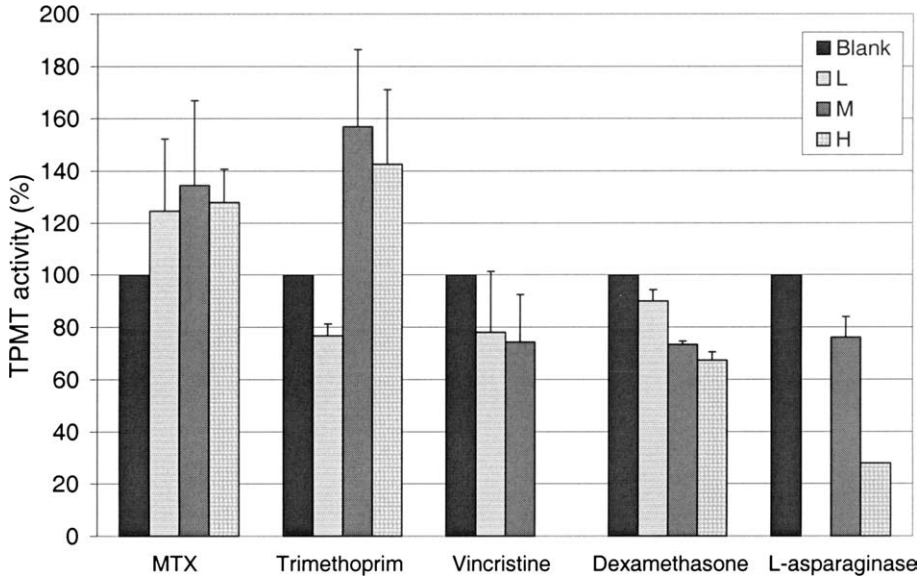

Fig. 4. TPMT activity after incubation of Molt F4-cells with various concentrations of several drugs. TPMT activity is given as mean percentage of blank (no drugs added) of triplicate measurements. Error bars represent the standard deviations. "H" = high concentration, "M" = medium concentration, "L" = low concentration of the drug administered. For the concentrations used: see Section 2. Viability of the cells was 75–98%, except for L-asparaginase incubations: this was 57% and 48% for "M" and "L", respectively.

counteracted by simultaneous addition of methionine (1.5 mM) to the medium (20.2 ± 2.2 pmol/10^6 Molt-F4 cells*hr, increase to approximately 112%). TPMT activity was increased to approximately 147% when cells were incubated with 0.01 μM MTX (25.5 ± 3.9 *vs.* 17.4 ± 3.6 pmol/10^6 Molt-F4 cells*hr in the blank: $n = 10$: $P < 0.001$). Methionine alone also resulted in an enhancement of TPMT activity in Molt F4-cells (23.6 ± 2.1 pml/10^6 Molt-F4 cells*hr). Vincristine, dexamethasone and L-asparaginase did not appear to have a stimulating effect on TPMT activity. On the contrary, at increasing concentrations these drugs inhibited TPMT activity.

4. Discussion

4.1. Increase of TPMT activity before 6MP administration

TPMT activities in children with ALL have been monitored from diagnosis throughout treatment and after cessation of therapy. In most patients, we found-in concordance with other authors-a very prominent increase in TPMT activity during ALL treatment. However, we have shown by *in vitro* experiments that the increase in TPMT activity was presumably attributable to antifolates, and not to thiopurine treatment.

Capdeville and colleagues [22] observed a higher TPMT activity at the start of the maintenance treatment, compared with that detected at a later phase of treatment. However, these patients were not monitored before the onset of chemotherapy. We have shown that the increase of TPMT activity already occurred during the first six weeks of induction treatment, i.e., before 6MP is administered to the patients.

In a subgroup of patients, we showed that median TPMT activity of the children who were transfused just before diagnosis was similar to that of children who did not get a RBC transfusion. This observation excluded the possibility that the TPMT increase was attributable to RBC transfusions.

It should also be taken into account that part of the observed increase of TPMT activity may be due to a recovered erythropoiesis by drug treatment, resulting that in a higher percentage of young RBC compared with at diagnosis. Lennard and colleagues [29] pointed out, younger RBC have higher TPMT activity than matured RBC.

After the initial increase in TPMT activity, we did not see a further rise of TPMT activity in ALL patients after the prophylactic treatment of the CNS with MTX infusions and during maintenance treatment (Fig. 3), indicating that a steady state had been reached. In the HR patients, we observed a transient decline of TPMT activity after the second intensification treatment with cyclophosphamide and cytosine arabinoside, although the activity was still higher than at diagnosis.

The mode of MTX administration (p.o. in the NHR group, i.v. in the HR group) did not lead to a difference in TPMT activity during maintenance therapy.

We could demonstrate that treatment with 6MP is not the cause of the rise in TPMT activity in our patients. Which of the drugs used during induction treatment would be responsible for the enhancement was investigated in Molt F4 cells. These cells were incubated

in vitro with drugs given during the first six weeks of treatment, i.e., the antifolates (trimethoprim used as Pneumocystis carinii pneumonia (PCP) prophylaxis, starting directly after diagnosis, and intrathecal MTX), vincristine, dexamethasone and L-asparaginase. The antifolates had an enhancing effect on TPMT activity, whereas the other three drugs did not increase TPMT activity. The rise in TPMT activity, caused by these antifolates, may be due to their inhibitory effect on the transmethylation pathway (Fig. 5). MTX as well as trimethoprim are known inhibitors of dihydrofolate reductase [23,24], and may inhibit synthesis of SAM. Moreover, the formation of adenosine triphosphate (ATP), necessary for the conversion of methionine to SAM, is inhibited by antifolates because these compounds can inhibit the purine *de novo* synthesis [25–28]. As a consequence, less SAM would be available for methylation reactions, resulting in a higher TPMT activity as a compensatory mechanism. This hypothesis is supported by our observation *in vitro* that methionine reduced the TPMT enhancement in Molt-F4 cells by trimethoprim. The enhancement was not completely reversed by methionine, probably because ATP remains the limiting factor. Whether this hypothesis is justified, should be studied further.

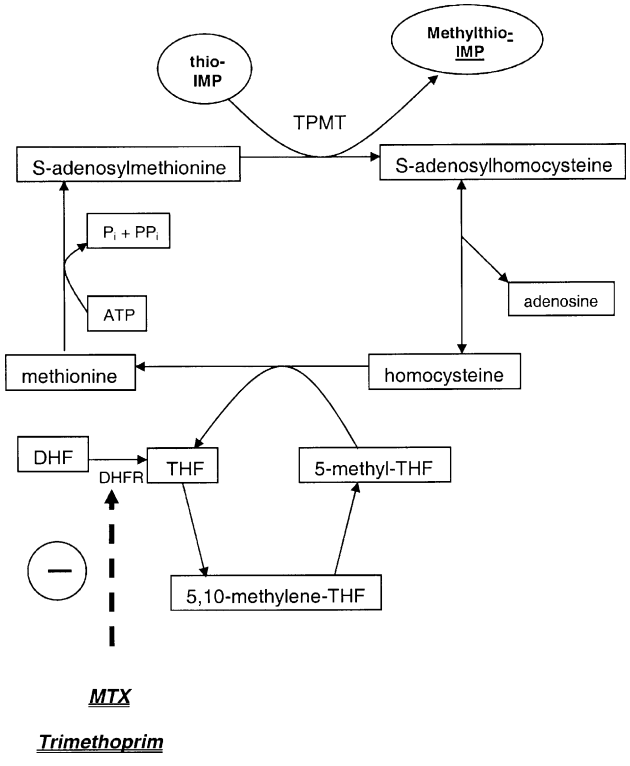

Fig. 5. The transmethylation pathway: thio-IMP, thioinosine monophosphate; methylthio-IMP, methylthioinosine monophosphate, THF, tetrahydrofolic acid, DHF, dihydrofolic acid, MTX, methotrexate, TPMT, thiopurine methyltransferase, DHFR, dihydrofolic acid reductase. ATP, adenosine triphosphate.

4.2. Differences in TPMT activity between a control population and ALL patients

The TPMT activity in our patients with ALL at diagnosis is significantly lower compared with controls. This may be explained by a different age distribution of RBC in leukaemic patients, containing a larger number of older cells compared with controls [29]. After fractionating RBC, other authors have reported that human TPMT activity varied with RBC age, with mature RBC having a lower TPMT activity than younger cells. RBC of leukaemic patients probably have a different age distribution, containing a larger number of older cells compared with controls due to the inhibition or cessation of erythropoiesis by leukaemic infiltration of the bone marrow. Earlier studies of Lennard and colleagues [14,30] did not show such a difference between TPMT activity at diagnosis in their patients with ALL compared with controls, which seems paradoxical to our observation. However, this may be explained by the different work-up of blood and expression of TPMT activity. They expressed TPMT activity as units ml^{-1} packed RBC. Using these units, dead cells were included in their calculation. We used isolated RBC, expressing TPMT activity in pmoles per 10^7 viable RBC, thereby excluding cells which were not viable before lysation.

The differences in TPMT activity between controls and ALL patients at diagnosis cannot be explained by differences in the frequency of mutant alleles, because we found no higher prevalence of mutant alleles in the ALL group compared with controls, in concordance with results by McLeod and colleagues [13]. This also gives a strong indication that children carrying *TPMT* mutations are not necessarily prone to leukaemogenesis.

4.3. The effect of RBC transfusions

During induction treatment, almost all patients with ALL receive (multiple) RBC transfusions. However, we did not observe any influence of RBC transfusions on the mean TPMT activity in a subgroup of wild-type patients at diagnosis. Nevertheless, we found an even higher TPMT activity after six weeks of treatment, compared with controls. So, it can be expected that the increase of TPMT activity is caused by therapy and cannot be attributed to RBC transfusions. It may even be that this rise in TPMT activity is underestimated, because of the mixture of patients' RBC with donor RBC, since the RBC TPMT activity in controls is lower than the activity in treated patients.

Prediction of the response of a patient to thiopurine treatment can be based either on measurement of TPMT activity or on mutational analysis or both. However, using the enzymatic activity measurements, TPMT deficiencies present at diagnosis or during treatment may be partly masked in individual cases, when these patients

receive RBC transfusions from a donor with a high TPMT activity. An explicit example here of has been described recently by Schwab and colleagues in Ref. [31] Therefore, both measurement of TPMT activity and a mutational analysis are important to detect TPMT deficiency.

In conclusion, we found a lower TPMT activity in patients at presentation of ALL, when compared with controls. This difference in TPMT activity could not be explained by differences in the frequency of mutant alleles between controls and our ALL patients.

Furthermore, it has been suggested previously that the increase of TPMT activity during ALL treatment could be ascribed to 6MP therapy. However, we demonstrated that TPMT activity is already increased before 6MP is administered. *In vitro* experiments indicated that this early increase could have been caused by the antifolates given during the induction treatment: i.e., intrathecal MTX and trimethoprim.

Conflict of interest statement

None declared.

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