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ORIGINAL ARTICLE

Role of TPMT and ITPA variants in mercaptopurine disposition

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Abstract

Purpose To explore the levels of thioguanine incorporated into DNA (DNA-TG), and erythrocyte levels of 6-thioguanine nucleotides (Ery-TGN) and methylated metabolites (Ery-MeMP) during 6-mercaptopurine (6MP)/Methotrexate (MTX) therapy of childhood acute lymphoblastic leukemia (ALL) and the relation to inosine triphosphatase (*ITPA*) and thiopurine methyltransferase (*TPMT*) gene variants.

Methods Blood samples were drawn during 6MP/MTX maintenance therapy from 132 children treated for ALL at Rigshospitalet, Copenhagen. The samples were analysed for thiopurine metabolites and compared to *TPMT* (rs1800460 and rs1142345) and *ITPA* (rs1127354) genotypes.

Results Median DNA-TG (mDNA-TG) levels were higher in *TPMT* and *ITPA* low-activity patients as compared to wildtype patients ($TPMT^{LA}$ 549 vs. 364 fmol/µg DNA, $p = 0.007$, $ITPA^{LA}$ 465 vs. 387 fmol/µg DNA, $p = 0.04$). mDNA-TG levels were positively correlated to median Ery-TGN (mEry-TGN)($r_s = 0.37$, $p = 0.001$), but plateaued at higher mEry-TGN levels. DNA-TG indices (mDNA-TG/mEry-TGN) were 42% higher in $TPMT^{WT}$ patients as compared to $TPMT^{LA}$ patients but no difference in DNA-TG indices was observed between $ITPA^{WT}$ and $ITPA^{LA}$ patients (median 1.7 vs. 1.6 fmol/µg DNA/nmol/mmol Hb, $p = 0.81$). DNA-TG indices increased with median Ery-MeMP (mEry-MeMP) levels ($r_s = 0.25$, $p = 0.001$).

Conclusions *TPMT* and *ITPA* genotypes significantly influence the metabolism of 6MP. DNA-TG may prove to be a more relevant pharmacokinetic parameter for monitoring 6MP treatment intensity than cytosolic metabolites. Prospective trials are needed to evaluate the usefulness of DNA-TGN for individual dose adjustments in childhood ALL maintenance therapy.

Keywords Acute lymphoblastic leukemia · Thiopurines · Pharmacology · Thiopurine methyltransferase · Inosine triphosphatase

Introduction

Genetic variation is of particular importance in drugs with a narrow therapeutic index, such as thiopurines, which are the most essential drugs in the maintenance treatment of childhood acute lymphoblastic leukemia (ALL). To date, there is only one widely accepted clinical guideline for dose modification based on gene status which is thiopurine dosing based on thiopurine methyltransferase (*TPMT*) (rs1800460 and rs1142345) genotype. Other gene variants such as nudix hydrolase 15 (*NUDT15*) (rs1168553232 and rs186364861)

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[1–7] and inosine triphosphatase (*ITPA*) (rs1127354) have been associated with 6-MP treatment-related toxicity [8–12].

6MP is a pro-drug and its cytotoxicity depends on conversion to 6-thioguanine nucleotides (TGN) which can be incorporated into DNA (DNA-TG). DNA-TG may mismatch during DNA replication, which activates the mismatch repair system. Continuous DNA-TG mismatching will eventually lead to apoptosis or mutations in the DNA copy strand [13]. TPMT methylates 6MP and thereby reduces the amount of drug available for TGN formation [14, 15]. However, the methylated metabolite methyl-thioinosine monophosphate (Me-TIMP) is a potent inhibitor of the purine de novo synthesis, resulting in a lower level of endogenous purines for incorporation into DNA, and a relatively higher level of thioguanine nucleotides (TGN), which in the end enhances the incorporation of TGN into DNA [16, 17].

ITPA catalyzes the hydrolysis of thioinosine triphosphate (TITP) to thioinosine monophosphate (TIMP). Through this loop, TITP is reconverted to TIMP, which can then increase TGN levels. NUDT15 is hypothesized to dephosphorylate the thiopurine active metabolite TGTP to TGMP, thus preventing the incorporation into DNA and reducing the desired cytotoxic effect of 6MP (Fig. 1).

SNPs in the *TPMT* gene result in approximately 90% of Caucasians being homozygous for high TPMT activity ($TPMT^{WT}$), 10% carry one low-activity allele ($TPMT^{LA}$),

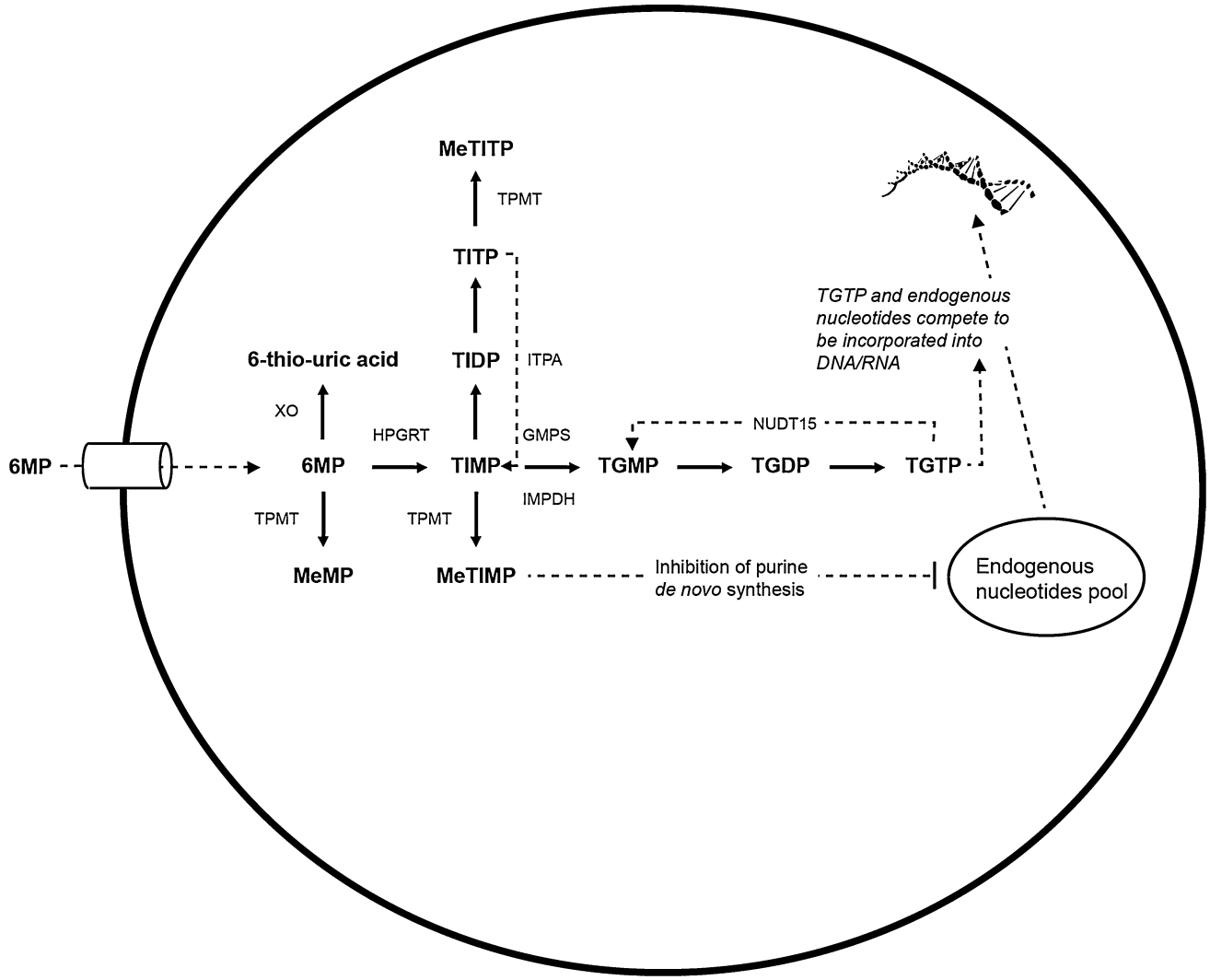

Fig. 1 Simplified scheme of the metabolism of 6MP and the enzymes involved. *6MP* 6-mercaptopurine, *HPGRT* hypoxanthine–guanine phosphoribosyltransferase, *TIMP* Thioinosine monophosphate, *IMPDH* inosine monophosphate dehydrogenase, *GMPS* guanosine monophosphate synthase, *TGMP* thioguanosine monophosphate, *TGDP* thioguanosine diphosphate, *TGTP* thioguanosine triphosphate, *ITPA* inosine triphosphate pyrophosphatase, *TPMT* thiopurine methyltransferase, *MeMP* methylmercaptopurine, *XO* xanthine oxidase, *TIDP* thioinosine diphosphate, *TITP* thioinosine triphosphate, *NUDT15* Nudix hydrolase 15. Deoxy forms ignored. Illustration made in Illustator

and 1 in 300 carries two low-activity alleles and are TPMT-deficient ($TPMT^{DE}$) [7]. The frequency of $ITPA^{LA}$ alleles is 5–7% in Caucasians and confers a reduction in enzyme activity by approximately 75 and 100% among heterozygous and homozygous individuals, respectively [18, 19]. ITPA and NUDT15 genetic variations are both most common in the Asian population and have both proved to significantly affect thiopurine toxicity [3, 7, 11, 20–22;7;11;20–22].

In a single-institution study, we explored the effect of TPMT and ITPA genotypes on the levels of the end point metabolite DNA-TG and its relation to levels of Ery-TGN and Ery-MeMP in 132 children with ALL undergoing 6MP/MTX maintenance treatment.

Patients and methods

Childhood ALL patients diagnosed at the University Hospital, Rigshospitalet, before December 31st, 2011 and treated according to the ALL2000 or ALL2008 protocols were eligible for this study. Infants and patients allocated to the high-risk treatment (HR) were excluded, as their treatment deviate substantially from standard risk (SR) and intermediate risk (IR) patients [23, 24]. Baseline parameters of the 132 patients who met the inclusion criteria and for which blood samples were available are shown in Table 1. Children diagnosed with ALL in the Nordic and Baltic countries are treated according to the common Nordic Society of Pediatric Hematology and Oncology (NOPHO) protocols, which are updated approximately every eighth year. In the NOPHO ALL2000 protocol, maintenance therapy was initiated at week 17 for SR patients and week 30 for IR patients; and in the ALL2008 protocol, at week 20 and 22, respectively. Regardless of risk group, the initial dose of oral 6MP was 75; 50 and 1–10 mg/m^2/24 h for $TPMT^{WT}$, $TPMT^{LA}$ and $TPMT^{DE}$ patients. No dose adjustments according to *ITPA* variants were done. The starting dose of oral MTX was 20 mg/m^2/week. 6MP and MTX doses were subsequently adjusted by a target white blood cell count (WBC) of 1.5–3.5 × 10^9/l for patients treated according to the NOPHO ALL2000 protocol and 1.5–3.0 × 10^9/l for patients treated according to the NOPHO ALL2008 protocol. During the first year of maintenance therapy, treatment intensifications were given at 4-week intervals, with alternating high-dose MTX (5 g/m^2/24 h with Leucovurin rescue) or Vincristine (2.0 mg/m^2)/Dexamethasone (6 mg/m^2/day for five days), until five high-dose MTX infusions had been given. The

Table 1 Descriptive statistics

	$TPMT^{WT}$	$TPMT^{LA}$	Total *p* value	$ITPA^{WT}$	$ITPA^{LA}$	Total *p* value
Gender			< 0.12			< 0.18
Female n. 60 (45)	51 (43)	9 (64)	60 (45)	51 (48)	2 (22)	53 (46)
Male n. 72 (55)	67 (57)	5 (36)	72 (55)	55 (52)	7 (78)	62 (54)
Total n. 132	118	14	132	106	9	115
Risk group			< 0.3			< 0.58
SR n. 70 (53)	64 (54)	6 (43)	70 (53)	56 (53)	5 (56)	61 (53)
IR n. 62 (47)	54 (46)	8 (57)	62 (47)	50 (47)	4 (44)	54 (47)
Total n. 132	118	14	132	106	9	115
TPMT genotype						< 0.69
$TPMT^{WT}$ n. 118 (89)				93 (88)	8 (89)	101 (88)
$TPMT^{LA}$ n. 14 (11)				13 (12)	1 (11)	14 (12)
Total n. 132				106	9	115

Data distribution on gender, risk group and genotype

WT wild type patients, *LA* low-activity patients

P values were calculated performing Chi-square test and show no significant difference between WT and LA patient bases on gender, risk group and genotype. Percentage on vertical total in brackets. ITPA genotype was not obtained in 17 patients. No TPMT-deficient patients were found in this cohort

second maintenance therapy phase (oral 6MP/MTX only, except for age-adjusted, intrathecal MTX at 8-week interval for IR patients) was initiated at week 56/70 (SR/IR, ALL 2000), or at week 58/66 (IR/IR, ALL2008). Treatment was discontinued 130 weeks after diagnosis in both protocols.

From January 2001 to January 2012, blood samples were collected from all patients treated at the Department of Pediatrics and Adolescent Medicine, The University Hospital Rigshospitalet, Copenhagen after approval by the Ethical Committee of Copenhagen (no. H-2-2010-002) and informed consent by the parents, according to the Declaration of Helsinki. For DNA-TGN analysis, 2.095 blood samples collected during maintenance therapy were available with a median of 23 samples per patient. Five patients had only one sample taken. Determination of Ery-TGN and Ery-MeMP concentrations had been performed in 2,636 samples, and 2,743 samples, respectively. All children diagnosed with ALL and treated according to the ALL2000 and ALL2008 protocols are routinely genotyped for *TPMT* polymorphisms (rs1800460 and rs1142345). As a part of this study, all patients were genotyped for ITPA polymorphisms (rs1127354) with TaqMan technology. ITPA genotypes were verified by independent analyses by a collaborating research group at Linköping University [25].

DNA-TG quantification

DNA-TG was quantified by derivatizing approximately 2 µg of whole blood DNA with chloroacetaldehyde in a phosphate buffer at 100 °C for 3 hours to produce etheno(ε)-TG and ε-guanine. The samples were cleaned on a strong cation exchanger (Strata-X-C, 30 mg, Phenomenex) and dried under flowing N2 (40 °C). After resuspension in 200 µl 0.1% formic acid (FA)/95% acetonitrile ε-TG and ε-guanine were quantified by hydrophilic interaction liquid chromatography (HILIC) on an aquity tandem mass spectrometry, as described by Jacobsen et al. [26].

Statistics

Data analyses were performed using SPSS version 22. Where indicated, arithmetic means (prefix m) of DNA-TG, Ery-MeMP and Ery-TGN were calculated for each patient based on all the samples available during maintenance therapy. DNA-TG indices were calculated as mDNA-TG/mEry-TGN. Correlation of continuous variables was assessed by Spearman's rank correlation coefficient (r_s). 6MP metabolite levels across genotypes were compared with Mann–Whitney *U* tests. Distribution of gender, risk group and genotype were assessed by Chi-square test. *P* values < 0.05 were regarded statistically significant.

Results

Patients' baseline parameters are presented in Table 1. DNA for ITPA genotyping was not available for 17 patients, but ITPA allele frequencies for the remaining 115 patients were in Hardy–Weinberg equilibrium ($p = 0.89$). Furthermore, distributions of patients with TPMT WT, LA and DE genotypes were in line with reported frequencies among Caucasians [27] and as expected the G460A and A719G variants were in strong disequilibrium. None of the included patients carried low-activity alleles for both ITPA and TPMT.

$TPMT^{LA}$ patients had significantly higher levels of mEry-TGN (median 491 vs. 232 nmol/mmol Hb, $p = 0.001$) (Fig. 2b; Table 2) and mDNA-TG (median 549 vs. 364 fmol/µg DNA, $p = 0.007$) (Fig. 2a; Table 2), but significantly lower levels of mEry-MeMP (median 6193 vs. 17,499 nmol/mmol Hb, $p = 0.001$) (Fig. 2c; Table 2) and DNA-TG indices (median 1.2 vs. 1.7 (fmol TG/µg DNA)/(nmol TG/mmol Hb), $p = 0.007$) (Fig. 2d; Table 2), when compared to $TPMT^{WT}$ patients.

We found significantly higher levels of mDNA-TG (median 465 vs. 387 fmol/µg DNA, $p = 0.04$) in $ITPA^{LA}$ as compared to $ITPA^{WT}$ patients (Fig. 2.a, Table 2). However, $ITPA^{LA}$ patients did not differ significantly from $ITPA^{WT}$ patients with respect to mEry-TGN (median 305 vs. 260 nmol/ mmol Hb, $p = 0.086$) (Fig. 2b; Table 2), mEry-MeMP (median 20,866 vs. 15,902 nmol/mmol Hb, $p = 0.12$) (Fig. 2c; Table 2), and DNA-TG indices (median 1.7 vs. 1.6 fmol/µg DNA/ nmol/mmol Hb, $p = 0.81$) (Fig. 2c; Table 2).

Gender, age, and number of samples available per patient were not significantly associated with metabolite levels. SR patients had higher levels of mEry-MeMP (17,759 vs. 14,286 nmol/mmol Hb, $p = 0.021$) and DNA-TG indices (1.8 vs. 1.5, $p = 0.041$), when compared to IR patients. Risk group was not significantly associated with mDNA-TG ($p = 0.138$) or mEry-TGN levels ($p = 0.69$) (Table 2).

Levels of mDNA-TG were positively correlated with mEry-TGN ($r_s = 0.37$, $p = 0.001$) (Fig. 3). In addition, mEry-MeMP levels were positively correlated to DNA-TG indices ($r_s = 0.33$, $p = 0.001$) (Fig. 4).

Discussion

It is well established that $TPMT^{LA}$ patients experience higher Ery-TGN levels and lower relapse rates as compared to $TPMT^{WT}$ patients, and some groups treat with reduced initial 6-MP doses to prevent unnecessary toxicity [28–31]. However, 6MP dose adjustments are guided by white blood

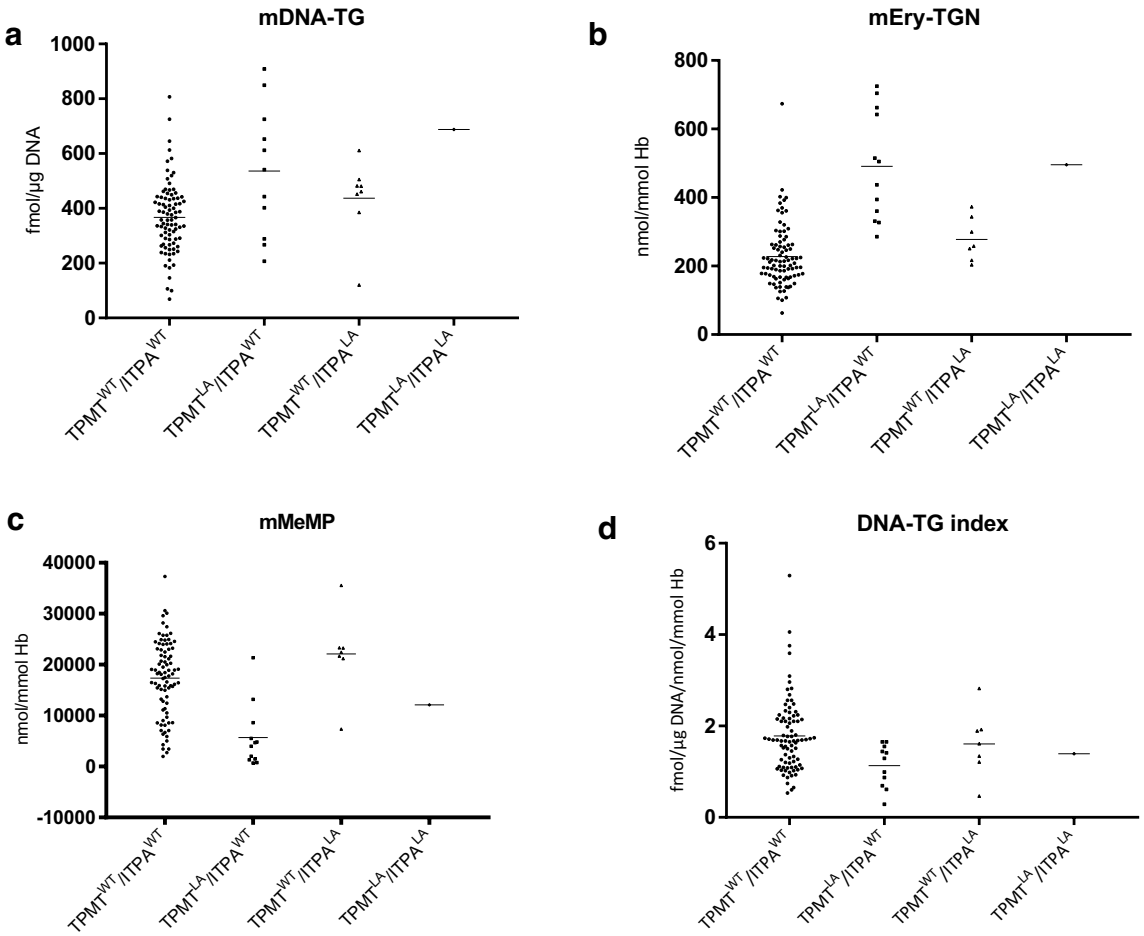

Fig. 2 Metabolites vs. genotype. Horizontal line is the median of the metabolite measurements in each patient group. Illustration made in GraphPad Prizm

cell count (WBC) aiming for WBC of $1.5-3.0\times10^9$ L [32] to reach equitoxic treatment [33–35], and it remains uncertain if patients benefit from this upfront dose reduction.

Findings indicate room for improvement to better tailor treatment with 6MP. DNA-TG is the principal cytotoxic metabolite, and we find significantly higher levels in $TPMT^{LA}$ patients as compared to $TPMT^{WT}$ patients. Contradictory to our findings, Ebbesen et al. found similar median levels of mDNA-TG in $TPMT^{LA}$ and $TPMT^{WT}$ patients, but non-significantly more $TPMT^{LA}$ patients had very high DNA-TG levels. The diverse findings may reflect the higher number of samples in the current study. In addition, our center extensively focusses on maintenance treatment, and the treatment intensity may overall have been higher than in the cohort of Ebbesen et al. [36].

The trend of DNA-TG levels to reach a plateau at high Ery-TGN levels indicates that with increasing Ery-TGN levels, proportionally less will be incorporated into DNA. This finding is in accordance with a previous study by Hedeland et al. [16]. This limit of incorporation may explain why $TPMT^{LA}$ patients have and tolerate higher levels of Ery-TGN without unacceptable myelosuppression, not least since they also have lower levels of methylated 6MP metabolites and thus less inhibition of purine de novo synthesis.

To achieve a more adequate understanding of the 6MP metabolism, we explored the impact of the enzyme ITPA, as recent studies have indicated that a SNP in the gene-encoding ITPA is associated with adverse drug reactions in thiopurine treatment [37, 38]. Moreover, $ITPA^{LA}$ has been associated with high levels of methylated 6MP metabolites [10, 39], higher risk of fever and neutropenia [10, 20, 40], hepatotoxicity [12, 20, 41], and possibly an increased risk of relapse [9, 42].

We found significantly higher levels of mDNA-TG, but only moderate, non-significant higher mEry-MeMP levels in $ITPA^{LA}$ patients as compared to $ITPA^{WT}$ patients. Methylated thiopurine metabolites has previously been shown to be significantly correlated to the incorporation of TGN into DNA [43]. $ITPA^{LA}$ patients may reconvert

Table 2 Results

	mDNA-TG, fmol/µg DNA	mEry-TGN, nmol/mmol hb	mEry-MeMP, nmol/mmol hb	DNA-TG index
Total	384 (272–458)	261 (178–305)	16,232 (9787–22,233)	1.7 (1.1–2.1)
$TPMT^{WT}$	364 (268–445)	232 (174–263)	17,499 (12,796–22,903)	1.7 (1.1–2.1)
$TPMT^{LA}$	549 (317–716)	491 (346–653)	6193 (1396–10,366)	1.2 (0.7–1.5)
p value	< 0.007	< 0.001	< 0.001	< 0.007
$ITPA^{WT}$	387 (287–448)	260 (175–304)	15,902 (8583–22,159)	1.7 (1.1–2.1)
$ITPA^{LA}$	465 (419–558)	305 (225–366)	20,866 (14,389–23,291)	1.6 (1.2–1.9)
p value	< 0.04	< 0.086	< 0.12	< 0.81
SR	393 (317–461)	254 (176–302)	17,759 (13,433–23,639)	1.8 (1.2–2.2)
IR	371 (255–450)	269 (185–318)	14,286 (7,358–20,517)	1.5 (1.1–1.8)
p value	< 0.14	< 0.69	< 0.021	< 0.041
Male	395 (312–461)	254 (192–263)	17,418 (12,426–21,818)	1.7 (1.2–2.1)
Female	369 (260–442)	268 (173–328)	14,823 (7024–22,469)	1.6 (1.0–2.0)
p value	< 0.23	< 0.91	< 0.24	< 0.22

Overview of median values of total and genotype, risk group and gender divided DNA-TG, Ery-TGN, MeMP and DNA-TG-index. Corresponding 25 and 75 percentiles in brackets. DNA-TG indices were calculated as mDNA-TG/mEry-TGN. *p* values from Mann–Whitney *U* test

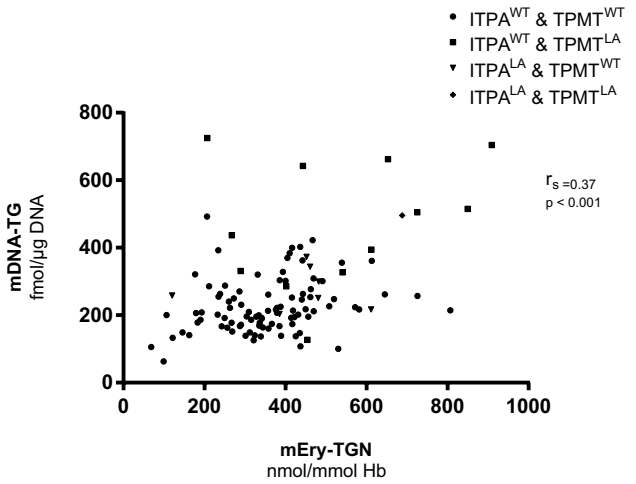

Fig. 3 Median Ery-TGN vs. median DNA-TG. The relationship between mEry-TGN and mDNA-TG. Median DNA-TG levels show a trend to reach plateau at high Ery-TGN levels. Spearman's rank correlation coefficient (r_s) was calculated. *p* values < 0.05 were regarded statistically significant. Circle: $ITPA^{WT}/TPMT^{WT}$ patients, square: $ITPA^{WT}/TPMT^{LA}$ patients, triangle: $ITPA^{LA}/TPMT^{WT}$ patients, diamond: $ITPA^{LA}/TPMT^{LA}$ patient. Illustration made in GraphPad Prizm

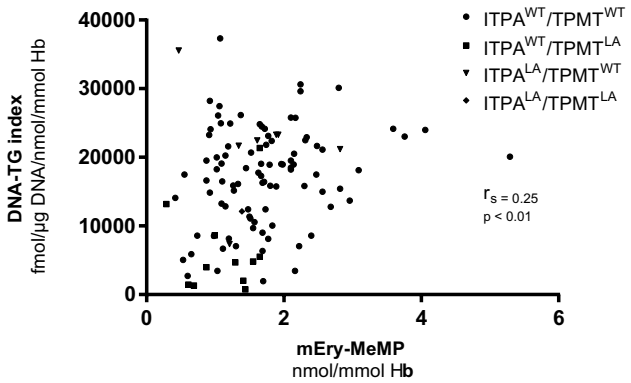

Fig. 4 Mean Ery-MeMP vs. DNA-TG index in relation to TPMT and ITPA genotype. Mean Ery-MeMP levels were significantly correlated to DNA-TG index indicating that with high levels of mEry-MeMP there was a significant increase in the relative incorporation of 6TGN into DNA. Spearman's rank correlation coefficient (r_s) was calculated. *p* values < 0.05 were regarded statistically significant. Circle: $ITPA^{WT}/TPMT^{WT}$ patients, square: $ITPA^{WT}/TPMT^{LA}$ patients, triangle: $ITPA^{LA}/TPMT^{WT}$ patients, diamond: $ITPA^{LA}/TPMT^{LA}$ patient. Illustration made in GraphPad Prizm

less TITP to TIMP resulting in higher levels of MeTITP. We speculate that these will add to the pools of methylated metabolites, inhibiting the purine de novo synthesis and leading to lower levels of endogenous purines to compete with TGN to be incorporated into DNA, as also suggested by Stocco et al. [8, 10]. Still, the difference in mDNA-TG levels observed for the ITPA genotype in this study is not explained by neither mEry-TGN levels nor mEry-MeMP levels alone. We speculate that the explanation could be found in the trends of $ITPA^{LA}$ patients having higher levels of mEry-TGN and mEry-MeMP metabolites; on their own the trends are too small to reach statistical significance, but their joint effect may result in the higher levels of mDNA-TG observed in $ITPA^{LA}$ patients.

As outlined above, this present study supports that polymorphisms in the genes encoding the enzymes TPMT and ITPA significantly modifies the metabolism of 6MP during

maintenance treatment of childhood ALL resulting in higher levels of mDNA-TG in both $TPMT^{LA}$ and $ITPA^{LA}$ patients.

A shortcoming of this study is that free cytosol 6TGN and methylated metabolite levels are measured in erythrocytes as a surrogate for nucleated cells, including lymphoblasts. This may not accurately reflect the metabolic status of the latter target population, as clinical studies have shown major differences between Ery-TGN and leukocyte cytosol 6TGN levels in the same blood samples [44;45]. This study is also limited by the fact that individual treatment doses and hematological parameters were not collected. The $TPMT^{LA}$ patients are treated with reduced doses as compared to $TPMT^{WT}$ patients to reach equitoxic treatment; but our results indicate that this may not be achieved.

In conclusion, our findings support that measurements of DNA-TG may prove to be a more relevant parameter for monitoring 6MP treatment intensity, since it combines the effects of TGN and MeMP levels.

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Compliance with ethical standards

Conflict of interest The authors of this article declare no conflict of interest.

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