MINIREVIEW

Pharmacogenetic studies of thiopurine methyltransferase genotype-phenotype concordance and effect of methotrexate on thiopurine metabolism

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Abstract

The discovery and implementation of thiopurine methyltransferase (TPMT) pharmacogenetics has been a success story and has reduced the suffering from serious adverse reactions during thiopurine treatment of childhood leukaemia and inflammatory bowel disease. This MiniReview summarizes four studies included in Dr Zimdahl Kahlin's doctoral thesis as well as the current knowledge on this field of research. The genotype-phenotype concordance of TPMT in a cohort of 12 663 individuals with clinically analysed TPMT status is described. Notwithstanding the high concordance, the benefits of combined genotyping and phenotyping for TPMT status determination are discussed. The results from the large cohort also demonstrate that the factors of gender and age affect TPMT enzyme activity. In addition, characterization of four previously undescribed TPMT alleles (TPMT*41, TPMT*42, TPMT*43 and TPMT*44) shows that a defective TPMT enzyme could be caused by several different mechanisms. Moreover, the folate analogue methotrexate (MTX), used in combination with thiopurines during maintenance therapy of childhood leukaemia, affects the metabolism of thiopurines and interacts with TPMT, not only by binding and inhibiting the enzyme activity but also by regulation of its gene expression.

1 | INTRODUCTION

The thiopurine drugs 6-mercaptopurine (6-MP), 6-thioguanine (6-TG) and azathioprine (AZA) were synthesized in the 1950s by Gertrude Elion and George Hitchings.[2] Soon after, 6-MP was successfully implemented in the treatment of acute lymphoblastic leukaemia (ALL), in combination with the folate analogue methotrexate (MTX). These drugs have been used for this purpose until the present day. In addition, thiopurine drugs are used for the treatment of inflammatory bowel disease and rheumatoid arthritis, and after transplant surgery.[3,4]

6-MP is an analogue for endogenous hypoxanthine, and the drugs are metabolized by several enzymes in the purine salvage pathway (Figure 1) to the active thioguanine nucleotides (TGN).[5] Thio-deoxy-GTP is incorporated into DNA [2,6]

Parts of the present MiniReview have been published previously in the PhD thesis of Dr Anna Zimdahl Kahlin.[1]
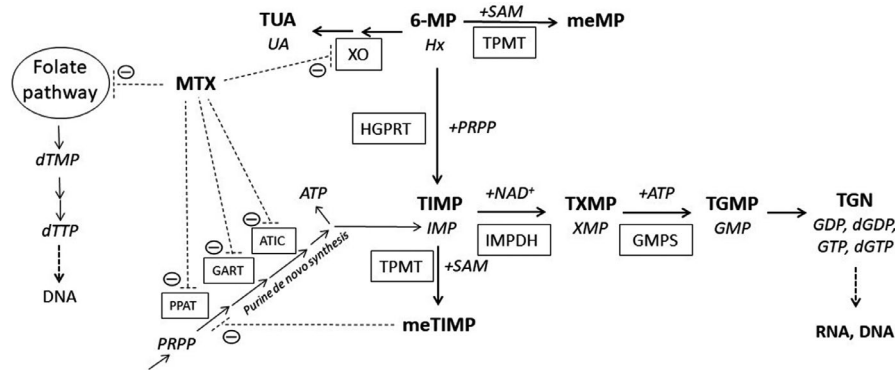
FIGURE 1 Simplified overview of the metabolism of 6-mercaptopurine (6-MP), the purine salvage pathway and the effects of methotrexate (MTX). Hx, hypoxanthine. ENZYMES: XO, xanthine oxidase; TPMT, thiopurine-S-methyltransferase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; PPAT, phosphoribosyl pyrophosphate aminotransferase; GART, glycinamide ribonucleotide transferase; ATIC, aminoimidazole carboxamide ribonucleotide formyltransferase; GMPS, guanosine monophosphate synthetase; IMPDH, inosine monophosphate dehydrogenase. COFACTORS: SAM, S-adenosyl-L-methionine; PRPP, phosphoribosyl pyrophosphate; NAD+, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate, METABOLITES: (T)UA, (thio)uric acid; meMP, methylated mercaptopurine; (T)IMP, (thio) inosine monophosphate; meTIMP, methylated TIMP; (T)XMP, (thio)xanthine monophosphate; (T)GMP, (thio)guanosine monophosphate; TGN (thioguanine nucleotides) includes TdGDP, TdGTP, TGDP and TGTP, thio-(deoxy)-guanosine-mono/di/triphosphate; endogenous nucleotides dGDP, dGTP, GDP and GTP. Reprinted with permission from reference 1.[1]

and activates the mismatch repair system,[7] causing apoptosis and thereby cytotoxicity. The immunosuppressive effect is thought to come from inhibition by thio-GTP of Ras-related C3 botulinum toxin substrate 1, important for T cell activation.[8] 6-MP is also converted into the active metabolite methylated thioinosine monophosphate (meTIMP), which inhibits the purine de novo synthesis pathway thereby causing a lack of endogenous purines as well as increasing the proportion of the TGN incorporated.[2,9]

An important enzyme in thiopurine metabolism is thiopurine methyltransferase (TPMT), which was one of the first and best examples of implemented pharmacogenetics. TPMT polymorphism can predict severe cytotoxic response that is preventable by thiopurine dose reduction.[10-14] TPMT deactivates 6-MP to the non-toxic methylated MP and is also responsible for the last step in the conversion to active meTIMP. About 10% of Caucasians and 2-4% of Asians carry a defective TPMT allele[4] that causes decreased TPMT enzyme activity and thereby increased levels of TGN metabolites. The TPMT alleles *2, *3A and *3C cause up to 95% of all low TPMT enzyme activities,[15] which makes genotyping for only these three positions favourable. However, in Caucasians, TPMT*3A is the most common defective allele,[15,16] and *3A is almost non-existent in East and South-East Asian and African populations in whom *3C is the most common allele.[4,17] Other than thiopurine metabolism, TPMT's natural function in the body is unknown, and deficient TPMT enzyme has so far not been correlated with any disease, although it has been proposed that TPMT has a function as a selenium-detoxification enzyme.[18]

MTX exerts its effect mainly by inhibition of the folate cycle enzyme dihydrofolate reductase (DHFR)[19] (Figure 1).

MTX can also be polyglutamated with up to seven glutamic acids (MTXPG). In these forms, the affinity for DHFR is stronger and the polyglutamates will in addition inhibit several enzymes involved in purine de novo synthesis. Furthermore, MTX is also known to inhibit the enzyme xanthine oxidase (XO), which deactivates 6-MP to thiouric acid (TUA); cotreatment with MTX therefore increases the bioavailability of 6-MP.[19,20] Despite the long and successful use of the combination of 6-MP and MTX during ALL treatment, the mechanism of this beneficial combination is still not fully understood.

This MiniReview is based on Zimdahl Kahlin's doctoral thesis.[1] Using a large cohort of clinical samples from nearly 16 000 individuals with requested TPMT status before or during thiopurine treatment, the objectives were to investigate concordance between TPMT genotyping and phenotyping and, in samples where TPMT genotype and phenotype was not in concordance, to describe and characterize previously uncharacterized TPMT alleles found by extended sequencing. In addition, the effect of MTX on the TPMT enzyme and thiopurine metabolism was investigated.

2 | FINDINGS

2.1 | Descriptions of TPMT enzyme activity and concordance of TPMT genotyping and phenotyping

In a large cohort available from the Clinical Pharmacology Division, Department of Medicine and Health Sciences, Linköping University, that included 15 968 individuals with TPMT status requested due to thiopurine treatment from year

2000 to 2015, the TPMT enzyme activity ranged from 0 to 40.8 U/mL packed red blood cells (pRBC).[21] Of all individuals, 88% showed TPMT enzyme activity within the normal range (≥9.0 U/mL pRBC), 11.6% had intermediate activity, and 0.4% had low activity (<2.5 U/mL pRBC). No distinct group of very high TPMT enzyme activity was identified in the material, previously identified by others as a separate group concerning TPMT enzyme activity level.[16] It has also been discussed that increased thiopurine doses will be favourable for these individuals.[22,23] So far, no established ultra-rapid TPMT allele or duplication of the TPMT gene has been identified to be of significance for tolerance of thiopurine treatment; however, variable tandem repeats (VNTRs) in the TPMT promoter have been described to affect the TPMT gene transcription levels.[24-27]

Of the diagnoses stated in the letters of referral, diagnoses from gastroenterological clinics were responsible for most (71%) of the TPMT status requests to the laboratory (Figure 2). Concordance of genotyping and phenotyping in 12 663 individuals (using data from both methods available) was 94.5% (Figure 3). The concordance rate of individuals with high TPMT enzyme activity detected as wild type by genotyping was 99.1%, while the intermediate metabolizers with heterozygous genotype had a lower concordance rate of 64.4% between genotype and phenotype. In a summary of genotype and phenotype concordance studies, Schaeffler et al[16] reported an overall concordance of 76-100% (n = 21-1214 individuals); in most of the studies, the concordance in the intermediate metabolizer group was lower.

2.2 | Findings of extended sequencing

Of 11 443 individuals genotyped as TPMT*1/*1 (wild type, wt) using the clinical routine genotyping method (analysing

for the three most common TPMT alleles [*2, *3A and *3C]), 579 had a TPMT enzyme activity lower than expected for the found genotype[21] (Figure 3). For these individuals, a second blood sample was requested, but not always obtained for re-analysis of the enzyme activity. A new sample was obtained from 310 individuals, and of those, 82 results were below 9 U/mL pRBC and were thus still considered to be discrepant according to the genotype result. Extended sequencing was performed on these samples, and 10 rare or novel TPMT alleles were found. By combining genotyping and phenotyping results and further investigation of discrepant samples, 15 individuals (of 12 663, 0.1%) were identified as carriers of a rare or novel TPMT allele. These would not have been detected if only genotyping of the three most common SNP positions had been performed. These results once again emphasize the importance of combined genotyping and phenotyping in clinical routine TPMT status determination. The allele frequencies of all 12 663 individuals are visualized in Table 1. The TPMT enzyme activity plotted in groups according to genotype showed a trimodal distribution with overlaps between the subgroups (Figure 4). After repeated measurements and extended sequencing, 90.3% were genotyped as TPMT*1/*1; 9.2% were carriers of one defective TPMT allele and 0.5% were carriers of two defective TPMT alleles. The overall concordance increased to 96.4%[21] after inclusion of the extended analysis of genotype and phenotype.

2.3 | Characterization of TPMT alleles *41-*44

Of the 15 identified rare or novel TPMT alleles,[21] three had not previously been characterized or registered by the TPMT Nomenclature Committee: TPMT*42, TPMT*43 and TPMT*44 (Table 2). In addition, a novel TPMT allele, which
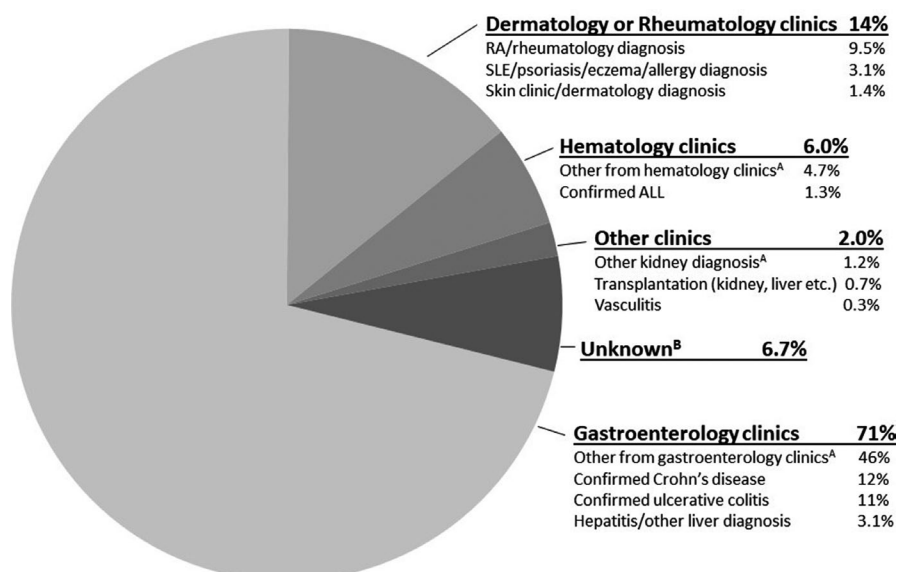

Dermatology or Rheumatology clinics	14%
RA/rheumatology diagnosis	9.5%
SLE/psoriasis/eczema/allergy diagnosis	3.1%
Skin clinic/dermatology diagnosis	1.4%

Hematology clinics	6.0%
Other from hematology clinics[A]	4.7%
Confirmed ALL	1.3%

Other clinics	2.0%
Other kidney diagnosis[A]	1.2%
Transplantation (kidney, liver etc.)	0.7%
Vasculitis	0.3%

Unknown[B]	6.7%

Gastroenterology clinics	71%
Other from gastroenterology clinics[A]	46%
Confirmed Crohn's disease	12%
Confirmed ulcerative colitis	11%
Hepatitis/other liver diagnosis	3.1%

FIGURE 2 Referring clinics for TPMT analyses. Diagnoses given in the letter of referral for clinical TPMT status determination requests, n = 15 975. ALL, acute lymphoblastic leukaemia; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus. These data have been published previously in text.[21] [A]Diagnosis not given in the referring letter or undiagnosed patient. [B]Not possible to categorize, for example sent via clinical chemistry laboratories

Concordance genotyping / phenotyping: 94.5 %

n=12,663

		TPMT enzyme activity (U/mL pRBC)		
		n, conditional proportions % (95% CI)		
		High	Intermediate	Low
TPMT genotype	Wildtype	**10,864** 99.1 (98.9-99.2)	**578** 35.4 (33.0-37.7)	**1** 1.6 (0.0-8.8)
	Heterozygous defective	**103** 0.9 (0.8-1.1)	**1,053** 64.4 (62.0-66.7)	**9** 14.8 (7.0-26.2)
	Homozygous defective	**0**	**4** 0.2 (0.1-0.6)	**51** 83.6 (71.9-91.8)

Total: **10,967** (100%) **1,635** (100%) **61** (100%)

FIGURE 3 Concordance of genotyping and phenotyping methods, n = 12 663. When comparing genotype (testing for TPMT*2, TPMT*3A and TPMT*3C) and the first analysed measurement of TPMT enzyme activity in patient samples referred for TPMT status determination, the concordance of the methods (white boxes) was 94.5% (95% CI; 94.1-94.9). However, heterozygous defective carriers had a TPMT enzyme activity of 0-14.0 U/mL pRBC and homozygous defective carriers an activity of 0-5.5 U/mL pRBC. Of individuals classified as wild-type TPMT*1/*1 by genotyping, 5.1% (n = 579) had an intermediate or low level of TPMT enzyme activity. In contrast, 0.9% (n = 103) of the individuals with high TPMT enzyme activity were detected as heterozygous defective. Reprinted from reference 21,[21] © 2019, with permission from Elsevier

TABLE 1 TPMT allele frequencies of 12 663 individuals after extended sequencing of discrepancies. Allele nomenclature according to the TPMT Nomenclature Committee. The TPMT allele data have previously been published as the number of individuals carrying each genotype[21]

TPMT alleles	Allele Frequency (%)	Alleles (n)
*1 (WT)	94.9	24 036
*2	0.08	19
*3A	4.5	1128
*3B	0.02	4
*3C	0.5	124
*9	0.02	4
*14	0.01	2
*15	0.004	1
*23	0.02	4
*31	0.004	1
*42	0.004	1
*43	0.004	1
*44	0.004	1
Total	100	25 326

was found in a patient treated at a hospital in Hong Kong, China, was characterized and named as TPMT*41.[28]

TPMT*41 was found in a patient with intermediate TPMT enzyme activity treated at a rheumatology clinic. It included a nucleotide substitution in c.719A > C (exon 10) causing an amino acid shift of p.Y240S. The common TPMT*3C allele had a substitution in the same position, c.719A > G. However, the amino acid shift of *3C is p.Y240C. TPMT*44 was found in a patient diagnosed with ALL, and the intermediate TPMT enzyme activity was

caused by a nucleotide substitution of c.497A > G in exon 8, which resulted in the amino acid substitution p.Y166C. The TPMT*3C, TPMT*41[28] and TPMT*44[21] variants and TPMT*1 (wt) were produced as recombinant proteins, expressed by *E coli* from a plasmid containing the (modified) human TPMT cDNA. Circular dichroism (CD) was used to analyse the thermal stability, and the thermal melting point (T_m) was obtained for the four proteins (Table 3), where TPMT*1 had the highest T_m and both *3C (p.Y240C) and *41 (p.Y240S) had a lower T_m, indicating decreased thermal stability. When comparing all four variants, recombinant TPMT*44 (p.Y166C) was shown to have the lowest T_m. The decreased thermal stability of TPMT*44 was probably due to disruption of the hydrogen bonding network in the TPMT core involving Y166-D151-Y180, which may result in increased degradation and thereby a shortened protein half-life in vivo.[29] In most of the so far studied TPMT alleles in which SNPs are found within the exons of the TPMT gene, a single amino acid substitution of the TPMT enzyme disturbs the enzyme structure and stability.[29-31] The low TPMT enzyme activity in defective TPMT allele is thereby commonly due to decreased quantities of TPMT enzyme, and for several TPMT alleles, correlation between enzyme levels and activity has been shown.[10,32] Increased aggregation formation and proteolysis are also consequences of TPMT alleles, resulting in low enzyme activity.[33-37]

By using isothermal calorimetry (ITC), the affinity of TPMT for the co-factor SAM was studied in TPMTw*1, TPMT*3C and TPMT*41.[28] The K_D between TPMTw*1 and SAM was determined to be 2.7 μmol/L. For TPMT*3C and TPMT*41, the K_D could not be determined because the signal-to-noise ratio was too high. However, by using the same settings for all three measurements and comparing the results,
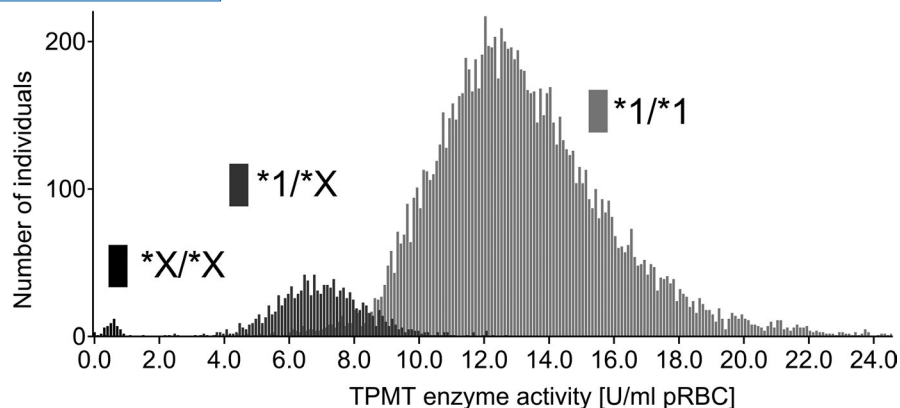
FIGURE 4 Histogram of TPMT enzyme activity of 12 663 individuals presented according to genotyping results. TPMT*1/*1 (wt), TPMT*1/*X (heterozygous defective) and TPMT*X/*X (homozygous defective). Range for intermediate metabolizers was 2.5-8.9 U/mL pRBC which normally corresponds to a heterozygous genotype. Eighteen activity measurements from individuals with TPMT enzyme activity between 25.1 and 40.8 U/mL pRBC were excluded from the figure. Reprinted from reference 21,[21] © 2019, with permission from Elsevier

TABLE 2 Summary of characterized TPMT single nucleotide polymorphisms and their consequences. Reprinted with permission from reference 1[1]

TPMT allele[a]	Diagnosis	Enzyme activity[b]	Nucleotide substitution	Protein consequence	Cause of low enzyme activity
*41	Rheumatological diagnosis	7.7	c.719A > C	p.Y240S	Decreased protein stability and weakened interaction with co-factor SAM
*42	Crohn's disease	7.7-8.4[c]	c.95_96insA	p.K32KfsX58	Insertion of extra A caused nonsense amino acid sequence and premature stop codon
*43	Systemic lupus erythematosus	7.5-8.5	c.243G > T c.262G > A	del. exon V	Deviating splicing pattern, deletion of exon V caused a non-functional enzyme
*44	Acute lymphoblastic leukaemia	.0-2.5	c.497A > G	p.Y166C	Single amino acid substitution decreased the stability of the resulting protein

[a]Published characterization in reference 28 (TPMT*41) and 21 (TPMT*42, TPMT*43, TPMT*44).

[b]U/ml pRBC.

[c]TPMT enzyme activity from relatives with genotype TPMT*1/*42 allele. The patient in whom the allele was found had extremely low enzyme activity caused by two defective alleles, TPMT*3C/*42.

TABLE 3 Thermal denaturation temperatures of recombinant TPMT*1, TPMT p.Y240C, TPMT p.Y240S and TPMT p.Y166C. The measurements were performed in the presence of tenfold molar excess of co-factor SAM

Thermal melting points, T_m	
rTPMT variant	T_m (°C)[a]
*1/ wt	49.4
*3C/ p.Y240C	42.4
*41/ p.Y240S	38.2
*44/ p.Y166C	36.6

[a]T_m has been published previously in references 21 (TPMT*44) and 28 (TPMT*3C and TPMT*41).

it could be concluded that both TPMT*3C and TPMT*41 have a lower affinity for SAM compared to TPMT*1. The loss of enzyme activity at prolonged incubation at 37°C was monitored for recombinant TPMT*1, TPMT*3C and TPMT*41, and the loss of enzyme activity was faster for both TPMT*3C and TPMT*41 compared to TPMT*1, with the most rapid decrease for TPMT*41. This correlates with thermal stability data, showing that TPMT*1 is more stable than TPMT*3C and TPMT*41.

TPMT*42 was found in a patient diagnosed with Crohn's disease who was found to be heterozygous carrier of TPMT*3A, determined by routine genotyping, although having a TPMT enzyme activity in the range of a homozygous defect individual. The TPMT*42 allele includes an insert of an extra A nucleotide in the DNA sequence resulting in a silent mutation (p.K32K). However, the extra nucleotide causes a frameshift in the open reading frame and a nonsense sequence of amino acids thereafter. In addition, a premature stop codon is created in amino acid position 58 which also resulted in a shorter amino acid sequence.[21]

Two nucleotide substitutions were detected in TPMT*43, both in exon 5.[21] When analysing the cDNA product of

FIGURE 5 Pedigrees of A, TPMT*42 and B, TPMT*44. TPMT enzyme activity and genotype after extended sequencing are given for each analysed individual. The patient in whom the allele was first identified is marked with a dotted line. Both figures are reprinted from reference 21,[21] © 2019, with permission from Elsevier
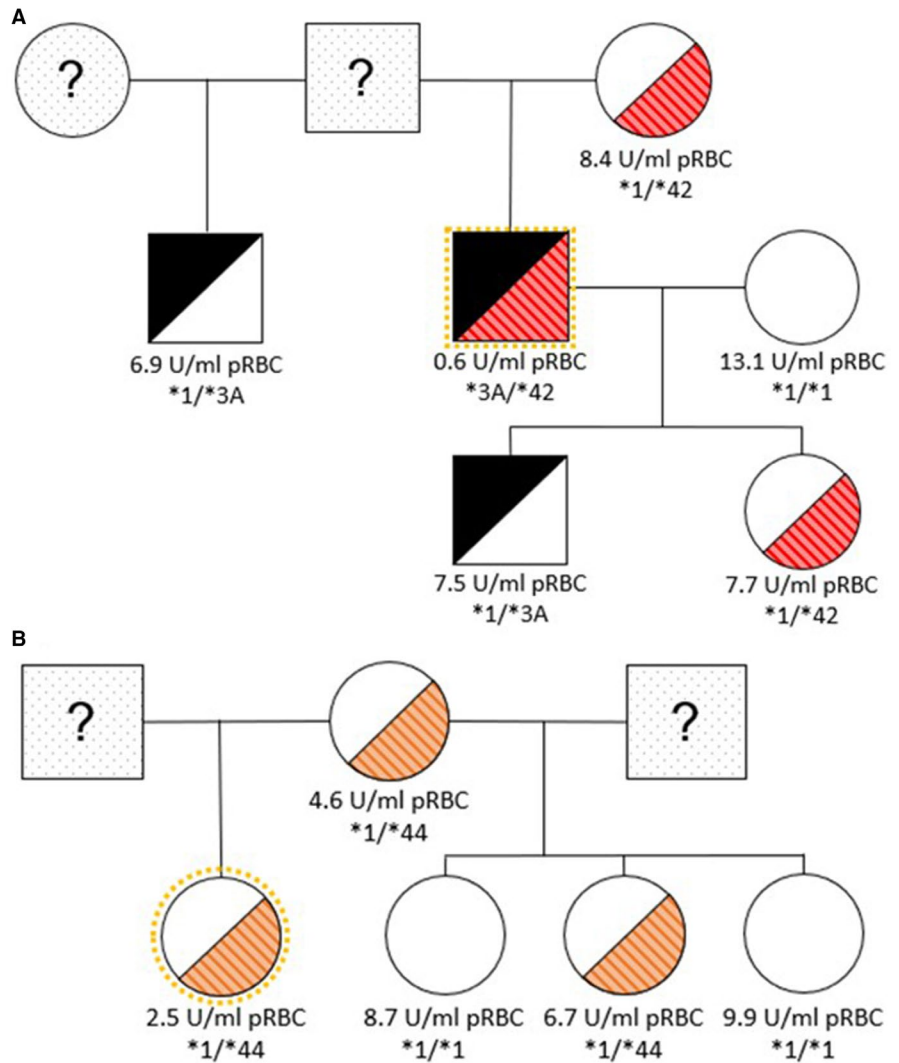

FIGURE 6 TPMT enzyme activity of unexplained discrepancies in the database, n = 451. The boundaries between low/ intermediate and intermediate/high TPMT enzyme activity are marked with dotted lines. Results from extended genotyping are included, and where more than one TPMT enzyme activity result was available, the result of the most recent measurement was used in the figure. Reprinted with permission from reference 1[1]
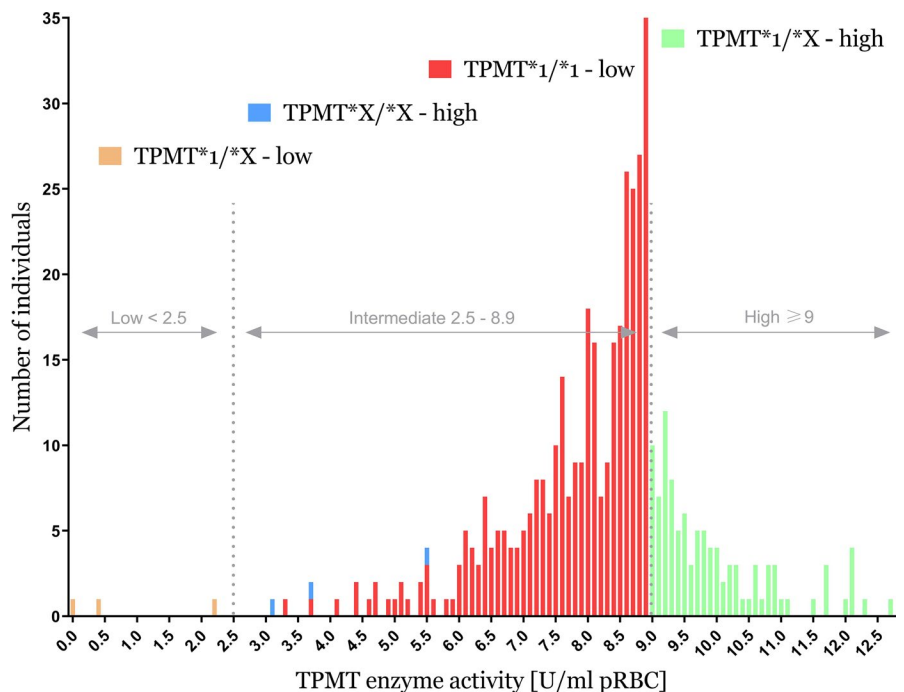
TPMT*43, three bands (instead of the normal one) were visible on the agarose gel. It was possible to sequence two of these. The band with the highest molecular weight gave the wild-type TPMT cDNA sequence, and the second band showed a TPMT cDNA sequence without exon 5. A TPMT enzyme without exon 5 will probably be dysfunctional and explains the intermediate TPMT enzyme activity measured in the patient sample in which TPMT*43 was found to be heterozygously expressed.

It was possible to analyse samples from the relatives of patients for both TPMT*42 and TPMT*44, and both alleles were shown to be inherited in the respective families[21] (Figure 5). Our research group have previously analysed heredity of novel TPMT alleles,[30,38] and in all investigated families, the TPMT alleles were in fact inherited.

2.4 | Unexplained TPMT discrepancies and other factors influencing TPMT enzyme activity

Discrepancies for which we could not find an explanation were found in individuals that were analysed with extended sequencing as well as in individuals where a second sample was requested but not obtained[21] (Figure 6). Recently, two separate large GWASs claimed that no factors other than TPMT genotype affected TPMT enzyme activity.[39,40] However, the fact that all discrepancies could not be explained, together with the large variability within the individuals defined as TPMT*1,[16,39,41] is driving many TPMT researchers to try to find other factors that affect TPMT enzyme activity. Factors available from the cohort, in addition to genotype, were gender and age (n = 12 654, mean ± SD; 39 ± 22 years). Analysing the data statistically using a general linear model, genotype and gender were found to be significant factors affecting TPMT enzyme activity.[21] Males had higher TPMT enzyme activity than females. Interactions were also found between gender and age and between genotype and age. This indicates that, when presenting the results according to genotype and gender, older individuals had higher TPMT enzyme activity than younger individuals (Figure 7). However, the differences are small, and it is doubtful whether the differences are of clinical importance. The small differences between the groups, in conjunction with relatively small sample size in other studies investigating these factors, may explain the variation in the resulting effect of these factors on TPMT activity and thiopurine metabolism.[39,42-47]

In addition, the TPMT enzyme activity during ALL treatment was studied by following 53 children from diagnosis and during treatment using the Nordic Society of Paediatric Haematology (NOPHO) ALL-2000 treatment protocol.[48] At diagnosis, 40% of the individuals with
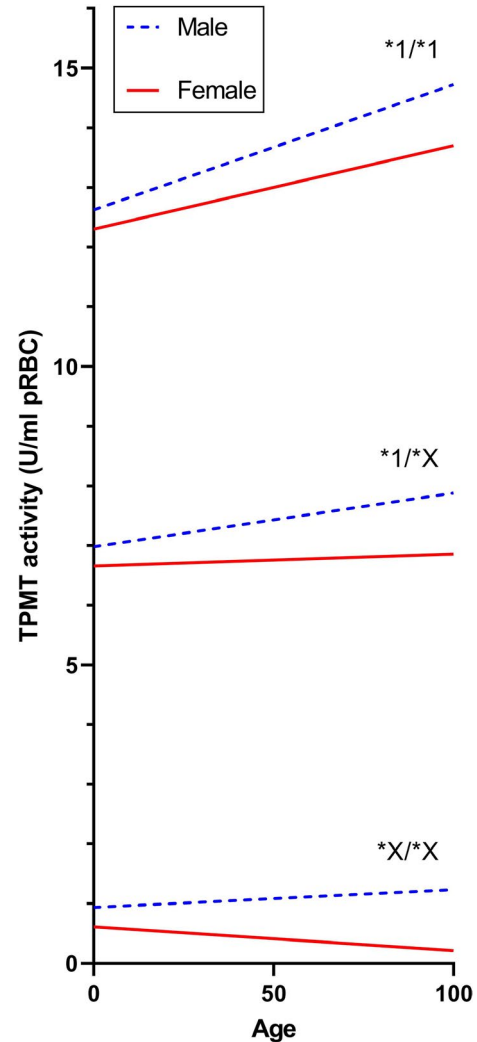

FIGURE 7 The effect of genotype, gender and age on TPMT enzyme activity. Regression lines derived from parameter estimates for TPMT enzyme activity and age for males and females in TPMT genotypes, based on the general linear model. Reprinted from reference 21,[21] © 2019, with permission from Elsevier

genotype TPMT*1/*1 had a TPMT enzyme activity < 9 U/mL pRBC (Figure 8). TPMT enzyme activity increased during treatment and reached its normal value on treatment day 106 from time of diagnosis. The false low TPMT enzyme activity at early ALL treatment was caused by disrupted haematopoiesis and an excess of older red blood cells (RBC), which have lower TPMT enzyme activity than younger RBC.[49] The false low TPMT enzyme activity in this patient group was also seen when selecting the haematology patients (n = 798) in the large cohort.[21] The concordance between genotyping and phenotyping in this group was only 70.7% (compared to 94.5% in the whole cohort). Remarkable in this group was that 78.7% (n = 225) of the individuals with intermediate TPMT enzyme activity had a genotype result of TPMT*1/*1 and were expected to have high TPMT enzyme activity.[21]

The levels of the co-factor SAM have been proposed to have an impact on TPMT enzyme activity by enhancing TPMT protein stability.[50,51] In 1017 donors, the SAM levels ranged from 1.6 to 50.9 nmol/g Hb and individuals with higher SAM levels had higher TPMT enzyme activity.[50] To study the SAM effect on TPMT protein stability, thermal stability measurements (T_m) were performed using recombinant TPMT*1 in the presence of a tenfold and 50-fold molar excess of SAM and in the absence of SAM.[28] The determined T_m without SAM was 47.2°C. In the presence of tenfold and 50-fold SAM, T_m was increased to 49.4°C and 52.5°C, respectively, demonstrating that the thermal stability of the TPMT protein was increased with the addition of SAM. Interestingly, by measuring T_m in the absence and presence of tenfold and 50-fold SAM using recombinant TPMT*3C and TPMT*41, where affinities for SAM were shown to be affected by the respective amino acid substitutions, higher concentrations of SAM were needed to get a significant increase in T_m than for the TPMT*1.[28]

Another factor that may influence the TPMT enzyme activity is recently received blood transfusions, since the patient's TPMT status could be affected by that of the donor.[52] The TPMT gene expression has been shown to be influenced by the thiopurine treatment[25]; however, the TPMT enzyme activity was overall unchanged in a study of thiopurine exposure during 20 weeks of IBD treatment.[53] Except for the established TPMT inhibitor allopurinol, other proposed inhibitors for TPMT are benzoic acid derivates, salicylic acid, diuretics, non-steroid anti-inflammatory drugs and the antibiotic trimethoprim-sulfamethoxazole.[54-59] In addition, variation in the PACSIN2 gene has been correlated to TPMT enzyme activity.[60]

2.5 | The effect of MTX on TPMT and thiopurine metabolism

The two drugs 6-MP and MTX form the cornerstone of the maintenance therapy phase—the longest phase of the ALL treatment protocol. During this phase, 6-MP is combined with weekly oral low-dose MTX (LD-MTX) and infusions with high-dose MTX (HD-MTX) followed by leucovorin rescue.[61] The effect of HD-MTX on TPMT enzyme activity and thiopurine metabolite levels (meTIMP and TGN) in vivo was studied by measuring these parameters before and approximately 66 hours after an HD-MTX infusion in a cohort of children treated for ALL.[48] Both TPMT enzyme activity and TGN levels were decreased after the MTX infusion (Table 4). The in vivo plasma concentration of MTX 23 hours after HD-MTX infusion was 84 µmol/L (median, range 57-102 µmol/L).[48] Plasma concentrations at LD-MTX treatment have been reported to be 0.02-0.2 µmol/L[62] and 1 µmol/L.[63] Consequently, 90 µmol/L MTX was used to simulate HD-MTX treatment and 0.01-0.5 µmol/L MTX was used to simulate the LD-MTX dose in in vitro studies.

A binding curve was obtained by titrating MTX to recombinant TPMT and measuring the fluorescence signal from the five tryptophans in the TPMT protein, and K_D was determined to be 24 µmol/L MTX.[48] In the presence of MTX (1, 50, 100 and 200 µmol/L MTX), recombinant TPMT enzyme activity was changed by 103, 85, 35 and 16%, respectively, compared to TPMT activity in the presence of a corresponding solvent.[48]

Incubations of T lymphoblastic MOLT4 cells with 90 µmol/L MTX for 24, 48 and 72 hours resulted in decreased TPMT enzyme activity after 48 and 72 hours[48] (Figure 9). Incubation of both MOLT4 cells and B-lymphoblastic
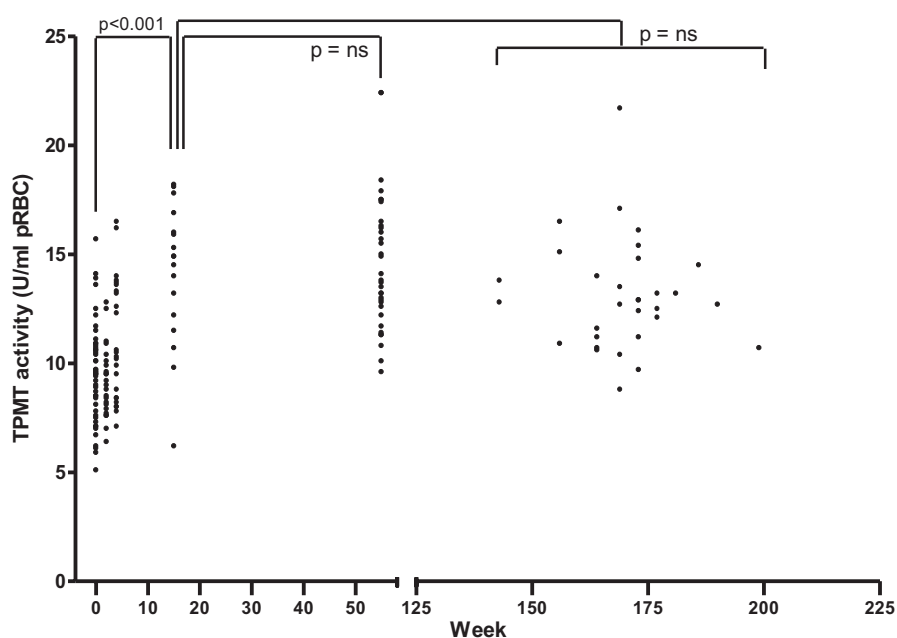

FIGURE 8 TPMT enzyme activity during ALL treatment. TPMT enzyme activity was measured in 47 children with genotype TPMT*1/1 during ALL treatment. Sampling was performed at diagnosis, days 15 and 29 (induction phase), day 106 (consolidation phase), approximately day 400 (maintenance therapy) and once after conclusion of treatment. ns = non-significant differences between time-points. Reprinted with permission from Springer Nature: EJCP reference 48,[48] © 2013

	0 h (pre-infusion)	Percentage change at approx. 66 h post-MTX infusion	Change vs pre-infusion values
TPMT	13.4[a] (11.4-16.2)	−9.2% (−13.0 to + 0.65)	$P = .013$, n = 21
TGN	111.5[b] (79-271)	−18.1% (−26 to − 14.6)	$P = .006$, n = 15
meTIMP	1609[b] (427-3900)	0% (−20.5 to + 36)	$P = .917$, n = 15

TABLE 4 Results of the effect of MTX on TPMT enzyme activity and metabolite levels after an HD-MTX infusion in vivo in children treated for ALL. Reprinted by permission from Springer Nature: EJCP reference 48,[48] © 2013

[a]Unit: U/mL pRBC.

[b]Unit: pmol/8×10^8 RBC. Metabolite levels are normalized to 6-MP dose (mg/d).

NALM6 cells with LD-MTX (0.01-0.5 µmol/L MTX according to clinical concentrations) alone or in combination with 6-MP at a fixed concentration of 2.2 or 1.7 µmol/L (according to IC50), respectively, for 16 or 26 hours, and after removing dead cells at the end of incubation, showed no effect on TPMT enzyme activity (authors' unpublished data; methods are described in Supplementals S1 and S2). Interestingly, TPMT enzyme activity was higher in NALM6 cells than in MOLT4 cells (27.7 and 9.2 cpm/µg total protein after 2 hours of incubation, respectively). TPMT gene expression was increased after 26 hours of incubation using the higher concentrations of MTX (Figure 10A,B). There were no significant

changes in TPMT gene expression after 16 hours of incubation with MTX with or without 6-MP.

The effect of MTX on the active thiopurine metabolites meTIMP and TGN as well as the intermediate metabolites TIMP and TXMP (Figure 1) was studied after 16 and 26 hours of incubation (Figure 10C–F). The metabolite levels were tenfold higher after 26 hours compared to 16 hours of incubation, indicating that the cells were alive and metabolizing the drugs. NALM6 cells produced higher levels of metabolites than MOLT4. At the lowest concentration of 0.01 µmol/L MTX, the metabolite levels appeared to be unaffected. In the presence of 0.05 µmol/L or higher concentrations of MTX, TGN levels were decreased. TIMP and TXMP levels decreased in a pattern similar to that for TGN in both cell lines. However, in NALM6 cells, there was a dramatic decrease in metabolite levels in the presence of 0.05 µmol/L or higher concentrations of MTX, while with increasing MTX concentrations in MOLT4, there appeared to be a more stepwise decrease (Figure 10C–F). For meTIMP levels, the pattern deviated from that for other metabolites and they appear to be less affected by the addition of MTX. This could potentially be due to meMP, which is indistinguishable from meTIMP after the acid hydrolysis step used in this method (Figure S1).

Even though the combination of 6-MP and MTX is strongly beneficial and has been used for decades, the reason for the positive effect is not yet fully understood.

Decreased TGN levels after MTX treatment, as described in the study results above, have also been reported from other studies[64-66]; however, the present study provides additional detail on the other metabolites meTIMP, TIMP and TXMP. The metabolite findings cannot be explained by the established mechanisms of MTX known today: except the already mentioned inhibition of XO, MTX treatment is known to accumulate PRPP (co-substrate to HGPRT) and decrease the available levels of ATP (co-substrate to GMPS) due to inhibited PDNS.[6,62,64,67,68] The described results instead indicate that the combination of 6-MP and MTX has other advantageous effects that require further studies to be fully understood. This hypothesis is further supported by the results of Zaza et al who compared gene expression in ALL cells after 6-MP treatment and 6-MP + MTX treatment, and demonstrated that these two treatments affected the expression of totally different genes.[64]
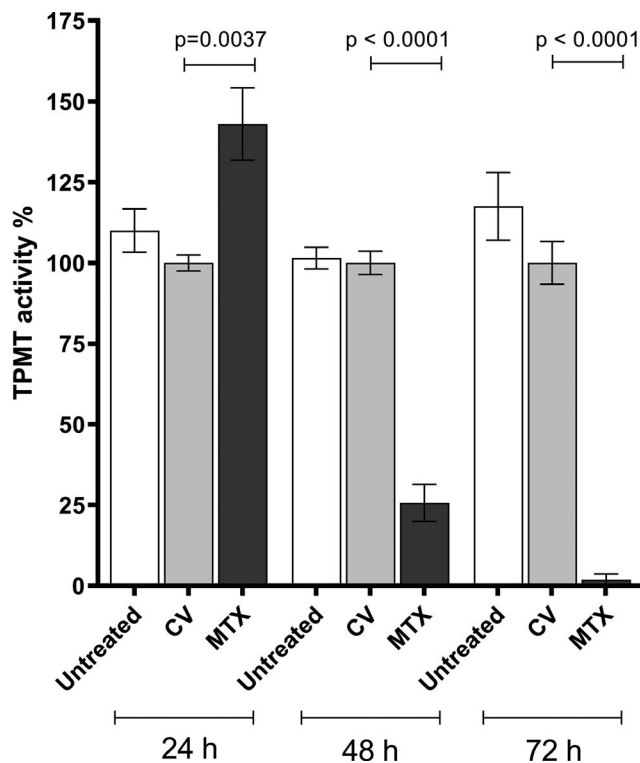

FIGURE 9 TPMT enzyme activity in T lymphoblastic MOLT4 cells incubated at 24, 48 or 72 h with the addition of MTX at a final concentration of 90 µmol/L (HD-MTX) in the medium. Results are shown as a mean percentage of resp. control vehicle (CV) ± SEM (n = 6). After 24-h incubation, TPMT enzyme activity was increased; after 48 and 72 h, only 26 and 1.8% of the corresponding CV TPMT enzyme activity remained, respectively. No significant difference between untreated and CV (NaOH) was detected. Reprinted with permission from Springer Nature: EJCP reference 48,[48] © 2013
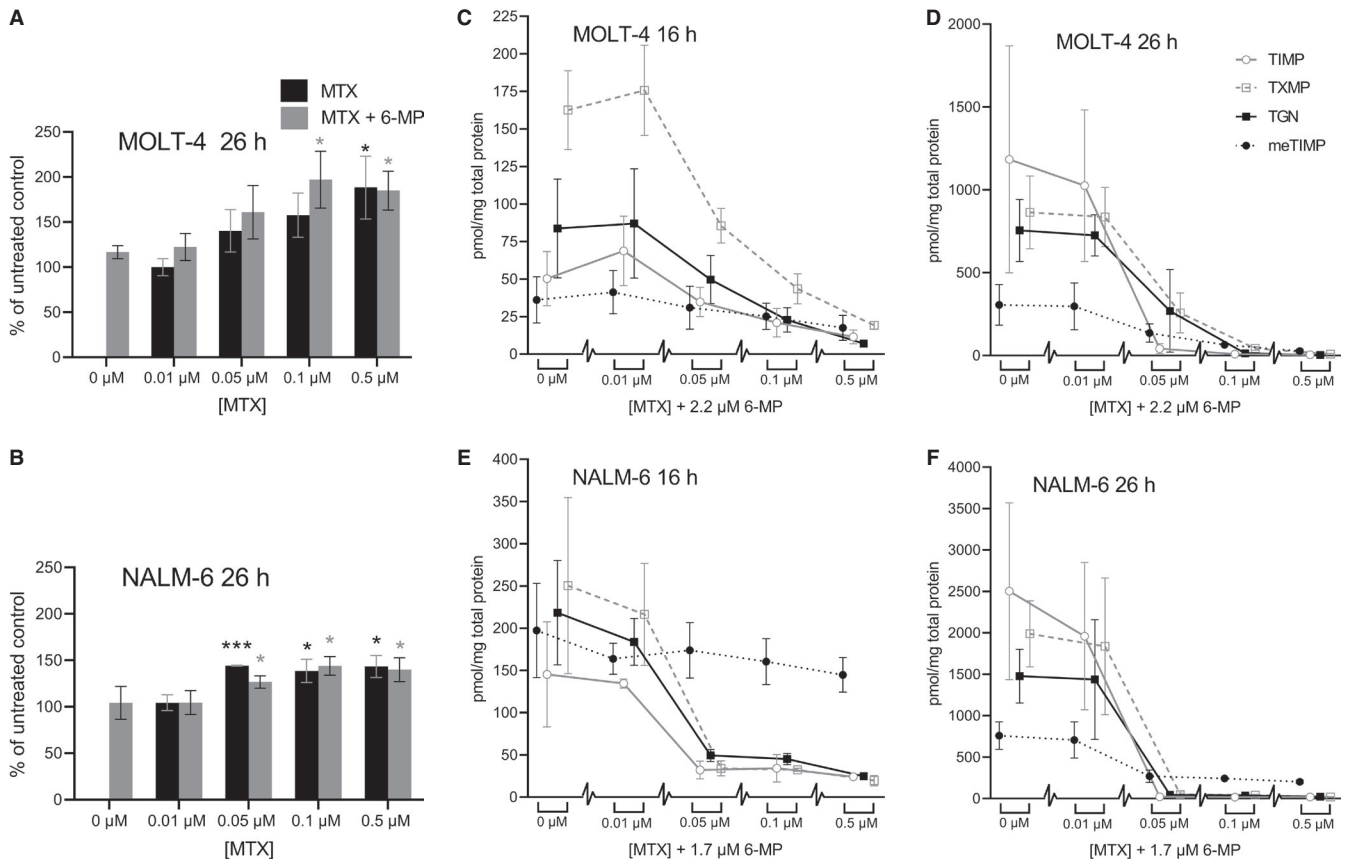
FIGURE 10 The effect of MTX on TPMT gene expression and thiopurine metabolite levels. A–B). Gene expression of TPMT in MOLT4 cells and NALM6 cells after incubation with various concentrations of methotrexate (MTX) (black) or in combination (grey) with 2.2 μmol/L or 1.7 μmol/L 6-mercaptopurine (6-MP), resp. Results are visualized as % of control vehicle (CV, cells treated with only NaOH, set to 100%) and mean ± SD. Stars refer to significant differences compared to CV. N = 3 A, Levels after 26-h incubation in MOLT4 cells. B, Levels after 26-h incubation in NALM6 cells. C–F, Thiopurine metabolite levels in MOLT4 cells and NALM6 cells after incubation with 2.2 or 1.7 μmol/L 6-mercaptopurine (6-MP), respectively, and various concentrations of methotrexate (MTX). TGN (■) thioguanine nucleotides, meTIMP (●) methyl thioinosine monophosphate, TXMP (□) thioxanthine monophosphate, TIMP (○) thioinosine monophosphate. Results are visualized as pmol/mg total protein, mean ± SD. N = 3 unless otherwise stated. C, Levels after 16-h incubation in MOLT4 cells. D, Levels after 26-h incubation in MOLT4 cells. E, Levels after 16-h incubation in NALM6 cells. F, Levels after 26-h incubation in NALM6 cells. Reprinted with permission from reference 1[1]

If and why decreased TGN levels are advantageous for the clinical effect or are a by-product of other favourable mechanisms is unclear. In either case, TGN levels appear to recover rapidly after HD-MTX treatment.[66] Differences in treatment response between B and T cell ALL have been reported to be due to the cell's ability to form MTXPG dependent on the levels of FGPS and DHFR and thereby the amount of MTX needed to inhibit the folate cycle.[69] The difference between LD-MTX and HD-MTX treatment has been suggested to be due to MTXPG formation and thereby inhibition of PDNS.[62,66] The above-described results indicate that an additional difference between LD-MTX and HD-MTX could be the MTX's ability to bind to TPMT and inhibit its enzyme activity. However, this mechanism cannot explain the effects of MTX on the thiopurine metabolism described above, in particular concerning the decrease of TIMP, TXMP and TGN.

MTX inhibition of HGPRT has previously been reported[70] and may deserve further attention in the future.

3 | CONCLUSIONS AND FURTHER PERSPECTIVES

The overall concordance between TPMT genotyping and phenotyping methods in 12 663 individuals with requested TPMT status for clinical purposes was 94.5%. However, it is important to note that when only the genotyping method was used to determine the TPMT status, with detection of the three most common variant alleles, the genotype of 15 individuals (of 12 663, 0.1%) would be reported incorrectly. Therefore, even though the concordance of the methods is high, combined genotyping and phenotyping is recommended

in clinical TPMT status determination because erroneous determination could result in patients being treated with the wrong dose of thiopurines.

With the increasing use of whole-exome and whole-genome sequencing for genotyping, the number of reported variants in the TPMT gene is increasing. However, without characterization of the variant alleles' effect on TPMT enzyme activity, dosage recommendations cannot be formulated. The described results (Table 2) show that the mechanisms of defective TPMT alleles differ and that the SNPs could affect TPMT function at both the mRNA splicing and transcription level, as well as at the amino acid and protein structure level. In addition, factors such as haematological disease, SAM levels, gender and age or drug interactions affect the levels of TPMT enzyme activity. Even so, many cases of unexplained discrepancies between genotype and phenotype exist and many cytotoxic events during thiopurine treatment cannot be explained only by altered TPMT enzyme activity. Recently, the impact of NUDT15 function in thiopurine metabolism and cytotoxicity was described and NUDT15 testing is currently being implemented in thiopurine treatment of childhood ALL. In contrast to TPMT, NUDT15 polymorphisms are more common in East Asian populations (allele frequency 10%) than in Caucasians (0.2-2%).[12,71,72] Recently, variations in the CRIM1 gene have been described to be an additional predictive factor for thiopurine toxicity.[73]

Recently, methods to measure the levels of DNA-incorporated TGN in WBC have been reported. Interestingly, erythrocyte TGN and white blood cell DNA-TGN levels correlate at lower erythrocyte TGN levels, but then reach a plateau, and at a higher level, higher erythrocyte TGN does not cause higher DNA-TGN levels.[74] Measuring DNA-TGN may be a more sensitive tool for treatment management and a factor with better concordance to the clinical effect of thiopurine treatment, measured in the target cells.

The combination of 6-MP and MTX is the cornerstone in the ALL maintenance treatment protocol. Our studies elucidate the MTX effect on thiopurine metabolism and on the TPMT enzyme in particular. MTX binds to recombinant TPMT and inhibits its enzyme activity.[48] In MOLT4 and NALM6 cell lines, the TPMT enzyme activity was unaffected at LD-MTX; however, at higher MTX concentrations and after 26 hours of incubation, gene expression increased. Using HD-MTX doses, the TPMT enzyme activity first increased after 24 hours and then decreased after 48 and 72 hours of incubation. In vivo, TPMT enzyme activity decreased after HD-MTX infusion.[48]

In addition, thiopurine metabolism was influenced by the combined treatment of 6-MP and MTX. In vivo TGN levels decreased, whereas meTIMP levels were unaffected after HD-MTX.[48] In MOLT4 and NALM6 cells, the metabolites TGN, TIMP and TXMP decreased dramatically with increasing MTX concentrations. Similarly to the in vivo

data, meTIMP was the least affected metabolite in cell lines. However, the results of the intermediate metabolites TIMP and TXMP challenge the currently established mechanisms of MTX. Apparently, the effect of MTX on the TPMT enzyme appears to be dose- and time-dependent. Despite the successful combination of MTX and 6-MP today, increased knowledge on the MTX effect on thiopurine metabolism may further improve the combined use of these drugs. Future in vitro studies should include measurement of DNA-TGN, which also could shed light on the effect of MTX on the thiopurine metabolism.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.