Polymorphic Variation in TPMT Is the Principal Determinant of TPMT Phenotype: A Meta-Analysis of Three Genome-Wide Association Studies

R Tamm¹,², R Mügi², R Tremmel³, S Winter³, E Mihailov², A Smid⁴, A Möricke⁵, K Klein³, M Schrappe⁶, M Stanulla⁶, R Houlston⁷, R Weinhilboun⁸, Irena Mlinarič Raščan⁴, A Metspalu¹,², L Milani², M Schwab³,⁹,¹⁰ and E Schaeffeler³

Thiopurine-related hematotoxicity in pediatric acute lymphoblastic leukemia (ALL) and inflammatory bowel diseases has been linked to genetically defined variability in thiopurine S-methyltransferase (TPMT) activity. While gene testing of TPMT is being clinically implemented, it is unclear if additional genetic variation influences TPMT activity with consequences for thiopurine-related toxicity. To examine this possibility, we performed a genome-wide association study (GWAS) of red blood cell TPMT activity in 844 Estonian individuals and 245 pediatric ALL cases. Additionally, we correlated genome-wide genotypes to human hepatic TPMT activity in 123 samples. Only genetic variants mapping to chromosome 6, including the TPMT gene region, were significantly associated with TPMT activity ($P < 5.0 \times 10^{-8}$) in each of the three GWAS and a joint meta-analysis of 1,212 cases (top hit $P = 1.2 \times 10^{-72}$). This finding is consistent with TPMT genotype being the primary determinant of TPMT activity, reinforcing the rationale for genetic testing of TPMT alleles in routine clinical practice to individualize mercaptopurine dosage.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC? ✓ Clinically, it is well known that patients with TPMT wild-type are still at risk for thiopurine-related hematotoxicity even after consideration of nongenetic factors. Prediction of TPMT phenotype is currently exclusively based on measurement of TPMT activity and/or genotyping.

WHAT QUESTION DID THIS STUDY ADDRESS? ✓ The largest meta-analysis of GWAS in a population cohort, pediatric ALL patients and human liver samples, was designed to assess whether TPMT genetics exclusively determines the marked interindividual variability of TPMT activity or if other genes contribute substantially.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE ✓ Only genetic variants mapping to the TPMT gene region at chromosome 6 are the primary determinants of TPMT activity in human. Notably, this could be demonstrated for human liver as the predominant site of thiopurine metabolism.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE ✓ Genetic testing of TPMT is sufficient to predict patient’s correct phenotype prior to thiopurine therapy promoting the clinical implementation process of preemptive testing. Future studies are warranted to identify so-far unidentified TPMT-independent factors to explain thiopurine-related hematotoxicity.

Mercaptopurine (MP) is important for maintenance therapy of childhood acute lymphoblastic leukemia (ALL).¹ The S-methylation of MP is catalyzed by the cytosolic enzyme thiopurine S-methyltransferase (TPMT). Over 30 polymorphisms in TPMT have been documented that have an effect on the enzymatic activity of TPMT.²–⁴ The most common alleles seen in most ethnicities are TPMT*3C (719A>G) and TPMT*3A (460G>A and 719A>G). There is a body of evidence that functional polymorphisms in TPMT are a determinant of MP-related hematotoxicity,⁵ with myelosuppression seen in patients with

¹Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia; ²Estonian Genome Center, University of Tartu, Tartu, Estonia; ³Dr Margaret Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany, and University of Tuebingen, Germany; ⁴Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia; ⁵Department of Paeiatrics, University Hospital Schleswig-Holstein, Kiel, Germany; ⁶Pediatric Hematology and Oncology, Hannover Medical School, Hannover, Germany; ⁷Division of Genetics and Epidemiology, Institute of Cancer Research, Sutton, UK; ⁸Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota, USA; ⁹Department of Clinical Pharmacology, University Hospital Tuebingen, Tuebingen, Germany; ¹⁰Department of Pharmacy and Biochemistry, University of Tuebingen, Tuebingen, Germany. Correspondence: E Schaeffeler (elke.schaeffeler@ikp-stuttgart.de) and M Schwab (matthias.schwab@ikp-stuttgart.de).

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TPMT deficiency receiving standard MP therapy.\textsuperscript{6,7} Furthermore, intermediate TPMT activity, only partly explained by TPMT heterozygosity present in 10% of individuals,\textsuperscript{8} is also associated with an increased risk of toxicity.\textsuperscript{5,6} Recently, a prospective clinical trial confirmed a lower incidence of leukopenia in pre-treatment TPMT heterozygous-tested patients with inflammatory bowel disease (IBD) after dose-adjusted thiopurine therapy.\textsuperscript{9}

While TPMT genotyping is advocated clinically by international guidelines using dosage individualization to limit MP-related toxicity,\textsuperscript{5,10} TPMT activity shows considerable variability even in those with low-risk TPMT genotypes, presumably as a consequence of as yet unidentified additional genetic or nongenetic factors.\textsuperscript{11} Although several studies have been performed trying to identify additional factors apart from the TPMT gene that influence TPMT activity,\textsuperscript{12–14} thus far the genome-wide association study (GWAS) approach has not been used to comprehensively investigate the relationship between constitutional genotype and TPMT activity in red blood cells (RBCs). Moreover, systematic data regarding the correlation between TPMT genotype and TPMT expression or function in human liver, as the predominant site of thiopurine metabolism, is still missing.

To address this deficiency we have conducted the so-far largest meta-analysis of three independent GWAS of TPMT activity, comprising 844 individuals of the Estonian population cohort and 245 pediatric ALL cases, as well as 123 human livers.

**RESULTS**

**GWAS in the Estonian population cohort**

We first investigated the relationship between genome-wide genotypes and TPMT activity in individuals from the Estonian cohort. TPMT activity measured in RBC of 844 individuals (414 males, 430 females; age range 18–87 years) of the Estonian cohort showed a bimodal distribution and none of the cohort were shown to be TPMT deficient (Figure 1a). Genome-wide single nucleotide polymorphism (SNP) genotyping was performed using microarrays. After quality control, European ancestry of all individuals was confirmed using 1000 Genomes as reference, as outlined in detail in the [Supplementary Methods](#). Further genetic variants, not covered by the microarrays, were imputed using 1000 Genomes as reference (details in Methods and Supplementary Methods).

Finally, 8,617,769 genotyped and imputed genetic markers were included in the subsequent genome-wide association analysis, using sex and age as covariates. As shown in the Manhattan plot (Figure 1b) and in the regional association plot (Figure 1c), the most significant association, by several orders of magnitude, was provided by genetic variants mapping to TPMT gene region at 6p22.3 in our genome-wide association analysis. In total, 169 genetic variants on chromosome 6 were significant at the genome-wide threshold ($P < 5.0 \times 10^{-8}$, Table S1) with a minimal $P$-value of $P = 2.73 \times 10^{-76}$. In addition, the most important TPMT alleles (TPMT*3A and *3C) were genotyped separately using real-time polymerase chain reaction (PCR) (Taq-Man) technology. Figure 1d shows the association of TPMT*3 alleles with TPMT activity, indicating that TPMT activity was significantly lower in heterozygous individuals (median 21, range 13–33 nmol 6-MTG $\times g^{-1} \text{Hb} \times h^{-1}$) compared to wildtype carriers (median 40, range 18–64 nmol 6-MTG $\times g^{-1} \text{Hb} \times h^{-1}$). Further GWAS analyses conditioning on the most frequent nonfunctional TPMT alleles (TPMT*3A and *3C) were performed to identify further genetic factors apart from TPMT. However, no significantly associated variants ($P < 5.0 \times 10^{-8}$) with TPMT activity in our cohort were found (Figure 1e). In addition, we investigated the contribution of SNP–SNP interactions, but could not identify any further significantly contributing variants (Figure S2) after correction for multiple testing. Moreover, previously identified candidate SNPs were only negligibly related to TPMT activity in the Estonian cohort based on our GWAS data (Table 1).

**GWAS in ALL patients**

Next we investigated whether the same relationship between genotypes and TPMT activity was shown in pediatric ALL. The ALL study cohort consists of 245 children of European ancestry (Figure S1) of the ALL Berlin-Frankfurt-Münster (BFM) trials,\textsuperscript{15} none of whom had been transfused within 3 months prior to blood sampling and samples were obtained before ALL maintenance therapy. The distribution of TPMT activity is depicted in Figure 2a. Two of the cases were previously shown to be TPMT-deficient.\textsuperscript{7,16} Next, a GWAS including sex and age as covariates was performed comprising 8,224,478 genotyped and imputed markers. As shown in the Manhattan plot and also in the regional association plot displayed in Figure 2b–c, only genetic variants within the TPMT gene region were significantly associated with TPMT activity. All other gene regions were only negligibly associated with TPMT activity. A list of all SNPs that were genome-wide significant ($P < 5.0 \times 10^{-8}$) is given in Table S2. The common variant 719A>G (rs1142345), which is part of the nonfunctional TPMT*3A and TPMT*3C alleles, displayed one of the lowest $P$-values (Figure 2c). Figure 2d shows the association of TPMT*3A-C alleles with TPMT activity. In addition, we previously genotyped the most important nonfunctional TPMT alleles using either TaqMan technology or matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and thereby identified one deficient patient as a carrier of the TPMT*3A/*11 genotype.\textsuperscript{7} TPMT activity in homozygous carriers of the TPMT*3A allele or in compound heterozygous carriers of two nonfunctional TPMT alleles (TPMT*3A/*11) displayed an enzyme activity $\leq$ 2 nmol 6-MTG $\times g^{-1} \text{Hb} \times h^{-1}$. TPMT wildtype carriers showed a significantly higher TPMT activity (median 30, range 9–69 6-MTG $\times g^{-1} \text{Hb} \times h^{-1}$) compared to heterozygous carriers of nonfunctional TPMT alleles (median 21, range 9–33 6-MTG $\times g^{-1} \text{Hb} \times h^{-1}$). Additional analyses conditioning on the TPMT*3A and *3C alleles revealed no significantly associated variants ($P < 5.0 \times 10^{-8}$) with TPMT activity in the ALL cohort (Figure 2e). Further, investigation of SNP–SNP interactions suggests lack of interaction effects between the investigated variants (Figure S2) after correction for multiple testing. In line with these observations, previously
Figure 1. (a) Histogram of TPMT activity in the Estonian cohort (n = 844 individuals included in the GWAS), indicating a bimodal distribution; none of the individuals were shown to be TPMT-deficient. (b) Results of genome-wide association analysis of TPMT activity in the Estonian cohort, using age and sex as covariates. The Manhattan plot shows the association P-values (−log_{10}-transformed) of genetic variants across chromosomes 1–22. Significantly associated genetic variants (P < 5.0 × 10^{-8}) are marked in green. (c) The regional association plot highlights the genomic region containing TPMT. Recombination rates and linkage disequilibrium estimates (r²) of variants with the TPMT variant 719A>G (rs1142345) are displayed. (d) Association of TPMT activity with common nonfunctional TPMT alleles (TPMT*3A-*3C). (e) Manhattan plot showing association P-values (−log_{10}-transformed) from conditional association analyses considering the most common TPMT alleles (TPMT*3A and TPMT*3C) and correcting for age and sex.
identified candidate SNPs were not associated with TPMT activity in the ALL cohort based on our GWAS data (Table 1).

**GWAS in human liver**

Following from these observations, we sought to identify genetic markers influencing TPMT activity in human liver, the most important thiopurine-metabolizing tissue. TPMT activity, determined in liver cytosol of 124 individuals, showed a bimodal distribution (Figure 3a) and none of the samples was TPMT-deficient. Subsequently, we performed a genome-wide association analysis corrected for age and sex including 7,481,872 genotyped and imputed markers. All individuals included in the GWAS were from European ancestry (Figure S1). As shown in the Manhattan plot and also in the regional association plot displayed in Figure 3b,c, only genetic variants within the TPMT region were significantly associated with TPMT activity ($P < 5.0 \times 10^{-8}$) (Table S3).

The TPMT 238G>C, 460G>A, and 719A>G polymorphisms were additionally genotyped by either TaqMan or MALDI-TOF MS technology.17,18 Carriers of TPMT*2 and TPMT*3A alleles showed a reduced hepatic TPMT activity (Figure 3d) (median 1.2, range 1.1–1.9 nmol h$^{-1}$ mg$^{-1}$) compared to TPMT wildtype patients (median 2.7, range 1.5–4.1 nmol h$^{-1}$ mg$^{-1}$). Moreover, by genotyping 22 functionally relevant TPMT alleles using an established and validated MALDI-TOF MS method,18 as well as next-generation sequencing (NGS) of the TPMT coding region, the presence of other functionally relevant TPMT variant alleles could be ruled out. Altogether, results from NGS were in agreement with imputed genotypes (Table S4).

In addition, analyses conditioning on the most frequent non-functional TPMT alleles (TPMT*3A and *3C) revealed no significantly associated variants ($P < 5.0 \times 10^{-8}$) with TPMT activity in human liver (Figure 3e). Subsequent investigation of SNP–SNP interactions suggests lack of interaction effects between the investigated variants (Figure S2) after correction for multiple testing. In addition, previously identified candidate SNPs were not associated with hepatic TPMT activity based on our GWAS data (Table 1).

Systematic data on TPMT expression and function in human liver are still missing and therefore we next correlated cytosolic TPMT activity with TPMT protein levels, determined by immunoblotting. As shown in Figure 4a, a highly significant correlation was observed ($r_i = 0.58, P < 2.2 \times 10^{-16}$) between protein expression and enzyme activity. In contrast, TPMT mRNA expression was not significantly correlated with either TPMT activity or TPMT protein level (Figure 4b,c) in human liver, even after exclusion of TPMT variant cases.

**Joint analyses of GWAS in the Estonian population cohort, ALL patients, and human liver**

Collectively our findings in three independent cohorts indicate that TPMT activity is primarily determined by genetic variation in the TPMT gene region in RBCs as well as in liver tissue. In total, 173 variants with $P < 5.0 \times 10^{-8}$ could be identified in at least one cohort. Of these, 89% were located on chromosome 6 in the genomic region 18.1–18.2Mb, which includes the TPMT gene (Figure 5a). The remaining 11%, which were also located on chromosome 6 (but outside the TPMT region), were only significantly associated with TPMT activity in the Estonian cohort (Figure 5a). Noticeably, no significantly associated variants were found on other chromosomes besides chromosome 6.

In addition to the separate analyses of our cohorts, we performed a joint meta-analysis of all three datasets to account, on the one hand, for potential differences in our cohorts, and on the other hand to increase power and reliability of GWAS analysis. Again, this joint meta-analysis of all three datasets only revealed significantly associated genetic variants on chromosome 6, including the TPMT gene region, with the sentinel marker (rs73726531, $P = 1.2 \times 10^{-72}$, effect size: $-2.2$; Figure 5b, Table S5). As shown in

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**Table 1** Results for previously identified candidate SNPs associated with TPMT activity and/or thiopurine-related toxicity

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Figure 2  (a) Histogram of TPMT activity in the pediatric ALL-cohort (n = 245), indicating two patients with TPMT deficiency. (b) Results of genome-wide association analysis of TPMT activity in the ALL study, using age and sex as covariates. The Manhattan plot shows the association P-values (−log10-transformed) of genetic variants across chromosomes 1-22. Significantly associated genetic variants (P < 5.0 × 10^-8) are marked in green. (c) The regional association plot highlights the genomic region containing TPMT. Recombination rates and linkage disequilibrium estimates (r^2) of variants with the TPMT variant 719A>G (rs1142345) are displayed. (d) Association of TPMT activity with common nonfunctional TPMT alleles (TPMT*2, TPMT*3A-*3C). (e) Manhattan plot showing association P-values (−log10-transformed) from conditional association analyses considering the most common TPMT alleles (TPMT*3A and TPMT*3C) and correcting for age and sex.
Figure 3  (a) Histogram of TPMT activity in human liver (n = 124), indicating a bimodal distribution; none of the individuals were shown to be TPMT-deficient. (b) Results of genome-wide association analysis of TPMT activity in human liver, using age and sex as covariates. The Manhattan plot shows the association P-values (−log_{10} transformed) across chromosomes 1–22. Significant genetic variants (P < 5.0 × 10^{-8}) are marked in green. (c) The regional association plot displays these values focusing on the genomic region on chromosome 6 (hg19), in which TPMT is located. Additionally, it displays recombination rate and linkage disequilibrium estimates (r^2) of variants with the TPMT variant 719A>G (rs1142345). (d) Association of TPMT activity with common nonfunctional TPMT alleles (TPMT*2, TPMT*3A-*3C). (e) Manhattan plot showing association P-values (−log_{10} transformed) from conditional association analyses considering the most common TPMT alleles (TPMT*3A and TPMT*3C) and correcting for age and sex.
the regional association plot, the sentinel marker is tightly linked to the TPMT variant 719A>G (rs1142345) (Figure 5b).

**DISCUSSION**

Based on several clinical studies and various disease entities (e.g., ALL in childhood, IBD, autoimmune diseases), pretreatment determination of the TPMT phenotype and subsequent pharmacogenetically guided dosing of thiopurines, at least in TPMT-deficient individuals, is recommended in routine clinical practice before commencing therapy. Very recently a landmark randomized clinical trial strongly corroborates the clinical utility of upfront genetic testing for TPMT in patients treated with thiopurines to avoid hematotoxicity. IBD patients who were identified as variant carriers of TPMT and subsequently received dose-adjusted thiopurine therapy showed a 10-fold reduction in hematologic side effects.

Nevertheless, there are still concerns with regard to whether TPMT genetics exclusively determines the marked interindividual variability of TPMT activity measured in RBC or whether other genes may contribute substantially, particularly in patients carrying the wildtype TPMT genotype. Clinically, it is well known that patients with TPMT wildtype are still at risk for thiopurine-related hematotoxicity and even after consideration of nongenetic factors (e.g., concomitant medication with the...
XO inhibitor allopurinol or viral infection by cytomegalovirus/parvovirus B19) underlying mechanisms are so far unknown to completely explain thiopurine-related hematotoxicity. Of note, in the retrospective study by Colombel et al., only 29.3% of 41 patients with IBD and azathioprine-related severe myelosuppression were carriers of \textit{TPMT} variant alleles.

Some studies have already been performed trying to identify additional genetic or nongenetic factors apart from the \textit{TPMT} gene that influence TPMT activity \textit{in vivo}. For example, \textit{S}-adenosyl-methionine (SAM), the global methyl donor in the human body, has been discovered as a modulator of TPMT activity. One previous study using HapMap CEU cell lines and two pediatric ALL-patient cohorts indicate that independent from \textit{TPMT} a second gene, \textit{PACSIN2}, which is a member of the “protein kinase C and casein kinase substrate in neurons” family of proteins, also modulates TPMT activity and is associated with the MP-related gastrointestinal toxicity in children with ALL. Additionally, a GWAS was performed and identified genes in the

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\textit{Figure 5} (a) Venn diagram depicting number of genetic variants significantly associated with TPMT activity ($P < 5.0 \times 10^{-8}$) in the three cohorts. All variants located on chromosome 6 in the chromosomal region 18.1–18.2 Mb are shown in blue, whereas those outside the region are shown in orange. No loci on other chromosomes were significantly associated with TPMT activity. (b) Manhattan plot showing association $P$-values ($-\log_{10}$ transformed) of the joint meta-GWAS of all three cohorts, revealing significant results within the TPMT locus only. Sentinel marker was chromosome 6:18103028 (rs73726531; MAF = 0.03, $P = 1.2 \times 10^{-72}$; effect size: $-2.2$), which is linked to TPMT variant 719A>G (rs1142345) (see regional association plot). Altogether, 148 markers on chromosome 6 exceeded the genome-wide significance threshold ($P < 5.0 \times 10^{-8}$) in the meta-GWAS (Table S5) and are displayed in the regional association plot.
thiopurine pathway that were associated with thiopurine metabolism in lymphoblastoid cell lines from individuals of different ethnic backgrounds and clinical responses in pediatric ALL patients. Altogether, conclusive information is still missing that demonstrates that TPMT genetics is the ideal predictor for TPMT phenotype and replication of novel identified non-TPMT candidate variants have not been performed in independent large-scale studies.

The present data from our meta-analysis of three independent GWAS clearly indicate that TPMT genetics seems to have the strongest impact on TPMT activity in humans and provides little support for the proposal that other genes may significantly contribute to interindividual variability of TPMT activity. Considering a genome-wide threshold of $P < 5.0 \times 10^{-8}$, our first GWAS for TPMT activity adjusted for age and sex in 844 volunteer-based samples of the Estonia resident adult population revealed 169 variants on chromosome 6, and 150 of those variants map to the genomic region 18.1–18.2 Mb including the TPMT gene. Since this population cohort comprises subjects with various ICD-10 diagnoses not associated with anemia, we can conclude that different diseases do not appear to significantly alter TPMT activity in RBC. Data from the second GWAS comprising 245 pediatric ALL children are in accordance with our findings from the Estonian cohort with 77 significantly associated variants mapping to chromosome 6 (18.1–18.2 Mb). Of note, we observed a TPMT genotype–phenotype discordance in ALL patients compared with the Estonia cohort attributed to the disease process of ALL and anemia of patients resulting in lower TPMT activities in RBCs due to degraded TPMT enzyme.

Additionally, we investigated for the first time TPMT activity in cytosols from 124 human liver samples. Again, the GWAS revealed that only variants on chromosome 6 (81 variants at region 18.1–18.2 Mb) were significantly associated with TPMT activity. Finally, data from our meta-analysis of all three datasets confirmed these findings, since once again all significantly associated genetic variants were located on chromosome 6, including the TPMT gene region. Noticeably, the genome-wide significant hits for TPMT activity extend to genes (e.g., KDM1B) located next to the TPMT gene. As shown in the regional association plot of the meta-GWAS, these variants are linked to the non-functional TPMT*3 alleles. Further analyses conditioning on the TPMT*3A and *3C alleles were performed, revealing no significantly associated variants ($P < 5.0 \times 10^{-8}$) with TPMT activity in all three cohorts. Moreover, analyses of SNP–SNP interactions in all three cohorts revealed no significant variants after correction for multiple testing, suggesting a lack of strong interaction effects among the loci tested.

Next we tried to replicate non-TPMT related candidate genes previously identified by a genome-wide study approach using HapMapCEU lymphoblastoid cell lines (LCLs). Of note, LCLs but even non-HapMap LCLs have been promised as useful model systems for cellular pharmacologic effects as well as biochemical effects and enzymatic reactions. Several studies have been reported using LCL resources including various whole-genome approaches (e.g., GWAS) to identify novel genetic variants that were subsequently associated with anticancer-related cytotoxicity (e.g., cisplatin, cytarabine). Matimba et al. have proposed a minor association ($P > 5.0 \times 10^{-4}$) between SNPs within “thiopurine-related” genes (XDH, IMPDH1, SLC28A3, ABCC4) and “nonthiopurine pathway” SNPs (FAM8A6P, AJG1/HIVEP2) with thiopurine cytotoxicity in LCLs with further validation in pediatric ALL patients. However, our findings provide little support for any effect mediated through TPMT, as there was only negligible correlation related to TPMT activity in our cohorts.

Moreover, SNPs localizing to PACSIN2, previously found to influence TPMT activity and be related to MP-related gastrointestinal toxicity were not associated with TPMT activity in our analyses. This finding supports Roberts et al. who failed to confirm an association between PACSIN2 genotype and thiopurine-related adverse drug reactions in IBD patients.

Furthermore, very recently the p.Arg139Cys (R139C) variant in the NUDT15 gene was associated with thiopurine-induced leukopenia, first in a retrospective cohort of Korean IBD patients, and confirmed by other studies including also children with ALL and MP intolerance. Very recently, Moriyama et al. provided mechanistic evidence of how NUDT15 variants independent from TPMT alter levels of active thiopurine metabolites, subsequently resulting in increased thiopurine cytotoxicity. No potential interaction of the NUDT15 R139C variant (rs116855232) with TPMT activity was found in any of our three cohorts, keeping in mind that the allele frequency is extremely low in non-Asians.

Additionally, a genome-wide approach was used to identify novel predictors of TPMT activity in LCLs, resulting in 96 genes ranked higher than TPMT itself. Except for SLC22A16, none of these genes were located on chromosome 6. Since based on our findings only genetic variants on chromosome 6 determine TPMT activity, these genes appear to be of minor importance to predict TPMT phenotype. Noticeably, no significantly associated variants in SLC22A16 ($P < 5.0 \times 10^{-8}$) with TPMT activity were found. Thus, use of LCLs in this investigation and the discovery of novel pharmacogenetic loci may have limitations, and validation of results in large-scale population/patient cohorts is needed.

In summary, for the first time we provide systematic data on TPMT expression (mRNA, protein) and function (activity levels) in a cohort of 124 human liver samples. Human liver is the predominant site of thiopurine metabolism and data are still lacking demonstrating a close correlation between hepatic TPMT protein expression and cytosolic enzyme activity. TPMT mRNA was not significantly correlated with either TPMT protein or TPMT protein level, which supports previous findings demonstrating that the common TPMT polymorphisms 460G>A and 719A>G affect TPMT activity by posttranslational modification and increased protein degradation. Moreover, no correlation was found between mRNA expression and either TPMT activity or TPMT protein expression measured by western blotting in subjects carrying the TPMT reference sequence confirmed by NGS. These data are in contrast to Lindqvist et al. reporting a significant correlation between normal/high TPMT enzyme activity in RBC and mRNA levels extracted from whole blood in
29 individuals. It remains questionable if the correlation of mRNA derived from white blood cells and TPMT enzyme activity measured in RBC is reasonable.

Limitations of our work may be that this study was designed based on SNP array data and did not investigate gene duplications/deletions or genomic rearrangements that might alter TPMT activity. In addition, to identify novel rare variants in genes other than TPMT that are associated with TPMT activity, whole genome NGS approaches are needed. Moreover, epigenetic regulation of TPMT expression, e.g., by noncoding RNAs such as miRNAs, was not investigated, which may in part be a plausible explanation of the missing correlation between TPMT mRNA expression and protein levels in human liver. Finally, our three study cohorts ethnically are restricted to individuals of European ancestry, as confirmed through genetic analyses (Figure S1), and therefore other ethnic groups like Asians and Africans are not covered. In addition, the present study was not designed to identify genetic variants associated with thiopurine-related toxicity. Thus, we cannot exclude that in addition to TPMT further genes are involved in risk of toxicity or treatment outcome.

In conclusion, based on the largest meta-GWAS including 1,212 subjects from different populations, we did not identify any novel associations for TPMT activity, thereby endorsing genetic testing for TPMT alleles as advocated by the Clinical Pharmacogenetics Implementation Consortium and the Dutch pharmacogenetics group for prediction of patient’s phenotype prior to thiopurine therapy in patients with ALL.

METHODS

Estonian population cohort
Individuals (n = 864; 422 males, age: median 34 years, range 18–87 years) were randomly chosen from a large cohort (52,000) of collected subjects from the Estonian Biobank, at the Estonian Genome Center, University of Tartu. The recruitment and sample collection has been described previously, and all participants signed a broad informed consent. One inclusion criterion for the TPMT study was that there is no regular use of medications associated with TPMT. The study was approved by the Ethics Review Committee on Human Research, University of Tartu, Estonia. The DNA was extracted from whole blood using the conventional salting out procedure. Hemolysates were prepared from RBCs according to established procedures, as described in the Supplementary Methods.

ALL-study cohort
ALL-children (n = 245; 144 males; age: median 6 years, range 1.1–17.4 years) of the BFM trials were included in the present study, none of whom had been transfused within 3 months prior to blood sampling and samples were obtained before ALL maintenance therapy. Sample collection was performed with informed consent in accordance with the principles of the Declaration of Helsinki and the study was approved by the Ethical Review Board. Genomic DNA, as well as RBC lysates, were prepared as previously described.

IKP liver cohort
Histologically normal human liver tissues as well as corresponding blood samples were collected from patients undergoing liver surgery at the Department of General, Visceral and Transplantation Surgery (University Medical Center Charité, Berlin, Germany) as previously described. The study was approved by the Ethics Committees of the Charité, Humboldt University (Berlin, Germany) and the University of Tübingen (Tübingen, Germany) in accordance with the principles of the Declaration of Helsinki. The tissue samples were stored at –80°C. Subcellular fractions were prepared according to standard procedures. Briefly, ~1 g of tissue was homogenized in 1 mM EDTA, 1 mM DTT, 10 mM HEPES pH 7.4, 0.2 mM Pefabloc (Roth, Karlsruhe, Germany) and 0.15 mM KCl and differentially centrifuged at 15,000g and 105,000g. The final cytosolic supernatant was immediately frozen in liquid nitrogen in aliquots and stored at –80°C until use. Cytosol used for TPMT activity measurements was available for liver samples of 124 patients (n = 124; 54 males, 70 females; age: median 58 years, range 7–85 years). Purification of genomic DNA from EDTA blood samples was performed as described previously, and DNA samples were available for 150 liver samples.

TPMT activity measurements from hemolyzed red blood cells and liver cytosol
TPMT activity was measured in hemolysates from all samples of the Estonian cohort, and the ALL-study cohort, and in all liver cytosols with a nonradioactive high-performance liquid chromatography (HPLC) method as described previously using 6-TG as a substrate. The cutoff for TPMT deficiency based on TPMT activity measurements in liver cytosol was suggested to be 0 nmol h⁻¹ mg⁻¹ and the cutoff for TPMT activity in RBCs is ≤2 nmol 6-MTG g⁻¹ Hb x h⁻¹.8

Genotyping of TPMT alleles
Genotyping of TPMT*2 (238G>C, rs1800462), TPMT*3B (460G>A, rs1800460), and TPMT*3C (719A>G, rs1142345) in the Estonian cohort was carried out by TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA) as previously described. Genotyping for TPMT*2 and TPMT*3 alleles in the ALL- and IKP-liver cohort were performed using TaqMan technology and MALDI-TOF MS as previously described.

Whole-genome genotyping and imputation analysis for individuals from the Estonian cohort, the ALL study, and the liver cohort
For the 864 samples of the Estonian cohort, the Illumina Human370CNV BeadChips were used for whole-genome genotyping. After quality control, recovery of untyped genotypes using 1000 Genomes as reference, and filtering, 8,617,769 markers and 844 individuals were included in the subsequent analysis. Genome-wide genotyping data for the ALL study was generated by Illumina Human Omni1-Quad arrays. After quality control, imputation, and filtering, 8,224,478 markers were included in the subsequent association analyses of 245 patients. Genome-wide genotyping data for liver samples were generated using HumanHap300 Genotyping BeadChips. After quality control, imputation, and filtering, 7,481,872 markers were included in the subsequent association analyses of 123 samples. Comprehensive details of the methods are provided in the Supplementary Methods.

Next-generation sequencing
In addition, the TPMT gene region was analyzed systematically in human liver samples for presence of genetic variations by next-generation sequencing (NGS). NGS was conducted at the Center for Genomics and Transcriptomics (CeGaT, Tübingen, Germany) as previously described. Details are provided in the Supplementary Methods.

TPMT mRNA quantification
High-quality RNA of 150 liver samples was extracted as described previously. TPMT mRNA was quantified with the TaqMan Gene Expression Assay Hs00990901m1 (Applied Biosystems). TPMT expression was normalized against β-actin expression, which was measured with the HUMAN ACTB (beta actin) Endogenous Control Assay (Applied Biosystems). The measurements were conducted on the Fast Real Time PCR System 7900HT (Applied Biosystems).
TPMT protein quantification

TPMT protein expression was quantified by immunoblot analyses of liver cytosol using a specific rabbit anti-TPMT antibody, which was kindly provided by Richard Weinshilboum (Mayo Clinic, Rochester, MN). TPMT protein levels, quantified through immunoblotting, were available for 122 samples.

Statistical analysis

Analyses of genome-wide association in individual studies. Association study of genome-wide imputed genotypes was performed with SNPTTEST v2.5.30 using the frequentist association test option with expected genotype counts. For this purpose, TPMT activity measurements were first power-transformed (Estonian cohort: $\lambda = 1.3$, ALL study: $\lambda = 0.5$, liver cohort: $\lambda = 1.65$) to fulfill Gaussian distribution assumption. Here, the transformation was determined via the MASS_7.3-35 package31 within R-3.1.1 (www.r-project.org). Distribution of transformed measurements was confirmed using a normal quantile–quantile plot and the Shapiro–Wilk test. Finally, the association between each of the genetic variants and the transformed TPMT activity measurements, corrected for sex and age, was analyzed in the additive genetic model. Subsequently, we additionally conditioned on additive dosages of the respective TPMT variants (TPMT*3A and TPMT*3C) to test for secondary signals. Details about the analyses of SNP–SNP interactions are provided in the Supplementary Data.

Meta-analyses. Results of all three GWAS datasets were combined into a joint meta-analysis using GWAMA software.42 Results for the Estonian study and the ALL study, where the TPMT activity was measured in blood, were combined in an inverse of variance weighted fixed effect meta-analysis and the results for the IKP liver cohort with activity measured in liver cytosol were added in a two degrees of freedom test allowing for the differential effect sizes between the two sample types (blood and liver).43

Visualization of the genome-wide association data. The genome-wide significance level was defined as $5 \times 10^{-8}$. Manhattan plots were drawn using the R-package qqman_0.1.1 and MANH.R script from the GWAMA software package.42 Regional association plots showing –log10 transformed (Estonian cohort: $k = 0.5$, ALL study: $k = 0.5$, liver cohort: $k = 1.65$) counts. For this purpose, TPMT activity measurements were first power-transformed (Estonian cohort: $k = 0.5$, ALL study: $k = 0.5$, liver cohort: $k = 1.65$) to fulfill Gaussian distribution assumption. Here, the transformation was determined via the MASS_7.3-35 package within R-3.1.1 (www.r-project.org). Distribution of transformed measurements was confirmed using a normal quantile–quantile plot and the Shapiro–Wilk test. Finally, the association between each of the genetic variants and the transformed TPMT activity measurements, corrected for sex and age, was analyzed in the additive genetic model. Subsequently, we additionally conditioned on additive dosages of the respective TPMT variants (TPMT*3A and TPMT*3C) to test for secondary signals. Details about the analyses of SNP–SNP interactions are provided in the Supplementary Data.

Further statistical analyses

Spearman’s correlation test was used to test association between TPMT activity and mRNA or protein expression. R-package beanplot_1.2.45 was applied to display the association between TPMT activity and common TPMT SNPs, with thick black lines representing the medians per group and the dotted line the overall median. All statistical tests were two-sided and statistical significance was defined as $P < 5\%$.

Additional Supporting Information may be found in the online version of this article.

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CONFLICT OF INTEREST

M. Schwab and E.S. are contributors to a patent filed on behalf of the Robert Bosch Gesellschaft für medizinische Forschung mbH related to polymorphisms in the human gene for TPMT and their use in diagnostic and therapeutic applications.

AUTHOR CONTRIBUTIONS


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