Comprehensive analysis of thiopurine *S*-methyltransferase phenotype–genotype correlation in a large population of German-Caucasians and identification of novel *TPMT* variants

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The thiopurine *S*-methyltransferase (TPMT) genetic polymorphism has a significant clinical impact on the toxicity of thiopurine drugs. It has been proposed that the identification of patients who are at high risk for developing toxicity on the basis of genotyping could be used to individualize drug treatment. In the present study, phenotype–genotype correlation of 1214 healthy blood donors was investigated to determine the accuracy of genotyping for correct prediction of different TPMT phenotypes. In addition, the influence of gender, age, nicotine and caffeine intake was examined. TPMT red blood cell activity was measured in all samples and genotype was determined for the *TPMT* alleles **2* and **3*. Discordant cases between phenotype and genotype were systematically sequenced. A clearly defined trimodal frequency distribution of TPMT activity was found with 0.6% deficient, 9.9% intermediate and 89.5% normal to high methylators. The frequencies of the mutant alleles were 4.4% (3A*), 0.4% (**3C*) and 0.2% (**2*). All seven TPMT deficient subjects were homozygous or compound heterozygous carriers for these alleles. In 17 individuals with intermediate TPMT activity discordant to *TPMT* genotype, four novel variants were identified leading to amino acid changes (K119T, Q42E, R163H, G71R). Taking these new variants into consideration, the overall concordance rate between *TPMT* genetics and phenotypes was 98.4%. Specificity, sensitivity and the positive and negative predictive power of the genotyping test were estimated to be higher than 90%. Thus, the results of this study provide a solid basis to predict TPMT phenotype in a Northern European Caucasian population by molecular diagnostics. *Pharmacogenetics* 14:407–417 © 2004 Lippincott Williams & Wilkins**

Pharmacogenetics 2004, **14**:407–417

Keywords: genotype–phenotype correlation, pharmacogenetics, population study, TPMT

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Sponsorship: This study was supported by the German Federal Ministry for Education and Science (BMBF grants 01 GG 9846 and grant 01 EC 0001) and by the Robert Bosch Foundation, Stuttgart, Germany.

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Received 23 February 2004
Accepted 22 April 2004

Introduction

Thiopurine *S*-methyltransferase (TPMT; EC 2.1.1.67) is a cytosolic enzyme that catalyses the *S*-methylation of aromatic and heterocyclic sulphydryl compounds such as 6-mercaptopurine, 6-thioguanine and azathioprine. These thiopurine drugs are used in the routine treatment of several diseases, such as acute lymphoblastic leukaemia in childhood (ALL), rheumatic diseases and inflammatory bowel diseases, and as immunosuppressants following organ transplantation [1–3].

TPMT enzyme activity in humans is controlled by a common genetic polymorphism at the *TPMT* locus and approximately 1 in 300 individuals has very low TPMT activity, 11% have intermediate activity and 89% show normal/high activity [4–6]. Inactivating mutations that are responsible for TPMT deficiency in a homozygous or compound heterozygous manner have been described (Fig. 1) and 17 variant *TPMT* alleles (*TPMT*2* to **15*) are known to date [5,7–15]. *TPMT*3A* was found to be the most prevalent deficient allele in Caucasians whereas the *TPMT*3C* allele is more frequently identified in African and South-east Asian populations [16,17]. All other inactive alleles are very rare in the populations studied, and have been described only in single cases [12]. Moreover, variable numbers of tandem repeats (VNTR) in the *TPMT* promoter have been identified [18]. However, the importance of *VNTR* alleles remains controversial

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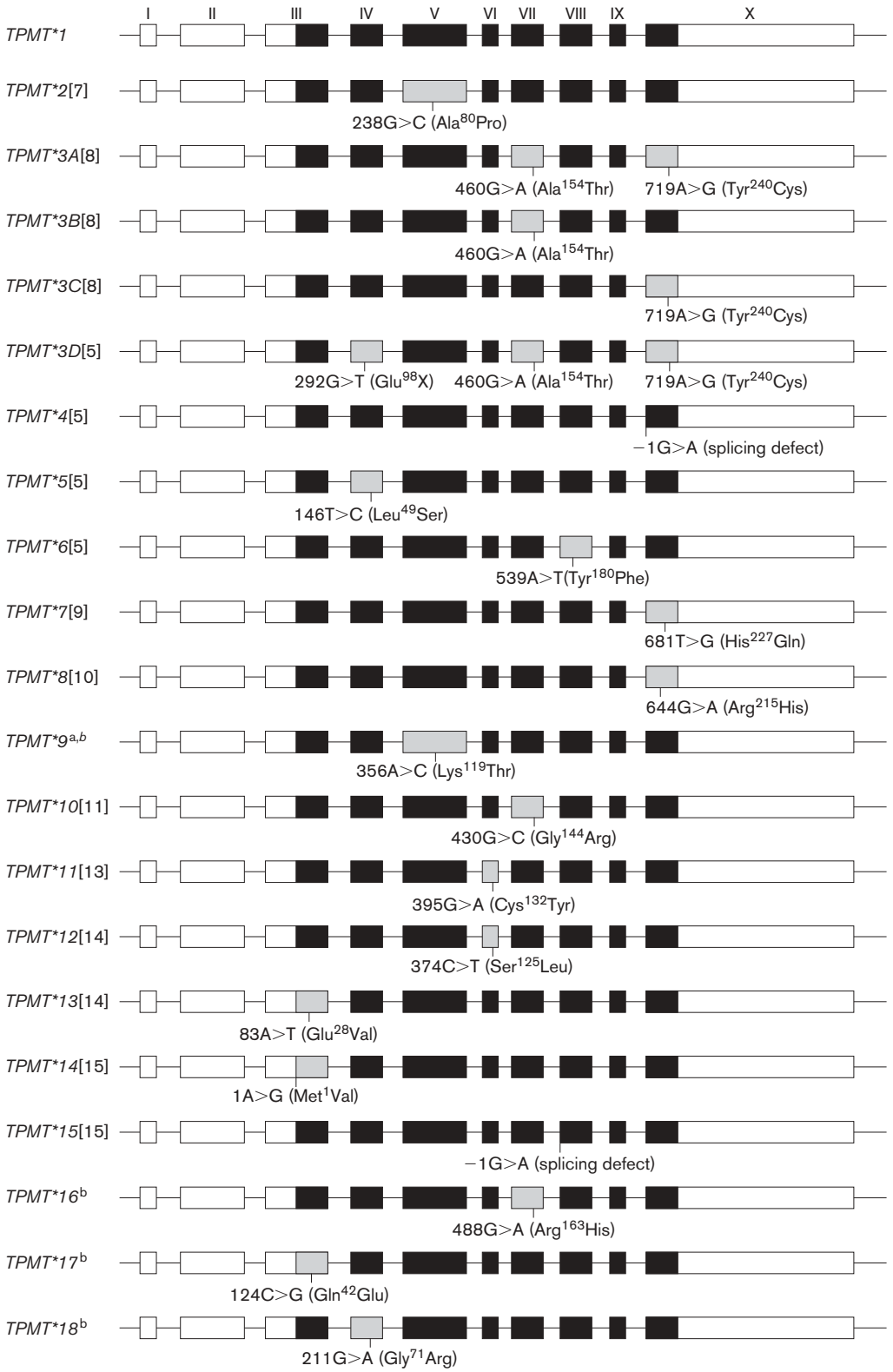
DOI: 10.1097/01.fpc.0000114745.08559.db

Fig. 1



Summary of the currently known thiopurine *S*-methyltransferase gene (*TPMT*) mutant alleles. Locations of the known and newly identified *TPMT* mutant alleles from the present study are shown. Black boxes represent exons that encode open reading frame (ORF) sequences; white boxes represent exons out of the ORF. Exons but not intron sizes are proportional to their relative lengths. References are listed in brackets. [a]R. Weinshilboum, personal communication; [b]present study.

because *VNTR* variations do not significantly modulate TPMT activity [19,20].

Numerous clinical studies have shown that TPMT-deficient patients are at high risk for severe and sometimes fatal haematotoxicity due to the accumulation of cytotoxic metabolites after treatment with standard doses of thiopurines [11,21–24]. In addition, patients who are heterozygous carriers of one inactive *TPMT* allele also have an increased risk for thiopurine-related toxicity [25–27]. Therefore, in both cases, genotype-guided dose adjustment is required [28–30].

Prospective determination of erythrocyte TPMT activity is advocated as a routine safety measure before therapy to avoid drug toxicity; however, the determination of the constitutive TPMT enzyme activity is time consuming and has a number of serious limitations. First, if a deficient or heterozygous patient has received transfusions with red blood cells (RBC) from a homozygous wild-type individual, which is not uncommon for newly diagnosed leukaemic children, TPMT activity cannot be reliably determined within 30–60 days after transfusion [23,31,32]. Second, thiopurine administration itself may alter TPMT activity in RBC with an increase of enzyme activity of approximately 20% compared to baseline, especially in heterozygous individuals [33,34]. Finally, some other clinically important drugs (e.g. sulfasalazine, olsalazine) have been identified as partly potent inhibitors of TPMT *in vitro* and *in vivo* [35–37] with the clinical consequence of an increased risk for developing leukopenia [38].

To avoid misclassification, genotyping has been proposed as a reliable method for identifying those patients who are at a high risk for developing toxicity and is already offered as a routine CLIA-certified molecular diagnostic from reference laboratories (e.g. http://www.prometheuslabs.com) to individualize treatment with thiopurine drugs. However, the prerequisition for replacing phenotyping by genotyping strategy depends on two requirements: (i) all relevant mutations must be known to explain the different phenotypes and (ii) when genotyping is used in clinical practice, the correct prediction of molecular diagnostics to determine the patients' phenotype should be higher than 90%, which can be estimated by the sensitivity and specificity of the genotyping test. Presently, on the basis of several studies, the overall level of concordance between *TPMT* genotype and phenotype varies between 76% and 100% [5,6,19,39–44]. The major limitation of these studies exists in the small sample size of individuals investigated because TPMT deficient subjects were usually not included. Moreover, the phenotype–genotype concordance in patients with intermediate TPMT activity is approximately 70–80%. Thus, it can be assumed that unknown mutations of *TPMT* may exist. This is corroborated by the identification of a novel missense mutation of *TPMT* (**11*) leading to TPMT deficiency in a compound heterozygous manner in a 5-year-old boy with ALL [13].

To investigate comprehensively phenotype–genotype correlation in a large population of healthy unrelated German-Caucasians, we determined the frequency distribution of TPMT activity in relation to *TPMT* genetics. In all cases of discordance between TPMT phenotype and genotype, sequence analysis of the complete open reading frame (ORF) of *TPMT* was performed to identify novel inactivating sequence variants. As endpoints of this study, sensitivity, specificity and the positive and negative predictive values for *TPMT* genotyping were estimated. Additionally, we elucidated the influence of age and gender on TPMT activity. Because environmental factors (e.g. nicotine) have been shown to be relevant determinants for the activity of various drug-metabolizing enzymes, such as CYP1A2 and UGTs [45,46], we have further investigated the effect of nicotine and/or caffeine intake on TPMT activity.

Subjects and methods

Study participants and protocol

A total of 1222 unrelated healthy German individuals of both genders (395 women, 827 men) with a mean (range) age of 38.1 (18–69) years were recruited among blood donors consecutively entering the Department of Transfusion Medicine, University Hospital Tuebingen. All individuals were of Caucasian origin. Individuals were only included in the study if they had no regular drug use (with the exception for oral contraceptives and/or vitamin pills). In addition, it was also recorded whether or not the volunteers were regular cigarette smokers (>5 cigarettes per day) or consumers of caffeine (coffee or tea). The study had been approved by ethics committees of the Landesaerzte Kammer Baden–Wuerttemberg and the University Hospital Tuebingen, Germany, and all volunteers provided their written informed consent.

All 1222 individuals were phenotyped for TPMT and 1214 individuals were simultaneously genotyped because, in eight cases, no DNA samples were available.

TPMT phenotyping

TPMT enzyme activity was measured in erythrocytes using a high-performance liquid chromatography (HPLC) method as described previously [27,47,48]. By contrast to the classical radiochemical method by Weinshilboum *et al.* [4] using 6-MP as substrate, 6-thioguanine (6-TG) was selected as substrate in our HPLC assay because a highly fluorescent product 6-methylthioguanine (6-MTG) is formed. This allows a very sensitive and highly specific quantification. The

higher TPMT activity levels measured with 6-TG in comparison to those obtained with 6-MP as substrate in the original radiochemical assay (< 5 = deficient; 5–10 = intermediate; > 10 = normal/high activity) [4,49] are caused by the use of 6-TG as substrate as previously shown by Weinshilboum *et al.* [50]. Laboratory staff were blinded to case status of the study participants.

Detection of *TPMT* sequence variations

Genomic DNA from peripheral leukocytes was isolated by standard methods. Exons of the *TPMT* gene and flanking intronic regions were amplified by polymerase chain reaction (PCR) and genotyping for the **2* and **3* alleles (**3A*, **3B*, **3C* and **3D*) was performed by a denaturing HPLC (DHPLC) method as described previously [51]. Samples with known sequence variations in exon 5, 7 and 10 were used as controls. All PCR reactions were performed with an MJ Research PTC-200 Peltier thermal cycler (MJ Research Inc., Watertown, Massachusetts, USA).

For individuals with an intermediate activity, not having any mutation in exon 5, 7 and 10 according to DHPLC analysis, direct sequencing was undertaken to examine the sequence of the entire *TPMT* ORF. Exons 3–10 (ORF) were amplified with primers as previously described [5]. Automated DNA sequencing was performed with the ABI BigDye Terminator sequencing kit and samples were analysed on an ABI310 Genetic Analyser (Applied Biosystems, Foster City, California, USA). To confirm new allelic variants, DNA was taken for a second PCR and the sequencing procedure was repeated.

Haplotyping of genomic DNA for the *TPMT*3* allele

Long range PCR was performed using the Expand Long Template PCR Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations with the following primers, which are located in intron 6 and exon 10 of the TPMT gene: 5′-ctccacacccaggtccacacatt-3′; 5′-catccattacattttcaggctttag cataat-3′. 8-kb PCR products, spanning the genomic region from exon 7 to exon 10, were cloned into the pGEM-T Easy vector Systems (Promega GmbH, Mannheim, Germany), to keep alleles separate. Plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and checked for insertions by restriction analysis. Sequencing of inserts was performed around the polymorphic sites G460A and A719G using the ABI BigDye Terminator sequencing kit and the samples were analysed on an ABI310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Data were analysed by parametric or nonparametric methods, depending on whether data distribution was normal or not normal. For paired comparisons, the Student's *t*-test or the Mann–Whitney *U*-test was used. To test whether the median TPMT activity among three or more groups differs, one-way analysis of variance (ANOVA) followed by the Bonferroni post test was performed. All statistical tests were performed two-tailed and $P < 0.05$ was considered as statistically significant. For all calculations, the GraphPadPrism software package version 3.0 was used (Graph Pad Software Inc., San Diego, California, USA).

Cumulative frequencies $F = n/N$ (with n = number of subjects with TPMT activity equal or below a certain activity; N = total number of subjects) were plotted versus TPMT activity. A sum of Rosin–Rammler–Sperling–Weibull (RRSW) functions was adjusted to the cumulative frequency data for TPMT activity [52,53]:

$$\mathrm{f(activity)} = \sum_{\mathrm{j}} \mathrm{a_j}(1 - \mathrm{e}^{-(\lambda_j(\mathrm{activity}-\tau_j))^{\beta_j}})$$

a_j = fraction of the total number of subjects assigned to the j_th distribution
λ_j = a scaling factor
β_j = slope factor
τ_j = shift of the RRSW function on the activity axis

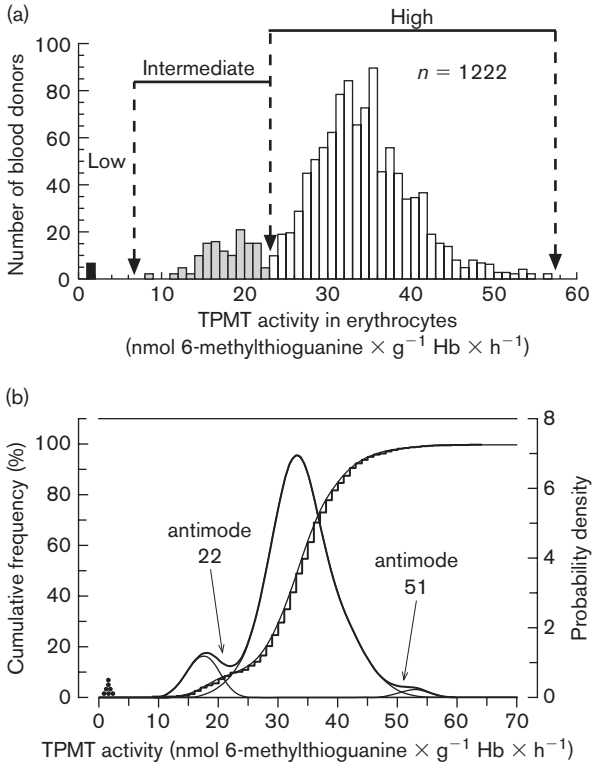
This adjustment was carried out for pooled data with increasing numbers of RRSW-functions; however, subjects with deficient activity were not included in the fitting procedure. HOEGIP-PC software [54], following the least square principle [55], was used for fitting. Each adjustment was carried out with β_j fixed to 3.57, for which the RRSW-function becomes a good approximation of the normal distribution function [56]. The decision for the appropriate number of RRSW-functions was guided by Akaike's information criterion [57] and by comparison of fitting results using the *F*-test for the quotient of the mean sum of squares computed for each adjustment.

Results

Distribution of TPMT activity in RBC

TPMT activity levels in the 1222 individuals showed a 32-fold variability, ranging from $\leqslant 2$ up to 65 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1}. The frequency distribution histogram and the distribution of the TPMT activity data with corresponding RRSW density functions are shown in Fig. 2. Best adjustment to the cumulative frequency data for the non-deficient individuals was achieved with three RRSW-functions, where two of these three strongly overlapped, together forming the dominant part of the distribution ranging from approximately 20–50 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1} (Fig. 2b). Thus, together with the deficient individuals, a trimodal dis-

Fig. 2



(a) Frequency distribution. Frequency distribution of the thiopurine *S*-methyltransferase (TPMT) activity among 1222 healthy individuals. (b) Analysis for cumulative frequency. Cumulative frequency of all subjects was adjusted by a sum of Rosin–Rammler–Sperling–Weibull (RRSW) functions. These functions are shown separately in terms of probability density functions (right *y*-axis). By fixing the slope factors of the RRSW functions to 3.57, the RSSW functions closely approximate the Gaussian distribution. An additional RRSW function was adjusted to the very high TPMT activity data, which indicates an additional phenotype subgroup of individuals with very high TPMT activity (approximately 50–65 nmol 6-MTG × g^{-1} Hb × h^{-1}).

tribution and, consequently, three different phenotype subgroups with very low, intermediate and normal/high activity can be clearly defined. Regarding the frequency distribution of TPMT activity, the pattern is quite similar to that first described by Weinshilboum and Sladek [4] in a study population of 298 randomly selected American subjects. Interestingly, a further subgroup of individuals with high activity was suggested by the model (Fig. 2b).

Seven individuals (0.6%) had TPMT levels ⩽ 2 nmol 6-MTG × g^{-1} Hb × h^{-1} and no overlap to subjects with intermediate activity was found. Based on probabilities, the antimode segregating normal and intermediate activity was estimated to be 22 nmol 6-MTG × g^{-1} Hb × h^{-1}. Consequently, all individuals with TPMT activity between 9 and 22 nmol 6-MTG × g^{-1} Hb × h^{-1} ($n = 125$, 10.2%) were characterized as intermediate metabolizers whereas 1090 individuals (89.2%) were classified as normal/high methylators. Between these two subgroups, only a slight overlap was observed. The subgroup of individuals with very high TPMT activity (51–65 nmol 6-MTG × g^{-1} Hb × h^{-1}) comprised 22 individuals (1.8%) of the total population.

Genotyping for *TPMT *2* and **3A* to *3D*

Genotyping of the 1214 individuals for the relevant mutations in exon 5, 7 and 10 defining the *TPMT* alleles **2* and **3A* to *3D* identified seven individuals as carriers of two mutant alleles, five in a homozygous mutant manner (**3A/*3A*, 0.4%) and two as compound heterozygotes (**3A/*3C*, 0.2%) (Table 1). The total number of heterozygous subjects was 111 (**1/*2*, 0.4%; **1/*3A*, 8.0%; **1/*3C*, 0.6%). Therefore, the frequencies of mutant alleles were 4.5% (**3A*), 0.4% (**3C*) and 0.2% (**2*). No individual was carrier of the *TPMT *3B* or **3D* allele.

Phenotype–genotype correlation and identification of novel sequence variants

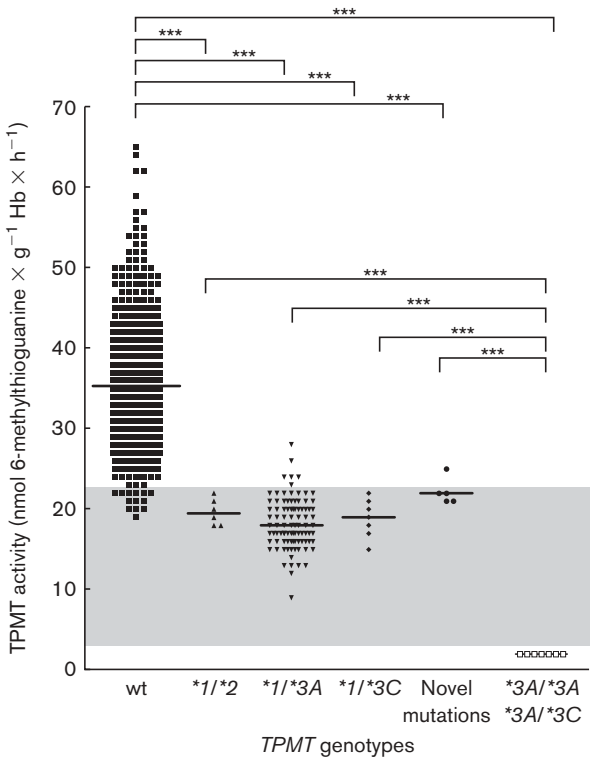
Figure 3 shows the distribution pattern of TPMT activity in relation to *TPMT* genotypes. On the basis of genotyping for the most frequent *TPMT* alleles **2* and **3A* to *3D*, all seven subjects with very low activity (⩽ 2 nmol 6-MTG × g^{-1} Hb × h^{-1}) were unequivocally identified as carriers of two non-functional alleles. Of the 111 heterozygote individuals, six had a normal activity with levels between 23 and 28 nmol 6-MTG × g^{-1} Hb × h^{-1}. Thus, TPMT activity varied among all heterozygous subjects between 9 and 28 nmol 6-MTG × g^{-1} Hb × h^{-1}. Conversely, 17 of the 1096 subjects carrying two functional alleles (**1/*1*) had intermediate TPMT activity ranging from 18 to 22 nmol 6-MTG × g^{-1} Hb × h^{-1}. The range of TPMT activity in all individuals homozygous for **1/*1* was between 18 and 65 nmol 6-MTG × g^{-1} Hb × h^{-1}. The median activity was significantly higher for the wild-type group (34 nmol 6-MTG × g^{-1} Hb × h^{-1}) com-

Table 1 Thiopurine *S*-methyltransferase (TPMT) genotypes in 1214 unrelated German-Caucasian blood donors

TPMT genotype[a]	Number	%	Median (range) activity (nmol 6-MTG × g^{-1} Hb × h^{-1})
**1/*1*	1091	89.8	34 (19–65)
**1/*2*	6	0.5	19.5 (18–22)
**1/*3A*	98	8.0	18 (9–28)
**1/*3B*	0	0	–
**1/*3C*	7	0.6	19 (15–22)
**1/*3D*	0	0	–
**3A/*3A*	5	0.4	< 2
**3A/*3C*	2	0.2	< 2
**1/*9* (A356C)	2	0.2	21, 25
**1/*16* (G488A)	1	0.1	22
**1/*18* (G211A)	1	0.1	21
**1/*17* (C124G)	1	0.1	22

[a]According to the nomenclature of *TPMT* alleles [5,7–15], including the novel defined *TPMT* alleles from the present study.

Fig. 3



Distribution of thiopurine *S*-methyltransferase (TPMT) activity among 1214 individuals in relation to their *TPMT* genotypes. The grey shaded area depicts the range of TPMT activity in red blood cells that defines intermediate TPMT activity (3–22 nmol 6-MTG × g^{-1} Hb × h^{-1}), which separates individuals with very low TPMT activity (TPMT deficiency; (≤2 nmol 6-MTG × g^{-1} Hb × h^{-1}) from normal/high TPMT methylators. ***$P < 0.0001$.

pared to heterozygous individuals (18 nmol 6-MTG × g^{-1} Hb × h^{-1}; $P < 0.0001$).

In the single case with a TPMT activity of 9 nmol 6-MTG × g^{-1} Hb × h^{-1}, a heterozygous *TPMT* genotype (**1/*3A*) was determined. To exclude the hypothetical case of compound heterozygosity (*TPMT*3B/*3C*), which cannot be unequivocally discriminated by conventional genotyping methods [58], haplotyping of genomic DNA for the *TPMT*3* allele was performed by sequencing of cloned PCR amplified genomic DNA. The heterozygous genotype **1/*3A*, and therefore an intermediate TPMT phenotype, was confirmed.

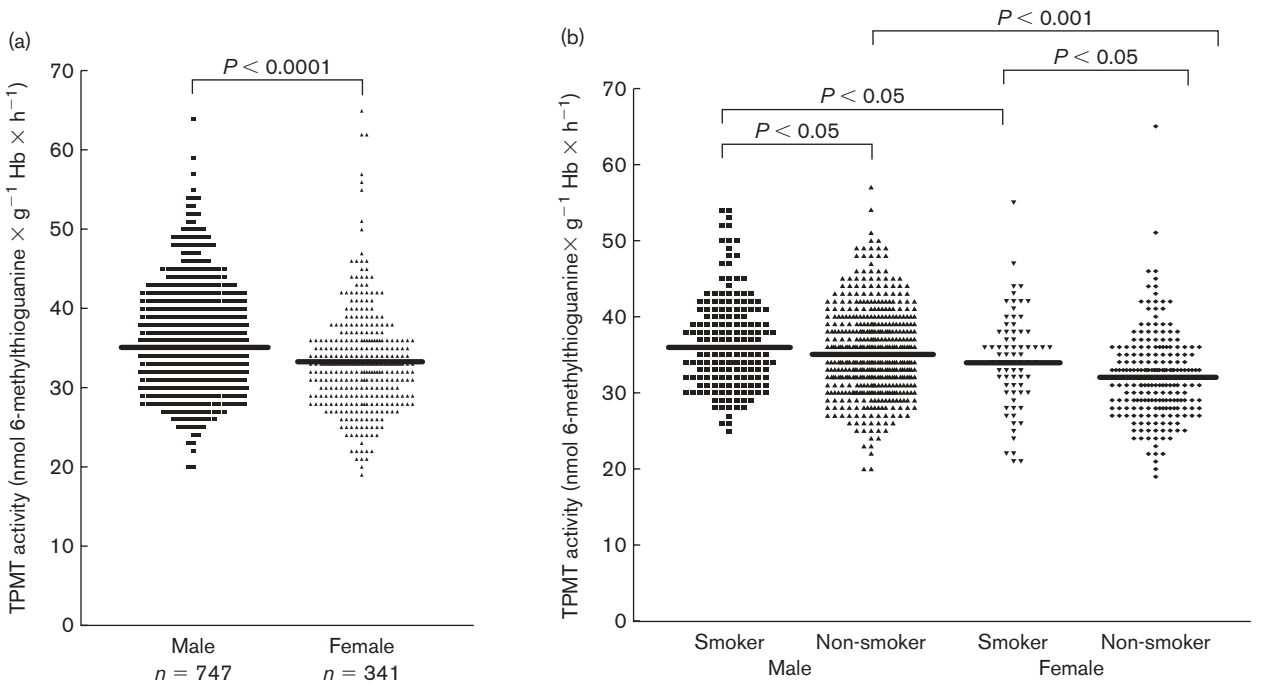
The discrepancy between genotype and phenotype in the present study may be explained by either one of the rare known or novel variants of *TPMT*. Therefore, we used two strategies to investigate systematically the ORF as well as all exon–intron boundaries of *TPMT* in all 17 individuals with intermediate TPMT activity and discordance to *TPMT* wild-type genotype. First, using the DHPLC method, which, in addition to genotyping strategy, allows simultaneous screening for unidentified mutations in exon 5, 7 and 10 [51], two novel sequence variants of *TPMT* in exon 5 (A356C) and exon 7 (G488A) were identified. Both mutations lead to an amino acid exchange (K119T and R163H). Second, direct sequencing strategy was used to examine the entire *TPMT* coding regions (exon 3–10), including all exon–intron boundaries. Two new variants in exon 3 (C124G) and exon 4 (G211A), respectively, were found which lead to amino acid changes (Q42E and G71R). The four individuals with a heterozygous genotype for C124G, G211A, A356C and G488A showed TPMT activites of 22, 21, 21 and 22 nmol 6-MTG × g^{-1} Hb × h^{-1}, respectively. Therefore, the discrepancy between phenotype and genotype in the 17 individuals could be explained in four cases by four novel non-synonymous mutations whereas, in the remaining 13 subjects, no further variants were identified.

Because DHPLC analysis was performed on all 1214 subjects of our study, we were able to investigate whether the novel variants at position 356 and 488 also occur in individuals with normal TPMT activity. Only one subject was found who was heterozygous for the A356C mutation and phenotypically defined as a normal methylator with a TPMT activity of 25 nmol 6-MTG × g^{-1} Hb × h^{-1}.

Taken together, we detected four novel non-synonymous *TPMT* variants. According to the present *TPMT* allele nomenclature, three of them were assigned the following designations: *TPMT*16* (G488A), *TPMT*17* (C124G) and *TPMT*18* (G211A). They were clearly associated with decreased TPMT activity leading to an intermediate TPMT phenotype (Table 1). The SNP at position 356 has been provisionally assigned to TPMT **9* allele at some scientific meetings; however, to the best of our knowledge, no further information is presently published (R. Weinshilboum, personal communication). We identified this variant (A356C) in two individuals, one of whom had an intermediate activity and a second who showed a normal TPMT activity.

By taking these new variants into consideration, estimation of genotype–phenotype correlation in our German-Caucasian population showed a concordance rate of 89.2% between *TPMT* heterozygosity and the intermediate methylator phenotype, and of 99.4% between *TPMT* wild-type and the normal/high methylator phenotype. The overall concordance rate between *TPMT* genotypes and phenotypes was 98.4%. The sensitivity and specificity for genotyping, including all identified mutations to predict the correct TPMT phenotype, was 90% and 99%, respectively. Correspondingly, the positive and negative predictive power was estimated to be 94% and 99%, respectively.

Fig. 4



Comparison of thiopurine *S*-methyltransferase (TPMT) activity levels with gender and smoking behaviour. (a) TPMT activity in male and female subjects with *TPMT* wild-type (**1/*1*). (Mann–Whitney *U*-test) ($P < 0.0001$). (b) Effect of smoking on TPMT activity in male and female subjects with *TPMT* wild-type (**1/*1*). $P < 0.05$ (ANOVA and Bonferroni post-test).

Effect of gender, age, nicotine and caffeine on TPMT activity

In addition to genetic factors, we investigated the influence of gender and age, as well as of the regular use of nicotine and caffeine, on TPMT activity. A direct comparison of average levels of enzyme activity in the different subgroups might not be appropriate because the presence of different numbers of individuals carrying mutant alleles could distort the average activity levels. Therefore, TPMT activities were compared only between individuals who were carriers of two wild-type alleles. With regard to nicotine and caffeine consumption, complete documentation was available in 1035 subjects.

A significant influence of gender and cigarette smoking was found. The average TPMT activity was 7% higher in males ($n = 747$; 35.86 ± 0.22 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1}) compared to females ($n = 341$; 33.32 ± 0.35 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1}; $P < 0.0001$; Fig. 4a). In addition, male and female smokers [male: 36.53 ± 0.42 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1} ($n = 192$); female: 34.26 ± 0.73 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1} ($n = 73$)] had shown a 4% and 6% higher TPMT activity, respectively, compared to non-smokers [male: 35.28 ± 0.24 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1} ($n = 535$); female: 32.06 ± 0.36 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1} ($n = 235$); $P < 0.05$; Fig. 4b]. By contrast, the age of individuals, as well as caffeine intake, did not significantly influence TPMT activity in either females or males (data not shown).

Discussion

This study was undertaken: (i) to assess the frequency distribution of TPMT phenotypes in German-Caucasians and (ii) to identify novel genetic variants of *TPMT* in cases which were discordant between phenotype and genotype.

To the best of our knowledge, this is the first analysis of phenotype–genotype correlation of the TPMT enzyme performed in a large-scale population of healthy individuals with the same ethnic origin, whereas all other studies comprised smaller sample sizes (Table 2). The commonly accepted frequency distribution of TPMT deficiency is 1 of 300 (0.3%) individuals, and therefore it is not surprising that only in four of the 11 populations listed in Table 2 were individuals with very low TPMT activity identified. In our large study, a clearly defined trimodal frequency distribution of TPMT was demonstrated, which is in line with the first description of TPMT polymorphism in 298 White Americans by Weinshilboum and Sladek [4]. This is noteworthy because, in two French populations with

Table 2 Summary of studies investigating the correlation between thiopurine S-methyltransferase (TPMT) phenotype and genotype

Study	Number of individuals (ethnicity)[a]	Concordance (%) of TPMT genotype with phenotype for				*TPMT* alleles investigated	Frequency of *TPMT*3B* allele (%)
		DM[b]	IM[2]	HM[2]	Overall		
Otterness *et al.* (1997)	25 (Norwegians)	No individual	100%	100%	100%	**2, *3A-*3D, *4-*6* + sequencing[c]	0
	21 (Koreans)	No individual	50%	100%	76.2%		
Yates *et al.* (1997)	48 (Northern Americans)	100%	95.2%	100%	97.9%	**2, *3A-*3C*	0
Spire-Vayron de la Moureyre *et al.* (1998)	191 (Europeans)	100%	53%	94%	87%	**2, *3A-*3D, *4-*7* + SSCP/sequencing[c]	0
Hon *et al.* (1999)	46 (African-Americans)	No individual	82.6%	100%	91.3%	**2, *3A-*3D, *8* + sequencing[c]	0
Alves *et al.* (2001)	143 (Portuguese)	No individual	78.6%	99.2%	97.2%	**2, *3A, *3C, *8* + HCSGE	Not investigated
Loennechen *et al.* (2001)	194 (Saami)	No individual	92.3%	100%	99.5%	**2, *3A, *3B, *3C, *6*	0
Rossi *et al.* (2001)	103 (Italians)	No individual	78.6%	100%	97%	**2, *3A-*3C*	0
Reis *et al.* (2003)	74 (Brazilians)	100%	86.7%	100%	94.6%	**2, *3A-*3C*	0
Larövere *et al.* (2003)	49 (Argentines)	No individual	57%	100%	81.6%	**2, *3A, *3B, *3C, *4* to **8*	0
Indjova *et al.* (2003)	76 (Bulgarians)	100%	63.6%	100%	89.5%	**2, *3A-*3D, *4, *6*	0
This study	1214 (German-Caucasians)	100%	89.2%	99.4%	98.4%	**2, *3A-*3D* + sequencing[c]	0

[a]Only subjects where phenotype and genotype were available. [b]DM: deficient metabolizer, IM: intermediate metabolizer, HM: high metabolizer; [c]Sequencing of ORF was performed in all cases with discordance between genotype and phenotype.

300 and 191 individuals, respectively, a subgroup of intermediate metabolizers could not be unequivocally distinguished [39,59].

Seven individuals in our study were TPMT deficient (0.6%), which substantiates a slightly elevated frequency distribution compared to the literature. The frequency of intermediate (10%) and normal metabolizers (89%) was no different to previous reports [60].

Moreover, it is interesting to note that the levels of normal/high activity in our population vary approximately three-fold with a maximum value of 65 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1}. When we analysed our cumulative frequency data for TPMT activities more intensively using RRSW functions, a small subgroup of individuals (1.8%) with very high activities was separated ranging from 51 to 65 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1} (Fig. 2b). This is in agreement with the observation by Yan *et al.* [61] who identified 123 individuals with a 'super high' TPMT activity in a set of 1211 clinical laboratory samples. Segregation analysis of RBC TPMT activities in 35 families including parent–offspring correlations and the correlation between siblings indicates that a proportion of the remaining variance in individuals with high TPMT activity is familial [62]. Thus, it may be speculated that in analogy to other drug metabolizing enzymes (e.g. cytochrome P450 2D6, glutathione *S*-transferase M1) a so-called ultra rapid metabolizer phenotype for TPMT may exist. As yet unidentified genetic factors may be partly responsible for this subgroup, but further detailed investigations are required to elucidate the molecular mechanism.

We focused our major interest on TPMT genotype–phenotype relationship because overall concordance rates from different population studies vary between 76% and 99% (Table 2). Especially in the case of intermediate metabolizers, the correlation is much less, as confirmed by two recent studies in Argentine and Bulgarian populations [43,44]. Here, only 57% and 63% of intermediate methylators, respectively, could be explained by known *TPMT* alleles, although rare alleles (e.g. **4, *6, *8*) have been considered. Moreover, some clinical studies have failed to demonstrate a homozygous mutant genotype in patients with thiopurine-related severe haematotoxicity and very low TPMT activity [24,63]. Thus, novel mutations could be expected provided that a representative sample size of individuals is investigated systematically.

Genotyping for the *TPMT* alleles in our population showed that the most frequent mutant alleles were **3A* (4.4%), **3C* (0.4%) and **2* (0.2%). This is completely in line with previous reports and confirms that the **3A* allele is the most relevant variant in Caucasians [64]. Because we did not identify any individual carrying the

3B* or **3D* allele, this demonstrates the extreme rare occurrence of these variants (Table 2). Therefore, the proposed diagnostic dilemma to distinguish with certainty between compound heterozygosity (3B/*3C*) and the **1/*3A* genotype, resulting in different phenotypes, appears to be not significantly relevant. Nevertheless, to provide an unequivocal genotyping method for **3B*, a molecular haplotyping method was recently described [58].

With regard to phenotype–genotype correlation, all seven TPMT deficient individuals could be explained unequivocally by genotyping for **2*, **3A* and **3C* alleles (Fig. 2). The difference between heterozygotes and subjects with no mutations was less clear-cut when the antimode of 22 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1} was applied. Seventeen of the 1096 homozygotes (1.6%) for the **1* allele showed a reduced activity ranging from 18 to 22 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1}. Systematic analysis of the coding region and intron–exon boundaries of *TPMT* revealed four novel heterozygous variants, leading to amino acid changes and were termed as *TPMT*9* and *TPMT*16* to **18*, which lead to amino acid changes. Strong evidence for the functional relevance of these mutations is the decreased activity *in vivo* in heterozygous individuals. Nevertheless, to completely characterize new alleles, expression studies in cell systems, such as mammalian cells or yeast, may be necessary. However, it is well-known that expression systems have some limitations as model systems for function *in vivo*, which has already been demonstrated for *TPMT*3C* with an enhanced proteolysis only in cos-1 cells but not in yeast [65,66]. Sequence comparison between human *TPMT* and the orthologues identified in primates, rodents, plants and bacteria showed that all the new polymorphic sites that we identified, as well as the residues with known clinical significance [A80P (**2* allele), A154T (**3B* allele) and Y240C (**3C* allele)], are located within the conserved regions of the polypeptide chain (data not shown). This observation corroborates the functional importance of the novel variants.

Considering the new alleles increased the concordance rate between intermediate phenotype and heterozygosity from 86% to 89%. However, it should be noted that none of the remaining 13 subjects with intermediate activity (1.1% of the overall population) discordant to *TPMT* wild-type genotype has shown any sequence variation in the ORF of *TPMT*. Conversely, seven individuals with one nonfunctional allele presented a TPMT activity above the cut-off of 22 nmol 6-MTG $\times$ g^{-1} Hb $\times$ h^{-1}, including one with the *TPMT*9* allele. Although sequencing of the 5′ and 3′ flanking region, as well as the entire intronic regions, was not performed, it appears to be unlikely that further variants may explain this discrepancy because it has been reported that additional screening for SNPs in the promoter region is unsuccessful in explaining differences between intermediate phenotype and *TPMT *1/*1* [24,39]. Additionally, previous studies have suggested that variation in *VNTR* allele frequency and the number of different *VNTR* repeats (type A and type B) influence TPMT activity [19,20,39,61]. However, contradictory results were reported, and the association with respect to the way that *VNTRs* influence TPMT activity is presently unclear.

Thus, alteration of TPMT by physiological or environmental factors may be the most likely explanation for the phenotype–genotype discrepancy observed not only in individuals with the **1/*1* genotype, but also in heterozygous individuals. For example, normal TPMT activities were determined in healthy subjects heterozygous for *TPMT*3A* [39], and it appears unsurprising that we found the *TPMT*9* allele both in an individual with intermediate and high activity. Consequently, we investigated the influence of gender and age on TPMT. Males had a 7% higher average TPMT activity than females ($P < 0.0001$) whereas age in our study (ranging between 18 and 65 years) did not alter TPMT activity. The impact of gender has been previously described for a healthy Saami population (8.3%) [67], and for average hepatic TPMT activity in a group of high methylators (11%) [68]. Nevertheless, RBC TPMT activity did not differ significantly between gender in some other ethnic populations, such as White Americans [4] and French [59,69]. This may be explained by the fact that, depending on the proportion of heterozygous individuals with intermediate activity, the average level of activity could be distorted. Thus, we compared only individuals carrying two wild-type alleles. The observed gender difference may be due to an inducing effect of testosterone on TPMT activity, which has already been demonstrated in rats. Male rats show an elevated renal TPMT activity compared to females [70]. With regard to smoking behaviour, male and female smokers showed significantly higher TPMT levels than non-smokers ($P < 0.05$). In this context, it is interesting to note that exposure to cigarette smoke extract in human lung epithelial-like cells leads to elevated levels of *S*-adenosylmethionine, which is the donor of the methyl group to the sulfur atom of thiopurines [71].

In consequence, additionally to disease status (e.g. impaired renal function), drug treatment (e.g. thiopurines, 5-aminosalicylates) and transfusion of RBC, which are important modulators of TPMT activity [27,34,39,72], environmental factors may also influence TPMT phenotyping results. Consequently, this can lead to a misclassification of the constitutional TPMT status. By contrast, genotyping offers some advantage. However, evaluation of each diagnostic test requires estimation of sensitivity and specificity, as well as the

positive and negative predictive value. Because all these parameters show values higher than 90% in our large-scale study, genetic testing for *TPMT* is worthy of adoption into clinical practice. Strikingly, testing for very rare mutant alleles does not significantly enhance the reliability of *TPMT* genotyping for intermediate methylators (86% versus 89%). Nevertheless, using automated genomic approaches, such as DNA-microarrays or Maldi-TOF technology, genotyping will allow a rapid, time and cost saving screening for all known *TPMT* inactivating mutations with maximal certainty for prediction. This may increase the routine clinical use of testing for TPMT to prospectively optimize thiopurine therapy.

Acknowledgements

The expert assistance of Monika Schick, Andrea Zwicker and Hans-Dieter Hayler is gratefully acknowledged.

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