# *Bayer Award*

# Whole-blood thiopurine *S*-methyltransferase activity with genotype concordance: a new, simplified phenotyping assay

Loretta Ford, Valerie Graham and Jonathan Berg

## Abstract

**Background:** We have developed a new thiopurine *S*-methyltransferase (TPMT) phenotyping method that measures TPMT activity in whole blood. To evaluate this assay, we compared it with conventional TPMT phenotyping, which uses a red blood cell (RBC) lysate and genotyping for analysis of common TPMT mutations.

**Methods:** Whole-blood and RBC lysates were prepared from 402 patients' samples received for routine analysis. The TPMT activity of lysates was determined using 6-thioguanine as substrate with high-performance liquid chromatographic (HPLC) analysis and fluorimetric detection. DNA was extracted from buffy coats using phenol–chloroform extraction. A multiplex amplification refractory mutation system (ARMS) strategy was used to screen for the common TPMT mutations TPMT*2 and TPMT*3 (TPMT*3A, TPMT*3C and TPMT*3D).

**Results:** TPMT activities were higher in the whole-blood (mean TPMT activity 51 nmol 6-MTG/gHb/h) compared with the RBC lysate (37 nmol 6-MTG/gHb/h). Overall, concordance with TPMT genotypic analysis was 97% for both the new whole-blood and standard RBC lysate methods. Between low TPMT activity and heterozygotes, both phenotypic methods gave a concordance of 79%.

**Conclusion:** Using multiplex ARMS testing for TPMT*2 and 3*C mutations to define the cut-off between low and normal TPMT activity, we have demonstrated that the new whole-blood TPMT phenotyping method performs as well as the conventional RBC lysate assay. This new method overcomes the need to prepare a RBC lysate, a process which is time consuming and increases analytical variation. The resulting assay is better suited to a regional or national TPMT phenotyping service.

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**Addresses**
Clinical Biochemistry Department, City Hospital, Dudley Road, Birmingham B18 7QH, UK

**Correspondence**
Dr L Ford
E-mail: Loretta.ford@swbh.nhs.uk

The Editorial team at the *Annals* would like to congratulate Dr Ford on winning the 2006 Bayer Award with a presentation based on this work

## Introduction

Thiopurine drugs such as azathioprine are widely used to treat autoimmune diseases by a number of clinical specialties, which can be attributed to their low cost and effectiveness as an alternative to steroid treatment.[1–3] Determination of thiopurine *S*-methyltransferase (TPMT) activity prior to treatment with azathioprine has, over the last couple of years, become established clinical practice.[4,5] This test identifies patients who, due to genetic variation, have undetectable TPMT activity and if treated with thiopurine drugs such as azathioprine experience severe adverse reactions.[6–8] Screening to identify patients with undetectable TPMT activity alone has been shown to be cost-effective,[7] and there is evidence that testing also enables dose modification in heterozygous patients, who exhibit reduced enzyme activity.[9] Patients with very high TPMT activity, who are at risk of hepatotoxicity caused by the increased production of methylated-thiopurine metabolites, may also benefit from dose modification.[7,10,11] A potential further role for testing may be in monitoring patients during treatment, allowing adjustment of thiopurine drug dosage in response to variation of TPMT enzyme activity.[12]

There are two strategies for determining patient TPMT status – phenotyping and genotyping – and for routine clinical use phenotyping is more firmly established.[7,13–15] Phenotypic analysis is typically performed using a red blood cell (RBC) lysate, the preparation of

which is a laborious process requiring several centrifugation and saline wash steps.[14,15]

We have provided a routine service for TPMT phenotypic analysis in the UK for three years, and have analysed approximately 16,500 patient samples and identified over 50 patients with undetectable TPMT activity (1:330). We have observed that TPMT activity for RBC lysates prepared from the same whole-blood sample can differ significantly, increasing analytical variation and on occasions giving falsely low results. We have therefore developed a new TPMT phenotyping method that uses a whole-blood lysate. To evaluate this new whole-blood method, we have compared it with both our established RBC lysate method and the TPMT genotype. Using the resulting phenotype–genotype concordance to define the cut-off between low and normal TPMT activity, we have determined reference intervals for this new whole-blood TPMT phenotypic assay.

### Study population and samples

Whole-blood samples ($n = 402$) collected into 4 mL EDTA tubes and received for routine analyses of TPMT activity were selected randomly over a four-week period in November 2005. Samples were stored for a maximum of two days at 4°C prior to analysis. As we have previously shown, TPMT activity is stable for up to eight days at room temperature. Samples more than eight days old at the time of analysis were therefore excluded from the study.

## Methods

### Preparation of whole-blood and RBC lysates

Whole-blood EDTA samples were mixed well by inversion, and two 200 μL aliquots were accurately pipetted into test tubes and capped. The tubes were then frozen for 15 min at −80°C to lyse the cells. The lysed cells were thawed in 600 μL of suspension buffer (0.1 M phosphate buffer, pH 7.4) and vortex mixed. RBC lysates were prepared in duplicate from the same EDTA blood samples as described previously,[14,16] except that the buffy coat was retained and added to a separate labelled test tube for DNA extraction and TPMT genotyping.

### TPMT enzyme reaction, haemoglobin concentration and high-performance liquid chromatographic (HPLC) analysis

The TPMT enzyme reaction, determination of haemoglobin concentration and HPLC analysis were all performed as described previously,[14,16] with the following modifications. Whole-blood lysate, RBC lysate, saline blank and 6-methylthioguanine (6-MTG, Sigma-Aldrich, Poole UK) controls (200 μL) were pipetted into glass vials and placed in a heating block at 37°C. TPMT incubation mixture (500 μL), containing 600 μmol/L of 6-thioguanine (6-TG, Alfa Aesar, Lancashire, UK) and 80 μmol/L of *S*-adenosyl methionine (SAM, Sigma-Aldrich, Poole, UK), warmed to 37°C, was added to each vial using an automatic pipette, and the vials were capped. After exactly 1 h, the reaction was stopped by placing the vials in a heating block set at 90°C for 10 min. The vials were then centrifuged at 4500 rotations per minute (rpm) for 5 min to remove the precipitated protein.

### TPMT genotyping

Total DNA (100–500 μg) was extracted from buffy coats using a standard phenol/chloroform extraction method.

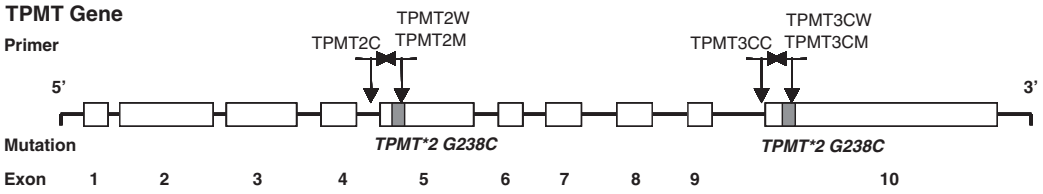
Multiplex amplification refractory mutation (ARMS)[17] analysis for the simultaneous identification of TPMT*2 and TPMT*3 alleles was performed based on the method described by Roberts *et al.*[18] To simplify interpretation, in this study, the individual ARMS reactions were combined into TPMT wild-type allele and mutant-specific multiplex ARMS reactions. For the TPMT*3 reaction, new wild-type and TPMT*3C mutant-specific primers were created and the specificity was checked using NCBI BLAST programme (www.ncbi.nih.gov/BLAST/). The sequences for all primers used in this study are shown in Table 1.

The multiplex polymerase chain reaction (PCR) was performed using QIAGEN Multiplex PCR kit (Qiagen House, West Sussex, UK). This PCR master-mix reduces the number of pipetting steps required to prepare each reaction, reducing the likelihood of errors. Reactions were prepared in 0.2 mL thin-walled tubes and contained a final concentration for each primer of 0.2 μmmol and approximately 1 μg of DNA. To guard against PCR failure, an internal control was included in each reaction (primers to the 574 bp sequence of the beta-2-microglobulin [B2M] gene).[18] Water blank controls for both the wild-type and mutant-specific ARMS reactions were prepared each time.

PCR reactions were performed in a TechneGenius Thermocycler (Techne, Cambridge, UK). The HotStarTaq DNA polymerase was activated by heating at 95°C for 15 min. Amplification was performed over 30 cycles of template denaturation at 94°C for 30 s, primer annealing at 62.5°C for 90 s and primer extension at 72°C for 30 s. After the last cycle, the reactions were incubated for an additional 2 min at 72°C.

Reactions were separated on 2% agarose gels prepared using 1 × TBE buffer (30 mmol Tris, 30 mmol boric acid and 0.1 mmol EDTA, pH 8.0) with 0.5 μg/mL of ethidium bromide. The resulting amplimers were visualized and images captured using a Gene Genius Gel Documentation and Analysis System (Syngene, Cambridge, UK).

Table 1 *Oligonucleotide primers used for multiplex ARMS TPMT analysis*




| Primer | Description | Direction | Primer sequence (5′–3′) | Product (bp) |
|---|---|---|---|---|
| BMF | Internal control | Sense | TgTAAACACTTggTgCCTgATATAgCTTgA | 574 |
| BMR | Internal control | Antisense | CATCAgTATCTCAgCAggTgCCACTAATCT | |
| TPMT2C | Common wild type | Sense | ATCTgCTTTCCTgCATgTTCT TTgAAACCC | 194 |
| TPMT2W | Wild type | Antisense | CACACCAACTACACTgTgTCCCCggTCTCC | |
| TPMT2M | TPMT*2 mutation | Antisense | CACACCAACTACACTgTgTCCCCggTCTCg | |
| TPMT3CC | Common wild type | Sense | ATTTTTAgTAgAgACAgAgTTTCACCATCT | 325 |
| TPMT3CW | Wild type | Antisense | TgTCTCATTTACTTTTCTgTAAgTAgTT | |
| TPMT3CM | TPMT*3C mutation | Antisense | TgTCTCATTTACTTTTCTgTAAgTAgTC | |

Primer positions are indicated relative to the sequence of human TPMT gene, Genbank accession number AB045146. Mismatched bases on primers are highlighted dark grey. New primers created in this study are shaded. Primers not highlighted are those described by Roberts *et al.*[18]
TPMT, thiopurine *S*-methyltransferase

### Statistics and calculations

The data were analysed using MINITAB Statistical Software Version 1.4 (MINITAB Inc, USA). For both the standard RBC lysate and whole-blood methods, TPMT activities were calculated from 6-MTG peak height, and are expressed in relation to haemoglobin concentration (nmol 6-MTG/gHb/h).

## Results

The distribution of RBC and whole-blood lysate TPMT activity compared with the genotyping results are presented in Figure 1.

### TPMT phenotyping

The results of phenotypic analysis of the 402 patient samples using the standard RBC lysate and new whole-blood method are shown in Table 2. TPMT activities using whole-blood lysates were higher compared to the standard RBC lysate method, with a relationship of $y = 0.6854 \times R^2 = 0.67$ and mean TPMT activities of 51 and 37 nmol 6-MTG/gHb/h, respectively.

The overall imprecision for both phenotyping methods was determined using EDTA whole blood collected from a volunteer. As shown in Table 3, the within- and between-batch imprecision was lower for the whole-blood method. The coefficient of variation between duplicate results for the whole-blood assay (5.1%) was half that using RBC lysate (10.4%).

Typical chromatograms for individuals with deficient, low and normal TPMT activities, using the two methods, are shown in Figure 2. An extra peak with a retention time of 1.35 min is seen on all whole-blood chromatograms, but it does not interfere with the measured product (6-MTG), which has a retention time of 1.8 min.

To ensure no interference from non-specific peaks with the same retention time as the measured product (6-MTG), the analysis of all high TPMT activity samples ($>55$ nmol 6-MTG/gHb/h using the standard RBC lysate method or $>80$ nmol 6-MTG/gHb/h for the whole-blood method) was repeated using an enzyme incubation of 0 min. No interfering peaks were detected in any of the patient samples with high TPMT activity.

### TPMT genotyping

In Caucasian, Asian and African-American populations, TPMT*2, TPMT*3A and TPMT*3C make up between 60 and 95% of mutant alleles for deficient TPMT activity.[19,20] Since many of the early reported TPMT*3B alleles are most likely the result of incomplete restriction digest,[17,21] a strategy of using TPMT*3C only to screen for all TPMT*3 mutations (TPMT*3A,[19] TPMT*3C[20] and TPMT*3D[22]) should be as effective as individual screening, and this is the approach taken here.

The results of TPMT genotyping are summarized in Table 4. Thirty-four patients (8.5% of total study

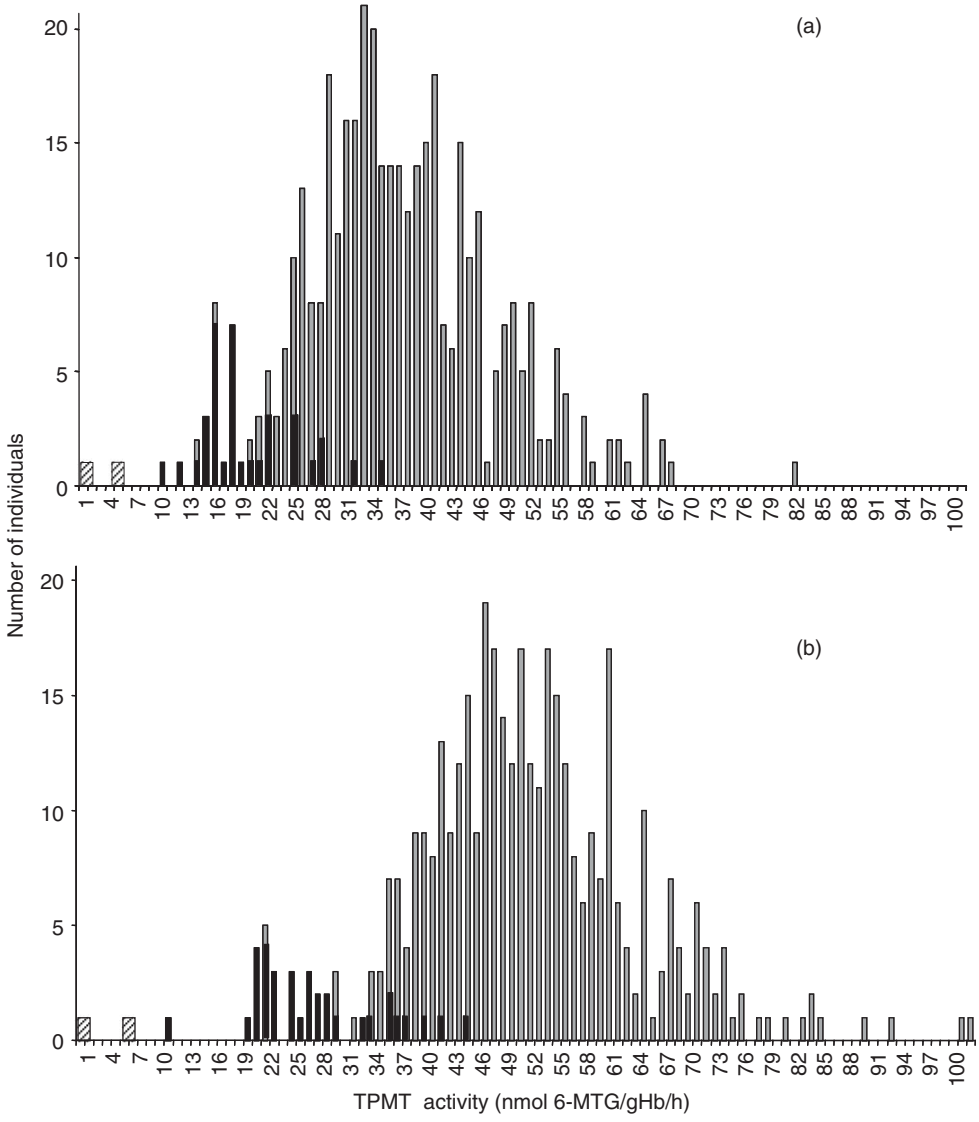



Figure 1 *Distribution of TPMT activity and genotype in 402 individuals. TPMT activity measured using (a) RBC lysate and (b) whole-blood lysate. Individuals with a TPMT*3/*3 genotype are shaded white with black lines, a heterozygous genotype; either TPMT*1/*2 or TPMT*1/*3 are shaded black and TPMT*1/*1 genotype grey. TPMT, thiopurine S-methyltransferase; 6-MTG, methylthioguanine*

Table 2 *TPMT phenotyping results using whole blood and RBC lysates*

| Method | Mean | Median | Standard deviation | Standard error mean | Min. | Max. |
|---|---|---|---|---|---|---|
| Standard RBC | 37.0 | 36.0 | 11.3 | 0.56 | 0.00 | 82.0 |
| Whole blood | 51.0 | 50.0 | 13.6 | 0.68 | 0.00 | 102.0 |

*n*=402 patients; All values are in nmol 6-MTG/gHb/h; RBC, red blood cell

population) were found to be heterozygous for either TPMT*2 or TPMT*3 mutation. Both patients found to have deficient TPMT activity were homozygous for the TPMT*3 mutation (TPMT*3/*3 genotype).

## Phenotype–genotype concordance

To define the cut-off between low and normal TPMT activity for both the whole-blood and RBC lysate phenotyping methods, the phenotype–genotype

Table 3 *Within-batch and between-batch imprecision data for whole blood and RBC lysate TPMT phenotyping methods*

| Method | Imprecision | Mean TPMT activity (nmol6-MTG/gHb/h) | CV (%) | *n* |
|---|---|---|---|---|
| Standard RBC | Within-batch | 39 | 3.6 | 10 |
| | Between-batch | 53 | 8.0 | 20 |
| Whole Blood | Within-batch | 45 | 2.7 | 10 |
| | Between-batch | 53 | 7.6 | 20 |

RBC, red blood cell; TPMT, thiopurine *S*-methyltransferase; 6-MTG, 6-methylthioguanine; CV, coefficient of variation

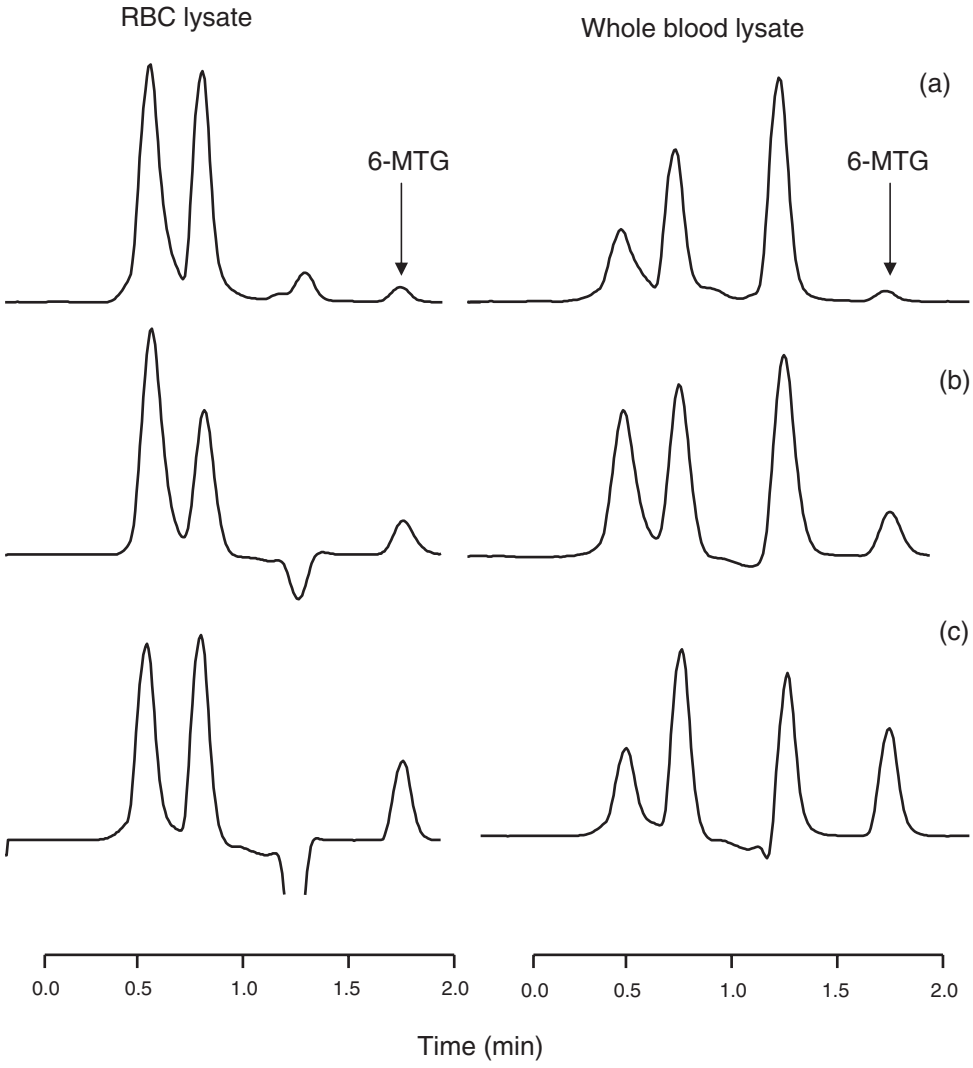



Figure 2 *Comparison of representative chromatograms of RBC and whole-blood lysate methods: (a) deficient TPMT activity, (b) low TPMT activity, (c) normal TPMT activity. TPMT, thiopurine S-methyltransferase; 6-MTG, methylthioguanine*

concordance was maximized by applying the heterozygote frequency of 8.5% and wild-type frequency of 85% found in this study. This approach assumes all mutations not screened for will result in similar activity to TPMT*2 and TPMT*3C mutations. It does not take into account patients with high TPMT activity which genotyping cannot identify.

The results of maximizing the phenotype–genotype concordance for the whole-blood and standard RBC lysate methods are presented in Table 5. For the standard RBC lysate TPMT phenotype assay, the cut-off between low and normal TPMT activity is $<23$ nmol 6-MTG/gHb/h and the overall phenotype–genotype concordance 96.5%. Applying the same heterozygote and

wild-type frequency to the whole-blood method, the cut-off between low and normal TPMT activity is higher, <35 nmol 6-MTG/gHb/h, but the overall concordance with genotyping is the same as that for the standard RBC lysate method at 96.8%.

## Discussion

The new whole-blood assay described here overcomes the need to prepare a RBC lysate, enabling a significant increase in sample throughput of the TPMT phenotype assay. This is important, as one key goal of a referral service is to minimize the delay to patient treatment by providing fast turn around of results. A further advantage to our new assay is that the whole-blood lysate is a more homogenous starting material than the RBC lysate. Depending on the operator, RBC lysates may contain varying amounts of other blood cells, as well as saline, and these variables increase the imprecision of the assay. Using whole blood overcomes this variation and is reflected in the lower intra- and inter-assay variation, and less variation between duplicate analyses compared with the RBC assay.

Comparison of the distribution of TPMT activity for the 402 patients studied using both phenotyping methods reveals that patients with deficient TPMT activity are discriminated from the rest of the population by both methods. However, the distinction between low and normal TPMT activity is better using the whole-blood assay.

In order to establish reference intervals, we used genotyping to screen for common TPMT mutations in the study population, and then applied the frequency of heterozygotes (TPMT*1/*2 and TPMT*1/*3 genotype 8.5%) and wild-type homozygotes (TPMT*1/*1 genotype 85%) obtained to both the standard RBC and new whole-blood assay results. The cut-off between low and normal TPMT activity (bottom 8.5% of TPMT activity results) for the new whole-blood assay is <35 nmol 6-MTG/gHb/h. For our standard RBC TPMT assay, the cut-off between low and normal TPMT activity of <23 nmol 6-MTG/gHb/h is slightly lower than the <25 nmol 6-MTG/gHb/h we reported previously.[16] Applying these reference intervals, overall, the phenotype–genotype concordance for both methods is identical (97%), as is the concordance between low TPMT activity and a mutant heterozygote genotype (79%). It is interesting to note that the concordance between a TPMT*1/*2 genotype and low TPMT activity for both phenotypic assays was 100%, whereas for a TPMT*1/*3 genotype the concordance was only 77.4%. It is therefore possible that specific TPMT mutations could be predicted from the phenotype, and depending on the enzyme activity expressed this could be associated with certain adverse reactions to thiopurine drug treatment.

The genotype–phenotype concordance between low TPMT activity and a heterozygote genotype reported here is lower compared with some recently published studies.[22–24] However, previous studies have typically been based on healthy controls. As such they do not reflect the population we routinely test, which includes patients of different ethnic origins and who may also be receiving multiple drug treatments. Our own audits have shown that it is not uncommon for patients to be treated with five different medications at a time, including azathioprine.[25] Unlike genotyping, phenotyping quantifies the biologically active enzyme and so more accurately reflects *in vivo* TPMT activity. Drug–drug interactions may potentiate thiopurine toxicity by inhibiting TPMT activity,[12,26,27] and treatment with thiopurine drugs can induce TPMT activity by 10–33% after just three months.[9,10] Therefore, genotyping offers an over-simplification of assigning TPMT activity status and could explain why the genotype–phenotype concordance between low TPMT activity and a heterozygote genotype is higher in studies that do not use routine patient samples.

Table 4 *TPMT Genotyping results for 402 population studied*

| Genotype | Number of patients | % of patient total |
|---|---|---|
| TPMT *3/*3 | 2 | 0.5 |
| TPMT*1/*3 | 31 | 7.7 |
| TPMT*1/*2 | 3 | 0.75 |
| TPMT*1/*1 | 366 | 91 |

TPMT, thiopurine *S*-methyltransferase

Table 5 *Phenotype–genotype concordance*

| Method | Cut-off level between low/normal TPMT activity (nmol 6-MTG/gHb/h) | % Concordance between phenotype and genotype | | | | | |
|---|---|---|---|---|---|---|---|
| | | Normal TPMT activity and TPMT*1/*1 | Deficient TPMT activity and TPMT*3/*3 | Low TPMT activity and TPMT*1/*2 | Low TPMT activity and TPMT*1/*3 | Low TPMT activity and all heterozygotes | Overall |
| Standard RBC | <23 | 98.1 | 100 | 100 | 77.4 | 79.4 | 96.5 |
| Whole blood | <35 | 98.4 | 100 | 100 | 77.4 | 79.4 | 98.5 |

RBC, red blood cell; TPMT, thiopurine *S*-methyltransferase; 6-MTG, 6-methylthioguanine

## Conclusion

We have developed a new phenotypic assay for TPMT that uses a whole-blood lysate. Using genotyping, we have demonstrated that our new TPMT whole-blood assay performs as well as, and has clear advantages over, the standard RBC assay. Sample preparation is fast and simple, resulting in reduced analytical variation. Whole blood, theoretically, has the potential to better reflect *in vivo* changes in TPMT enzyme activity such as induction caused by thiopurine drug treatment, making it more suited for routine clinical use. Future work will focus on monitoring changes in TPMT activity before and during thiopurine treatment to determine what effect, if any, induction has on patient TPMT activity, in order to create new simple treatment protocols, further improving thiopurine drug efficacy.

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