

# Determination of Thiopurine S-methyltransferase Activity using Reverse-Phase High-Performance Liquid Chromatography Assay with Ultraviolet Detection: Reference Values for the Indian Population

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## Abstract

**Objective:** To determine thiopurine S-methyltransferase (TPMT) activity and the range of TPMT activity values for the Indian population. **Methods:** An isocratic reversed-phase high-performance liquid chromatography method with ultraviolet detection for the determination of TPMT activity in red blood cells was developed and validated. TPMT activity (nmol of 6-methylmercaptapurine [6-MMP]/h/ml erythrocytes) was measured based on the conversion of 6-mercaptopurine to 6-MMP with S-adenosyl-L-methionine. The stability of erythrocyte lysate at  $-20^{\circ}\text{C}$  and the whole-blood specimens for TPMT activity were determined at  $4^{\circ}\text{C}$ . TPMT enzyme activity was measured in 150 patients who were not on azathioprine (AZA), and population difference in TPMT enzyme activity was evaluated. **Results:** The assay was linear from 1.0 to 60.14 nmol 6-MMP concentrations. The chromatogram peaks obtained were baseline separated for 6-MMP and the internal standard. The bias and precision of low-, medium-, and high-quality controls (QC) were within acceptable limits. Stock and lysate specimens were found to be stable in  $-20^{\circ}\text{C}$ . The Indian populations have a considerable number of patients with low- and intermediate-TPMT activity. The wide range of TPMT activity in the study population (3.85–35.68 nmol/h/ml erythrocytes) is suggestive of high inter-individual variability in the formation of 6-thioguanine nucleotide formation, and therefore clinical efficacy and safety of AZA. **Conclusion:** A simple isocratic method was developed and validated for measuring TPMT activity in erythrocytes. Measuring TPMT activity before initiating AZA may help to decide the initial dose of AZA in patient management.

**Keywords:** 6-mercaptopurine, 6-methylmercaptapurine, azathioprine, Thiopurine S-methyltransferase

## INTRODUCTION

Thiopurine S-methyltransferase (TPMT) enzyme is a biotransformation phase II cytosolic enzyme responsible for the S-methylation of aromatic and heterocyclic sulfhydryl compounds. Thiopurine medications such as azathioprine (AZA) and mercaptopurine [MP] wherein prodrug MP form the cornerstone of therapy in inflammatory bowel disease (IBD), autoimmune bullous diseases, and multiple sclerosis. Both MP and AZA (a MP prodrug) are metabolized to 6-thioguanine nucleotide (6-TGN) and 6-methylmercaptapurine (6-MMP) nucleotide which is responsible for both cytotoxic and immunosuppressant effects.<sup>[1,2]</sup>

There is a good correlation between the serum concentration of 6-TGN with both the therapeutic efficacy and adverse

effects of AZA.<sup>[3,4]</sup> The major adverse effects of AZA include myelosuppression, hepatotoxicity, and pancreatitis.<sup>[5-7]</sup> One of the most serious dose-dependent adverse drug reactions is myelosuppression, which is primarily related to 6-TGN. Toxic concentration of 6-TGN is attributed not only to overdosing but also to a decreased inactivation of the drug through the TPMT enzyme pathway.

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TPMT enzyme activity can be phenotypically determined by the quantification of 6-MMP formed in the conversion reaction, using 6-MP as the enzyme substrate.<sup>[8-10]</sup> TPMT enzyme activity of individuals is largely determined by genetic constitution, and the genetic variations cause wide inter-individual variability in metabolism of AZA and thereby the concentration of 6-TGN. In a article published by Weinshilboum and Sladek, erythrocytic TPMT enzyme activity was categorized into low, intermediate, and normal/high activity in 0.3%, 11%, and 89% of population, respectively.<sup>[11]</sup> The study published by Chun *et al.* in IBD pediatric patients treated with AZA required discontinuation of the therapy in up to 20% of patients due to the occurrence of various adverse events, which are dose-dependent such as leukopenia (27%) and myelosuppression (1.5%–5%), and nondose-dependent such as nausea, vomiting (15.5%), and pancreatitis (7.5%).<sup>[12]</sup> In patients with low TPMT activity, 6-TGN concentration will be higher which may lead to potentially fatal bone marrow suppression. TPMT enzyme activity status of patients is indirectly a major determinant with potential to predict the clinical efficacy and toxicity of AZA.

It is crucial to individualize the dose based on TPMT to achieve maximum clinical efficacy and minimum adverse drug reactions. It is highly recommended that this phenotype test should be estimated before initiating AZA for any clinical conditions,<sup>[13]</sup> followed by monitoring the metabolite concentration to maximize efficacy and minimize toxicity.

### Aims

The aim of our study was to develop and validate an assay to determine the activity of TPMT enzyme in red blood cells and to study the distribution of this activity in a heterogeneous Indian population.

## MATERIALS AND METHODS

### Settings and design

The study was carried out in the Department of Pharmacology and Clinical Pharmacology in collaboration with Department of Gastroenterology in Christian Medical College, Vellore, Tamil Nadu.

### Reagents and chemicals

6-MP, 6-MMP, S (5'-Adenosyl)-L methionine iodide (SAM), dithiothreitol (DTT), internal standard (aminoacetophenone), trichloroacetic acid, acetonitrile, and methanol (high-performance liquid chromatography [HPLC] grade) were obtained from Sigma-Aldrich (India). Phosphate-buffered saline (PBS, pH 7.4; 1M) was an in-house prepared solution.

### Sample collection

Blood samples were collected from volunteers in K2 ethylenediaminetetraacetic acid (EDTA) tubes. Each volunteer

donated 10 ml of blood in five microtubes (2 ml each) at the start of the assay development.

### Erythrocytes lysate preparation

The erythrocyte isolation procedure was performed as follows: 2 ml of blood collected in K2 EDTA tube was centrifuged at 6400 rpm for 3 min. Plasma, leukocytes, and the upper layer of the erythrocytes (buffy coat) were removed. The remaining erythrocyte pellet was washed two times with PBS and centrifuged for 3 min at 6400 rpm. About 1ml of washed erythrocytes was mixed with 4 ml of millipore water and centrifuged at 13,000 rpm for 10 min after determining the hematocrit using the microhematocrit method. The supernatant was separated and stored at – 20°C in microtubes till analysis for a maximum period of 8 weeks. TPMT activity is reported to be stable in this storage condition.

### Mobile phase preparation

40 mM double salt phosphate: dipotassium phosphate+monopotassium phosphate ( $K_2HPO_4 + KH_2PO_4$ ) buffer was prepared in Millipore water, filtered before use.

### Principle of TPMT assay

The assay is based on the principle of TPMT catalyzed conversion of 6-MP to 6-MMP using SAM as the methyl donor. The amount of 6-MMP produced depends on the TPMT enzyme activity in the processed patient's blood specimen. One unit of TPMT activity represents the formation of 1 nmol of 6-MMP/1.5 h of incubation. The quantification of 6-methylmercaptapurine was performed by an isocratic HPLC assay. The enzyme activity was normalized per hour per milliliter of packed erythrocytes.

### Preparation of standard samples

Two separate primary stocks of 6-MMP (1 mg/ml) in Millipore® water were prepared for the calibration standards and the quality controls (QC). The required concentrations of standards and QC were obtained by secondary and tertiary dilution in human erythrocyte lysate from the primary stock to get a final concentration of 60.14, 40.09, 20.05, 10.02, 5.03, and 1.0 nmol/mL for calibration standards and 50.12, 15.05, and 2.0 nmol/ml for QC, respectively.

### Extraction method

#### Extraction method for calibration standards

For extraction of standards, 15 µl of 3 mM dithiothreitol (DTT) in phosphate buffer was mixed with 200 µl of lysate to simulate the extraction method of patient blood specimens and to this was added 35 µl of SAM (0.25 mM) and DTT (1 mM) and mixed well. After adding stop solution (4 µg/ml aminoacetophenone as internal standard prepared in 2 M trichloroacetic acid in ACN), the solution was kept in – 20°C for 10 min and then centrifuged at 13,000 rpm for 10 min. The supernatant was separated and 80µl was injected to HPLC.

#### Extraction method for patient sample

6-MP (10 µL; final concentration: 150 mmol/L) was added to 200 µL of red cell lysates containing 105 µL of 3 mM DTT

in phosphate buffer and preincubated for 5 min at 37°C. DTT prevents oxidation of thiol group of 6-MP.<sup>[14]</sup> The reaction was started by adding 35 µL of a mixture of SAM and DTT (final concentration of SAM, 0.25 mmol/L; final concentration of DTT: 1 mmol/L). The tubes were incubated for 1.5 h at 37°C, and the reaction was stopped by adding stop solution (4 µg/ml of aminoacetophenone as an internal standard in 2 M trichloroacetic acid in Acetonitrile) and mixed for 10 s. Samples were kept in -20°C for 10 min. After cooling, the tubes were centrifuged at 13,000 rpm for 8 min, and 80 µl of the supernatant was analyzed by HPLC.

### High-performance liquid chromatography conditions

HPLC system used for assay development and validation was Shimadzu LC-2010A HT, and resolution was achieved with reverse-phase analytical column, Capcell Pack C18 (250 mm × 4.6 mm, i.d. 5 µm, Shiseido) at a flow rate of 1.3 mL/min and column temperature of 35°C. The mobile phase was an isocratic flow of 40 mM double salt phosphate buffer and methanol (HPLC grade) at a ratio of 79:21. The total run time was 16 min, and typical injection volume was 80 µl with analytes detected at 290λ. Blank samples, prepared without adding 6-MP and SAM, were analyzed with each patient specimen. Peak areas of the spiked sample were subtracted from the peak areas of blank samples to get the 6-MMP concentration and the TPMT enzyme activity was determined.

### Measurement of TPMT phenotype activity

The TPMT activity of patients was measured by comparing the 6-MMP formed after incubation, against 6-MMP calibration curve (nmol 6-MMP formed per hour per ml of packed erythrocytes). All the patient samples were extracted in two sets; with and without SAM to quantify the 6-MMP generated without the addition of methyl donor. The amount of 6-MMP generated without adding SAM is subtracted from the amount of 6-MMP generated after adding SAM.

### Assay validation

Assay validation was performed based on guidelines issued under the United States, Food and Drugs Administration guidance for the bioanalytical method of validation.

A 6-point calibration curve was assessed using 6-MMP concentrations from 1.0 to 60.14 nmol/ml and three QC 2.0 to 15.05 nmol/ml. Standard samples were used to determine the analytical recovery, accuracy, and precision of the method over the entire concentration range. The linearity was calculated on six replicates performed over a 2-month period. The slope, the intercept, and the correlation coefficient of each calibration curve were estimated by plotting the ratio of 6-MMP peaks area and internal standard I.S. peak area.

### Bias and precision of quality controls

Bias and method precision (intra-day and inter-day precision) – as the degree of agreement of different extractions of QCs was determined. The formulas used to calculate bias and precision are mentioned below.

Bias =

$$\frac{\sum_{n=1}^x \left( \frac{|\text{Observed value} - \text{Expected value}|}{\text{Expected value}} \right)}{x} \times 100$$

where,  $x$  = total number of observations

Precision (relative standard deviation [SD]) = (SD/mean) × 100

To calculate bias and precision of the assay, we performed five different extractions of low-, medium-, and high-QCs on day 1 and another five different extractions on day 3. The calibration standards used were same on day 1 and day 3. The days were counted from the time of preparation of QC.

### Bias and precision for the lower limit of quantitation

Bias and precision were determined for the lower limit of quantification (1.0 nmol/mL erythrocyte concentration). Five different extractions of lower limit of quantitation (LLOQ) were performed and were compared against the calibration curve prepared on the same day [Table 1].

### Linearity of calibration curve

The linearity of calibration curve was determined for 6-MMP concentrations ranging from 1.0 to 60.14 nmol/ml and was validated using three QCs ranging from 2.0 to 50.12 nmol/ml [Table 1].

### Reproducibility (reinjection)

We checked the reproducibility of QCs by performing five repeat injections from the same extract. The low, medium, and high concentrations QC were compared against the calibration curve to determine the accuracy and precision of 6-MMP extraction methods.

### Stability

Stability of primary stock of 6-methyl mercaptopurine, 6-thioguanine, and S (5'-Adenosyl)-L methionine iodide/dithiothreitol

Stability of 6-MMP primary QC stock was evaluated by preparing low and high QC from stock stored over a period of 34 days in -20°C against calibration standards prepared from

**Table 1: Bias and precision of quality controls and lower limit of quantification**

| Different extractions                           | Bias (%) | Precision |
|-------------------------------------------------|----------|-----------|
| Low QC (2 nmol 6-MMP)                           | 7.0      | 0         |
| Medium QC (15 nmol 6-MMP)                       | -5.6     | 0.35      |
| High QC (50 nmol 6-MMP)                         | -6.9     | 1.25      |
| LLOQ (1.0 nmol 6-MMP)                           | 5        | 0         |
| Reproducibility (reinjection - same extraction) |          |           |
| Low QC                                          | 7        | 0         |
| Medium QC                                       | 10.57    | 0.84      |
| High QC                                         | 3.92     | 0.22      |

QC=Quality controls, 6-MMP=6-Methylmercaptopurine, LLOQ=Lower limit of quantification

newly prepared primary stocks. 6-MP stability over a period of 58 days at  $-20^{\circ}\text{C}$  was determined indirectly by determining the TPMT activity of five patients. The stability of SAM/DTT stored as multiple aliquots in  $-20^{\circ}\text{C}$  was determined over a period of 75 days by indirectly measuring TPMT activity of patients using newly prepared SAM/DTT and compared against extracts using stored SAM/DTT at  $-20^{\circ}\text{C}$ .

#### Autosampler stability

The stability of extracted sample in the autosampler was determined over a period of 5 h at  $4^{\circ}\text{C}$ .

#### Stability of TPMT enzyme activity in the lysate at $-20^{\circ}\text{C}$ and whole blood at $-4^{\circ}\text{C}$

The stability of TPMT enzyme activity in the whole blood was determined at  $4^{\circ}\text{C}$  for 24 h, and in the lysate was determined at  $-20^{\circ}\text{C}$  over a period of 30 days, respectively, by comparing 6-MMP concentrations against newly prepared calibration standards.

### TPMT enzyme activity in the Indian population

#### Inclusion criteria

- All patient specimens received in the Clinical Pharmacology Unit for the estimation of TPMT activity.

#### Exclusion criteria

- Patients who received AZA.

To determine the distribution of TPMT enzyme activity (nmol/h/ml of erythrocytes) in the Indian population, enzyme activity of 150 patients who were not on AZA were measured. The distribution of TPMT enzyme activity was determined, and the population were classified into low, intermediate, normal, high, and very high TPMT activity according to 1<sup>st</sup>, 5<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup>, 95<sup>th</sup>, and 99<sup>th</sup> percentile,

respectively. Based on patient demographics, ethnicity was broadly classified as Dravidian, Indo-Aryan, and North East. People residing in the South Zone of India were considered as Dravidian in origin, and those residing in East, West, North, and Central zones were categorized as Indo-Aryan origin. The North East zone of India was considered as of different ethnic dissent.

#### Statistical analysis used

The median and interquartile range were determined, and graphs were generated using R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria) and Microsoft Excel.

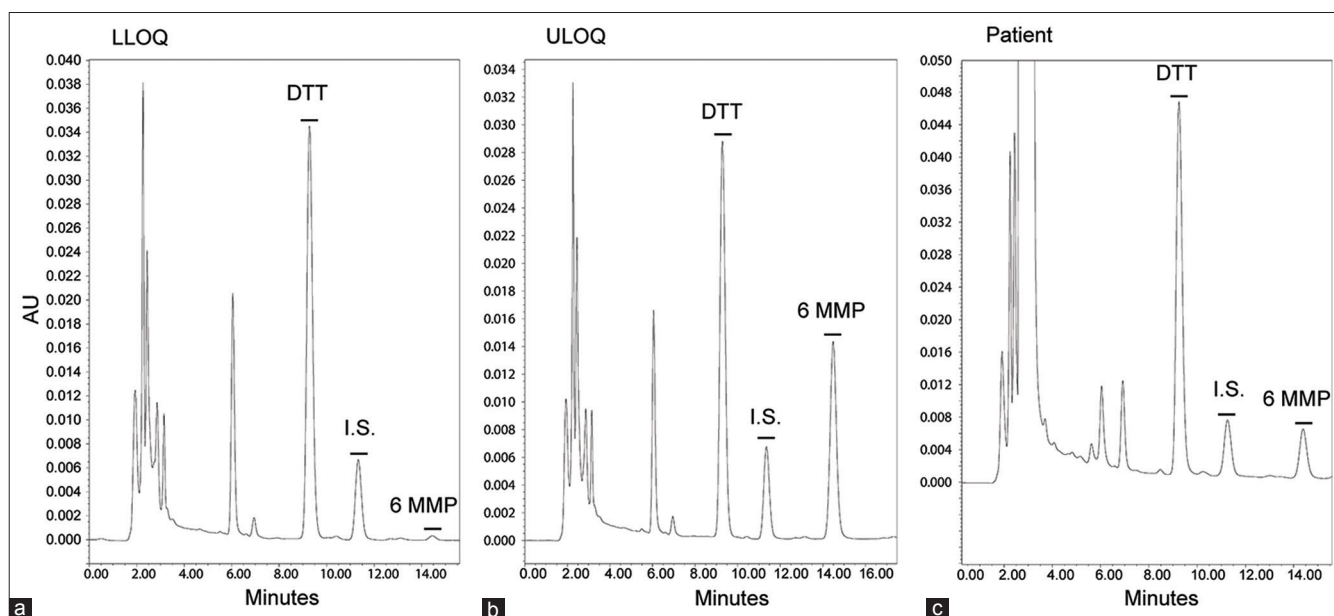
## RESULTS

### Chromatogram

The isocratic assay developed was reliable to quantify erythrocytic TPMT enzyme activity in patients with a total run time of 16 min. The representative chromatogram is shown in Figure 1 for a patient with a TPMT activity of 11 nmol/h/ml erythrocytes. The retention time of the internal standard I.S. was 11.3 min and that of 6-MMP was 14.5 min. The chromatogram peaks were symmetric and were fully baseline separated. The assay was linear from 1.0 to 60.14 nmol 6-MMP concentrations.

### Bias and precision of quality controls and lower limit of quantitation

The intra-day accuracy and precision of low-, medium-, and high-QC were in agreement with the calibration curve. The imprecision of five different extractions of LLOQ was 0.0%. The precision and bias of QC and LLOQ and the reproducibility are shown in Table 1.



**Figure 1:** Chromatogram showing (a) lower limit of quantitation, (b) upper limit of quantitation (c) Patient (with a TPMT enzyme activity of 11 nmol/hr/ml erythrocytes)

Stability

The stock was found to be stable for 6-MMP, 6-MP, and SAM/DTT for a period of 30 days, 58 days, and 75 days, respectively, when stored at – 20°C. Blood lysate was stable for 36 days, and lysate calibration standards of 6-MMP were stable for 43 days when stored at – 20°C. The TPMT activity in whole blood was stable for a period of 24 h when stored at 4°C. TPMT enzyme activity did not deteriorate in an extracted sample over a period of 5 h when stored in the autosampler at 4°C.

TPMT enzyme activity in the Indian population

The distribution of TPMT enzyme activity was determined (range: 3.85–35.68 nmol of 6-MMP/h/ml erythrocytes) in the Indian population [Figure 2]. Around 5.3% of population had TPMT activity <8.3 nmol/h/ml erythrocyte. Most of the patients (90%) were in the range of 8.33–27.24 nmol 6-MMP/h/ml erythrocytes enzymatic activity [Table 2]. The mean (SD) of TPMT activity for males (*n* = 85) was 15.31 (5.48) and that of females (*n* = 65) was 16.99 (6.0). Since there were only three patients from North East zone, only Dravidian (*n* = 53) and Indo-Aryan (*n* = 93) population were analyzed for their distribution of TPMT activity [Figure 3]. The mean (SD) of TPMT activity for Dravidian and Indo-Aryan population was 16.49 (5.84) and 15.80 (5.82), respectively.

Discussion

An isocratic method has been developed and validated for the quantification of TPMT enzyme activity using 6-MP as a substrate in isolated human erythrocytes, using a modified method (isocratic mobile phase flow) published by Indjova *et al.*<sup>[9]</sup> The chromatogram peaks obtained were baseline separated for 6-MMP and the internal standard. The bias and precision of low-, medium-, and high-QC, and LLOQ were satisfactory. The calibration curve range of 1.0–60.14 nmol 6-MMP was adequate to cover the range of TPMT enzyme activity in a population. The use of an isocratic mobile phase method decreased the run time required to determine the TPMT activity.

The TPMT enzyme activity in isolated erythrocytes maintained at 4°C and – 20°C over a period of 24 h and 47 days, respectively, showed no loss of enzyme activity when the 6-MMP concentrations were compared against newly prepared calibration standards. This finding was similar to that reported by Lennard and Singleton.<sup>[15]</sup> Whole-blood specimens should be transported

to laboratory centers for determination of TPMT activity within 24 h of blood collection with the temperature maintained at 4°C.

The proportion of patients with normal/high TPMT activity is similar in our study compared to those reported by Zhang *et al.* and Chouchana *et al.*<sup>[1,16]</sup> [Table 3].

Table 2: Classification of thiopurine S-methyltransferase enzyme activity

| Classification | TPMT enzyme activity (nmol/h/ml erythrocytes) | Proportion of patients (%) |
|----------------|-----------------------------------------------|----------------------------|
| Very low       | <4.89                                         | 1                          |
| Low            | 4.89-8.33                                     | 4                          |
| Intermediate   | 8.33-12.37                                    | 20                         |
| Normal         | 12.37-18.61                                   | 50                         |
| High           | 18.61-27.24                                   | 20                         |
| Very high      | >27.24                                        | 5                          |

TPMT=Thiopurine S-methyltransferase

Table 3: Study comparison according to thiopurine S-methyltransferase enzyme activity

| Study                                                    | TPMT activity*    |                            |                           |
|----------------------------------------------------------|-------------------|----------------------------|---------------------------|
|                                                          | Low, <i>n</i> (%) | Intermediate, <i>n</i> (%) | Normal/high, <i>n</i> (%) |
| Comparison 1                                             | <5.1              | 5.1-13.7                   | >13.7                     |
| Lowry <i>et al.</i> ( <i>n</i> =170) <sup>[17]</sup>     | None              | 14                         | 83                        |
| Prabha <i>et al.</i>                                     | 1.3               | 37.3                       | 61.3                      |
| Comparison 2                                             | <4.89             | 4.89-12.37                 | >12.37                    |
| Zhang <i>et al.</i> ( <i>n</i> =9) <sup>[16]**</sup>     | 5.2               | 26.3                       | 68.4                      |
| Prabha <i>et al.</i>                                     | 1                 | 24                         | 75                        |
| Comparison 3                                             | <4.3              | 4.3-8.7                    | >8.7                      |
| Chouchana <i>et al.</i> ( <i>n</i> =4911) <sup>[1]</sup> | 0.7               | 10.4                       | 88.8                      |
| Prabha <i>et al.</i>                                     | 1.3               | 6                          | 92.67                     |

\*TPMT activity classified according to cited literature, \*\*TPMT activity measured after initiating azathioprine. TPMT=Thiopurine S-methyltransferase

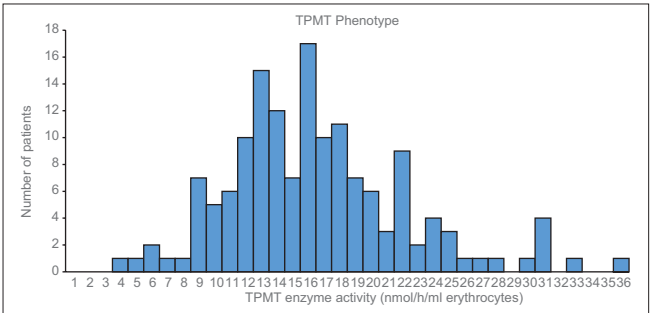


Figure 2: Distribution of TPMT enzyme activity in the Indian population

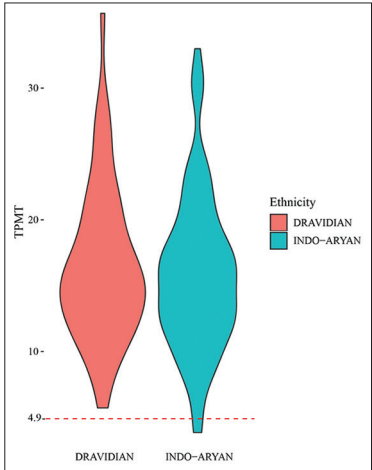


Figure 3: Violin plot showing difference in the distribution of TPMT activity for Dravidians and Indo-Aryans. Mean ± standard deviation = 16.49 ± 5.84 (Dravidian, *n* = 53) and 15.80 ± 5.82 (Indo-Aryan, *n* = 93)

There was no significant difference in TPMT activity for the Dravidian population versus Indo-Aryan population (mean  $\pm$  SD = 16.49 (5.84) and 15.80 (5.82), respectively), although a trend toward a lower activity is seen in the Indo-Aryan category. We acknowledge that this finding can only be confirmed with more number of patients.

The Indian population have a considerable number of patients with low and intermediate TPMT activity. The wide range of TPMT activity in the study population (3.85–35.68 nmol/h/ml erythrocytes) is suggestive of high inter-individual variability in the formation of 6-TGN formation, and therefore efficacy and safety of AZA. Therefore, routine measurement of TPMT enzyme activity may play a role in the determination of the initial dose of AZA in the Indian population. Further studies are planned to investigate the role of individualizing the dose of AZA using a combined approach of TPMT enzyme activity, sequential monitoring of 6-TGN, and clinical-based outcome.

## CONCLUSION

A simple isocratic method was developed and validated for measuring TPMT activity in erythrocytes. The range of TPMT activity measured in 150 Indian patients is 3.85–35.68 nmol/hr/ml erythrocytes. Measuring TPMT activity before initiating AZA is important to decide the initial dose of AZA in patient management.

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## Conflicts of interest

There are no conflicts of interest.

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