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ORIGINAL ARTICLE

Thiopurine metabolite level and toxicity in Indians with inflammatory bowel disease

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Abstract

Background and Aim: A lower dose requirement and higher toxicity of thiopurine is reported in Asian patients with inflammatory bowel disease (IBD) as compared with Caucasian patients. These reports are based on thiopurine methyltransferase measurement studies rather than metabolite estimation.
We studied the utility of thiopurine metabolite estimation in Indian patients with IBD and compared dose and toxicity with Asian and Caucasian patients.
Methods: In this prospective study, 6-thioguanine nucleotide (6-TGN) and 6-methylmercaptopurine levels were determined by HPLC in 76 IBD patients treated with thiopurines. The levels were correlated with dose, disease activity, and toxicity. The dose-related metabolite levels and toxicity were compared with Caucasian and Asian patients reported in literature.
Results: Of the 76 patients (32 women, mean age: 35.9 [SD: 14.54] years, 36 Crohn's disease and 40 ulcerative colitis), 1 non-compliant patient had undetectable level of metabolites. Of the 75 patients, 21(28%) had therapeutic level of 6-TGN, 37(49%) had subtherapeutic level and 17(23%) had supratherapeutic level. The 6-methylmercaptopurine levels ranged up to 4971 pmol/8 $\times$ 10^8 red blood cells. Six (8%) patients showed toxicity. Thiopurine dose was optimized in 20 (26.31%) patients. Dose-based metabolite levels were comparable to Asian and Caucasian patients. The toxicity (8%) observed in our patients was less than that reported (12–39%).
Conclusion: Half of the patients in this study had low and a quarter had high 6-TGN levels. One-fourth of the patients needed dose modification. The dose-based metabolite levels were comparable and the toxicity was less than that reported in Asian and Caucasian patients.

Introduction

Inflammatory bowel disease (IBD) is a chronic, immunologically mediated, often progressive disease in a genetically susceptible host. The immunosuppressants, azathioprine (AZA) and 6-mercaptopurine (6-MP), are used as a second-line therapy in almost 60% of patients with IBD to induce and maintain remission.[1–6] The recommended dose of AZA is up to 2.5 mg/kg and that of 6-MP is 1–1.5 mg/kg/day. The drug is metabolized by multistep, multienzymatic pathways leading to wide interindividual variability. It is difficult to clinically optimize thiopurine therapy in IBD patients and 28% of patients have hepatotoxic and myelotoxic adverse reactions, and 9% of patients are resistant to therapy.[7–9]

To optimize the drug, enzyme activity and molecular status of the AZA and 6-MP metabolizing pathway are assessed. This primarily includes thiopurine methyltransferase (TPMT) enzyme activity and molecular status. The prevalence of TPMT mutation is 10% in Caucasian, 2% in Southwest Asians, and 5% in Chinese.[10] The prevalence of TPMT genetic variant in India is reported to be up to 4.7%.[11–13] Hence, the role of TPMT mutation may not be very relevant amongst Asians. Recently, nucleoside diphosphate-linked moiety X-type (NUDT-15) genetic variant has been found to be significantly associated with thiopurine toxicity amongst Asians.[14,15]

In spite of the molecular screening, it is difficult to predict therapeutic efficacy and hence estimation of thiopurine metabolites, 6-thioguanine nucleotide (6-TGN) and 6 methylmercaptopurine (6-MMP), is widely used. 6-TGN is an active metabolite in majority of patients. Approximately 15% patients preferentially metabolize thiopurine toward 6-MMP (and are called shunters).[16] A disproportionate increase in 6-MMP may result in poor therapeutic efficacy and predispose to hepatotoxicity. Ooi *et al.* have suggested 6-MMP:6-TGN ratio >11 as a discriminator for shunters. Identifying these shunters early in treatment followed by dose escalation and addition of allopurinol have been reported by them to be helpful in gaining desired response in 61% of their patients.[16] Several other studies have stated that optimal use of thiopurine metabolite levels may improve the efficacy of thiopurine therapy by 15–30%.[17]

It is reported that the thiopurine metabolism differs in Asian patients who do not tolerate full dose of thiopurine.[10,18–21] However, these studies were based on TPMT genotype and activity rather than metabolite estimation.

We aimed to study the utility of thiopurine metabolite measurement in Indian patients with IBD. We looked at the levels of thiopurine metabolites and whether the metabolite estimations led to change in clinical management of the patients. We also studied if Indian patients needed lower doses and had more toxicity as compared with Caucasian patients and also compared them with Asian studies reported in literature.

Methods

Patients. This was a prospective study conducted in Division of Gastroenterology and Department of Biochemistry of the Hospital from September 2015 to March 2017. The study was approved by the Institutional Review Board. Consecutive patients with IBD on AZA or 6-MP, with active disease or patients with stable disease who were considered for dose modification were included. Non-consenting patients were excluded. The patient's demographic details were entered in a prospectively maintained database, which includes age, gender, disease onset, diagnosis, investigations, treatment details, and follow up. The disease activity was determined by clinical symptoms, stool examination, C-reactive protein, stool calprotectin, and imaging: abdominal ultrasound, computed tomography (CT) scan, and/or magnetic resonance enterography. Seventy-three patients were on 5-ASA (5-amino salicylic acid) therapy. TPMT and NUDT-15 genotype status analyses were carried out for 37 and 12 patients, respectively. The patients who started on thiopurine before referral and were tolerating the drug did not undergo TPMT genotype analysis. The change in the management after the thiopurine metabolite report was documented in the database.

Thiopurine metabolite measurement

Chemicals. 6-Thioguanine (6-TG), 5-bromouracil (5-BU) and D, L-dithiothreitol (DTT) were obtained from Sigma Aldrich(St Louis, MO, USA). 6-MMP was obtained from Santa Cruz Biotech (Dallas, TX, USA). Potassium dihydrogen phosphate, acetonitrile, and perchloric acid were obtained from Merck (Kenilworth, NJ, USA). Milli Q water was obtained from Water purification system 2089 Model 27c Milli Q Biocell (Millipore, Billerica, MA, USA).

Sample collection and storage. Blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes. Red blood cells (RBCs) were isolated by centrifugation at approximately 1740 g. The plasma and buffy coat were separated and RBCs were washed thoroughly with phosphate buffer saline (PBS). The cell count was determined on Sysmex XN-2000 Model & Serial No: 1369/13640/11538 (Millipore) and samples were stored at −20°C until analysis.[22,23]

Metabolite estimation. Thiopurine metabolite 6-TGN and inactive metabolite 6-MMP were estimated using HPLC. The stock standards for 6-TG and 6-MMP were prepared in 0.1 mol/L sodium hydroxide while internal standard 314 μmol/L 5-BU was prepared in milli Q water. These standards are stable for 4 weeks when stored at −20°C. Solution of 1.1 mol/L DTT in milli Q water was prepared prior to use each time. Calibration standards and controls were prepared by spiking pooled RBCs with known concentration of 6-TG and 6-MMP.

Erythrocyte thiopurine metabolite levels were determined by using acid hydrolysis.[6] The RBC count in calibration standards, controls, and patients was normalized to 8×10^8 RBCs/250 μL. Internal standard 5-BU (314 μmol/L) was added to reaction mix along with 1.1 mol/L DTT and milli Q water. After vortex mixing the samples, 70% perchloric acid was added to the mixture. The samples were then centrifuged at 3000 g for 10 min and the supernatant was further hydrolyzed in boiling water bath for 1 h. Hydrolysate was filtered with 0.2-μm syringe filters and 50 μL of it was injected into HPLC system.[23,24]

Chromatographic conditions. The samples were analyzed using a binary HPLC pump Model No. 1525 connected to UV detector model No. 2487 from Waters Corp, Milford, MA, USA. For chromatographic separation of 6-TGN, 6-MMP, and 5-BU, symmetry C18 column (Waters Corp; 150 × 3.9 mm; particle size of 5 μm) was used as the stationary phase. The mobile phase consisted of solution A (20 mmol/L KH_2PO_4 in 3% acetonitrile pH: 3.5) and solution B (100% acetonitrile). The analytes were eluted at a flow rate of 1.1 mL/min with a linear gradient for 10.5 min followed by stabilization with mobile phase A. The column was maintained at 42°C. All the three analytes were estimated on UV detector by switching wavelengths between the peaks within same run. 6-TGN was detected at 343 nm, 6-MMP at 303 nm, and 5-BU at 280 nm.[22]

From the literature, 6-TGN level of 235–400 pmol/8×10^8 RBCs was considered to be in therapeutic range. Inactive metabolite 6-MMP level of >5700 pmol/8×10^8 RBCs was considered above the tolerable range.[25]

Statistical analysis. MedCalc Statistical Software version 16.8.4 (Ostend, Belgium) was used for statistical analysis. Mann–Whitney test was used to compare non-parametric variables. Spearman's rho test was used to assess correlation between variables. All calculated *P*-values were two-sided and a value of <0.05 was considered statistically significant.

Results

Seventy-six patients (36 Crohn's disease [CD] and 40 ulcerative colitis [UC]; 32 women) with a mean age of 35.9 (SD: 14.54; range: 8–73) years underwent thiopurine metabolite estimation. The disease activity was as follows: active CD 33 patients (13 women), active UC 28 patients (14 women), CD in remission 3 patients (3 women), and UC in remission 12 patients (2 women).

Thiopurine metabolite level in study population. Both metabolites showed a wide interindividual variability (Fig. 1) with the level ranging from 56.4 to 1048 pmol/8×10^8 RBCs for 6-TGN and up to 4971 pmol/8×10^8 RBCs for 6-MMP. One patient had undetectable 6-TGN and 6-MMP (she admitted non-compliance on confrontation by the clinician). Of the 75 patients, level of 6-TGN in therapeutic range was observed in 21 (28%) patients while 37 (49%) had low level and

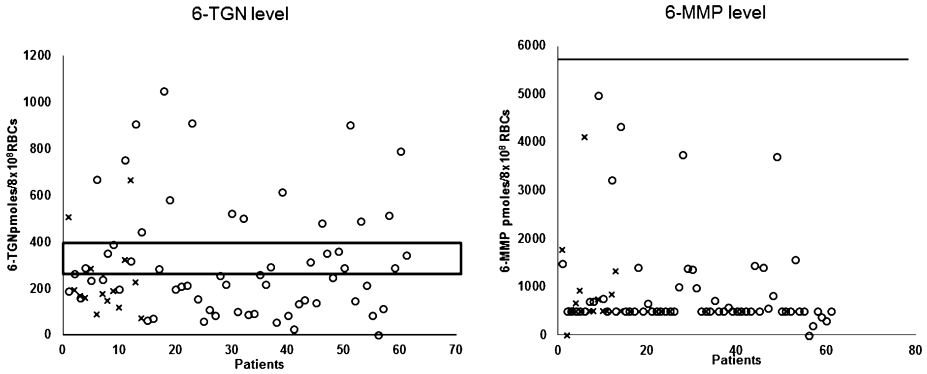

Figure 1 Thiopurine metabolite levels in patients (○, active status ; ×, remission status).

17 (23%) had a high 6-TGN level. None of the patients had a 6-MMP level in the toxic range. The metabolite levels and thiopurine dose in the study population are mentioned in Table 1. Median (interquartile range [IQR]) 6-TGN level and dose in the study population were 227 (212) pmol/8 × 10^8 RBCs and 1.36 (0.92) mg/kg body, respectively. Only four patients (5%) of our study population had 6-MMP:6-TGN ratio > 11 suggesting minimal shunting.

Dose-based levels. We attempted to correlate the levels with the dose <2 mg/kg (low dose) or >2 mg/kg (high dose). The median (IQR) level in patients with low dose (n = 57) was 214 (232) pmol/8 × 10^8 RBCs. Amongst these patients, 31 had low, 13 had therapeutic, and 13 had high 6-TGN level. The median dose (IQR) in this group was 1.06 (0.69) mg/kg. Amongst the patients with high thiopurine dose (n = 18), median (IQR) 6-TGN level was 300 (176) pmol/8 × 10^8 RBCs. In this group, there were eight patients with therapeutic, six with subtherapeutic, and four with supratherapeutic level of 6-TGN. The median dose (IQR) in these patients was 2.2 (0.22) mg/kg.

A Mann–Whitney U-test conducted to determine the difference in 6-TGN level between high- and low-dose IBD patients indicated a statistical difference ($P < 0.05$), with 6-TGN level being higher in high-dose patients. However, on Spearman rho analysis, thiopurine dose and 6-TGN level showed a weak correlation (r = 0.316).

Disease status-based metabolite level. The patients with active disease (n = 61) had high median (IQR) 6-TGN level, that is 246 (250) pmol/8 × 10^8 RBCs while those in remission (n = 14) had 181 (116) pmol/8 × 10^8 RBCs. Mann–Whitney U-test in these groups indicated that there was no difference ($P > 0.05$) in 6-TGN level.

Change in clinical management. Subsequent to metabolite testing, thiopurine dose was changed in 20 (26.31%) patients while in the remaining 55 (75%) the dose was not altered for variable reasons. The drug was discontinued in 1 patient, the dose was reduced in 8 patients, and increased in 11 patients.

Seventeen patients had high 6-TGN level. Complete cessation of drug was required in one patient who had very high 6-TGN level, that is 910 pmol/8 × 10^8 RBCs, on a dose of 0.96 mg/kg body weight. Amongst the eight patients for whom the dose was reduced, seven had high 6-TGN level ranging from 500 to 1048 pmol/8 × 10^8 RBCs and were on a dose ranging from 1.25 to 2.51 mg/kg body weight. None of these patients had cytopenia. One patient was on a dose of 2.5 mg/kg body weight and had 6-TGN level of 324 pmol/8 × 10^8 RBCs. This patient was clinically responding and had a low (<30 μg/g) level of fecal calprotectin. His hemoglobin was low (9.8 g/dL) and hence the dose was reduced. All the patients who had dose reduction continued to maintain the earlier clinical status (six in remission and two continued to have active disease and were switched to biological therapy). Amongst the remaining eight patients in whom dose was not changed, one underwent surgery and was off medication while one patient had a dose reduction only a week prior to the blood collection for metabolite estimation and hence was allowed to stabilize. Five patients had only a marginal increase in 6-TGN level which was not associated with any toxicity and hence dose was not altered while the remaining one patient needed a subsequent dose reduction on further follow up.

Subtherapeutic drug level was obtained in 37 patients. The dose was increased in 11 patients of whom 9 had low 6-TGN levels ranging from 58 to 220 pmol/8 × 10^8 RBCs with a dose of 0.78–2.3 mg/kg body weight. Amongst these 11 patients, 7 showed therapeutic response, 3 were switched to biological agents, and 1 could not afford biologic therapy. Of the remaining 26 patients, 17 were clinically stable and hence did not need dose escalation, 4 were intolerant to higher dose while 1 had an infection due to which the dose could not be increased, 3 were noncompliant, and 1 underwent surgery.

Adverse events. Adverse events occurred in six patients (8%) in the study group. These patients had cytopenia. Thiopurine was discontinued in four and reduced in two patients. Amongst these patients, one had high 6-TGN level, four had therapeutic, and one had subtherapeutic 6-TGN level. Analysis for NUDT-15 gene variation could be performed only in two of the six patients mentioned above. Both these patients had therapeutic 6-TGN level and were heterozygous for NUDT-15 variation. The 6-MMP levels in all our patients were within the limit (<5700 pmol/8 × 10^8 RBCs). They also did not present with any hepatotoxicity.

Table 1 Correlation of dose and 6-TGN levels

	6-TGN level (pmol/8 × 10^8 RBCs)		Dose (mg/kg)	
	Mean ± SD†	Median (IQR)	Mean ± SD†	Median (IQR)
Study population				
All patients	298 ± 233	227 (212)	1.4 ± 0.6	1.36 (0.92)
Drug levels				
Therapeutic levels	295 ± 45	288 (66)	1.56 ± 0.61	1.53 (1.09)
Low levels	132 ± 59	139 (100)	1.22 ± 0.6	1.06 (0.78)
High levels	664 ± 188	616 (286)	1.58 ± 0.5	1.5 (0.56)
Disease type				
CD	328 ± 267	246 (246)	1.34 ± 0.6	1.17 (1)
UC	272 ± 200	217 (224)	1.38 ± 0.6	1.37 (0.87)
Disease status				
Active	312 ± 245	246 (250)	1.38 ± 0.59	1.25 (0.91)
Remission	236 ± 164	181 (116)	1.46 ± 0.63	1.4 (0.99)
Dose				
Low dose	288 ± 240	214 (232)	1.13 ± 0.41	1.06 (0.69)
High dose	330 ± 211	300 (176)	2.24 ± 0.2	2.2 (0.2)
Clinical status				
Clinical response	288 ± 240	223 (216)	1.37 ± 0.6	1.3 (0.91)
No clinical response	312 ± 233	234 (290)	1.43 ± 0.6	1.36 (1.06)

†Mean 6-TGN level and mean dose showed correlation of $P < 0.05$ in all subgroups.
6-TGN, 6-thioguanine nucleotide; CD, Crohn's disease; IQR, interquartile range; RBC, red blood cell; UC, ulcerative colitis.

Discussion

Although thiopurines remain important second-line therapy in patients with IBD, up to 60% of the patients do not respond to conventional dosing and about one-third develop myelotoxicity or hepatotoxicity.[26] Optimal use of thiopurines may improve their efficacy by 15–30%.[17] There is a poor correlation between weight-based dosing of thiopurines and 6-TGN and 6-MMP levels.[26] Previous studies have suggested that Asian patients do not tolerate full doses of thiopurines and have more toxicity.[10,18–21] However, as mentioned above, these studies were based on estimation of TPMT genotype and activity rather than drug metabolites. Presently, prior to administration of a thiopurine drug, TPMT (and in some Asian centers, NUDT-15 genotype) testing is done and dose is adjusted according to the TPMT genotype or activity status. In this study, we estimated thiopurine metabolites in 76 patients with IBD and compared the findings with previously reported studies.

The 6-TGN levels in our patients showed wide variability. The levels did show an overall correlation with drug dose; however, there was a wide variability in individual cases. A high 6-MMP level predisposes to the risk of hepatotoxicity.[8] None of our patients had 6-MMP level >5700 pmol/8 × 10^8 RBCs or hepatotoxicity. Increased shunting, that is 6-MMP:6-TGN ratio > 11, was observed only in four (5%) of our patients while Ooi *et al*. have reported 39% shunters amongst Australians.[16] The variation may be due to larger sample size ($n = 343$) in their study or a population difference.

The median dose (IQR) in this study was 1.36 (0.92) mg/kg. At this dose, a high 6-TGN level was obtained in 23% of patients, 49% had subtherapeutic level while 28% had a therapeutic level. The other studies in literature where measurement of thiopurine metabolites has been performed are shown in Table 2.[27–35] The comparison of different studies is complicated by the fact that some have used mean, whereas others have used median dose and drug levels.

Two Australian studies used a median dose of 2 mg/kg. In both these studies, 52% and 72% were in subtherapeutic range.[28,30] In one of these studies by Goldberg *et al*.,[28] mean 6-TGN level was 273 pmol/8 × 10^8 RBCs. In a study from USA by Dassopoulos *et al*., the mean dose in patients with normal TPMT activity was 2.5 mg/kg and the mean 6-TGN level was 230 pmol/8 × 10^8 RBCs.[35] In our study, patients who received the dose of >2 mg/kg ($n = 18$) (median thiopurine dose of 2.2 mg/kg), median (IQR) 6-TGN level was 300 (176) pmol/8 × 10^8 RBCs and mean (SD) 6-TGN level was 330 (211) pmol/8 × 10^8 RBCs. In this group, 8 of 18 patients had therapeutic and 6 patients had subtherapeutic and 4 patients had supratherapeutic level of 6-TGN.

A dose of 1.8 mg/kg body weight was used by Gilissen *et al*. in the Dutch population. At this dose, median 6-TGN level was 235 pmol/8 × 10^8 RBCs and approximately 50% of the patients were in subtherapeutic range.[33] Gupta *et al*. from the USA used a mean dose of 1.5 mg/kg of AZA in patients with active disease and 1.3 mg/kg body weight in patients with remission. At this dose, median 6-TGN level was 213 pmol/8 × 10^8 RBCs in active disease and 173 pmol/8 × 10^8 RBCs in patients with remission.[29] A still lower dose, that is 1.2 mg/kg body weight, was used by Koreans, wherein the mean 6-TGN level was 328 pmol/8 × 10^8 RBCs.[34] In our study, patients on low dose of AZA (mean dose: 1.13 mg /kg, $n = 57$) had mean 6-TGN level of 288 ± 240 pmol/8 × 10^8 RBCs and median 6-TGN level of 217 (224) pmol/8 × 10^8 RBCs. At this dose, 55.18% had subtherapeutic level and 22.41% had each therapeutic and supra-therapeutic 6-TGN level. From these studies, we can interpret that the dose in Indian population is not different

Table 2 Comparison of various reported studies on thiopurine metabolites

First author	Number of patients	Mean dose (mg/kg)	Median dose (mg/kg)	Toxicity (%)	Low 6-TGN levels (%)	Therapeutic 6-TGN levels (%)	High 6-TGN levels (%)	Mean 6-TGN level	Median 6-TGN level	Mean 6-MMP	Comments
Goldberg[28]	169	2	NA	18	52	34	14	273			
Gupta[29]	101	1.5 in active	NA	14.85					213	Undetectable in 60%	
		1.3 in remission	NA						173	19% of patients > 5700	2/101 Patients had hepatotoxicity
Ooi[30]	56 Pediatric patients and 326 metabolite measurements	2	NA	21.4	72	28			160 (10–732)		Pediatric population
Gearry[31]	216	NA	NA	25.9							
Dubinsky[32]	92	NA	1.25	39							
Gilissen[33]	100	1.8	NA	NA	50	50			235		
Kim[34]	109	1.2	NA	34.8				328			
Dassopoulos[35]	69	1 mg/kg intermediate TPMT activity	NA	11.59							
		2.5 mg/kg normal activity						230		3407	
Parkar (present study)	76	1.4	1.36	8	49	28	23	298	227	875	

6-MMP, 6-methylmercaptopurine; 6-TG, 6-thioguanine; 6-TGN, 6-thioguanine nucleotide; TPMT, thiopurine methyltransferase.

from Asian and Western populations, although there are some variations.

Irrespective of the dose used in all these studies, the therapeutic level was attained in 28–50%, majority of patients (49–72%) had subtherapeutic level and 14–20% had supratherapeutic level.

Adverse events. In our study, thiopurine toxicity was seen in six (8%) patients, which is the lowest amongst the studies stated in Table 2. The reported toxicity in these studies varied between 12% and 39%. In our study, only one patient had high 6-TGN level and the remaining had normal or low level. NUDT-15 genotype status was available for two patients. Both these patients had 6-TGN levels in therapeutic range and were heterozygous for NUDT-15 genotype, suggesting this genotype status to be a contributor to drug toxicity.

Does thiopurine metabolism differ in Asian as compared to Caucasian? Do Asian patients need a different dose of thiopurines? Are they more susceptible to thiopurine toxicity?

There are clearly some differences in Asian as compared with Caucasians concerning thiopurine metabolism. Asians are less likely (approximately 3%) to have TPMT mutation as compared with Caucasians (10%).[10] In Asians, NUDT-15 mutation is more relevant for thiopurine-induced toxicity than TPMT (risk allele frequency of 0.2% in Europeans,[36] 7.2% in Indians,[15] 8.5% in Thai,[37] 10.2–16.3% in Japanese,[38] 11.6% in Taiwanese,[39] and 23.2% in Koreans[14]). Although it is said that Asian patients need a lower dose, almost half of our patients (31/57) on low dose and 5 of 17 on high dose (>2 mg/kg body weight) had subtherapeutic level. Many patients with high level tolerated this metabolite level. It is previously reported that Asians have higher likelihood of thiopurine toxicity.[21,34] We had lowest toxicity as compared with that reported in the literature and did not observe any hepatotoxicity.

In conclusion, this study showed a wide variability in 6-TGN level, with minimal 6-MMP shunting. Level of 6-TGN in therapeutic range was observed in 21 (28%) patients while 37 (49%) had low levels and 17 (23%) had high 6-TGN level. None of the patients had 6-MMP level in the toxic range. Based on metabolite levels and clinical condition, dose was modified in about one-fourth of patients. The comparison of dose-based levels with prior reported studies in the West and Asia showed no difference in metabolite levels. The thiopurine toxicity in this study was low (8%) as compared with Caucasians and Asians (12–39%).

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