# Normal Ranges of Thiopurine Methyltransferase Activity Do Not Affect Thioguanine Nucleotide Concentrations With Azathioprine Therapy in Inflammatory Bowel Disease

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**Background:** Thiopurine methyltransferase (TPMT) activity influences azathioprine conversion into active metabolite 6-thioguanine nucleotide (6-TGN). Low TPMT activity correlates with high 6-TGN and risk for myelosuppression. Conversely, normal-to-high TPMT activity may be associated with low 6-TGN and drug resistance, the so-called hypermetabolizers. Our aim was to identify the effect of normal-to-high TPMT activity on 6-TGN concentrations in an inflammatory bowel disease population.

**Methods:** A retrospective chart review of patients aged ≥18 with inflammatory bowel disease, on azathioprine, with documented TPMT activity and 6-TGN concentration was performed. Correlations were evaluated via the Spearman rho correlation coefficient. Linear regression was used to determine the effect of TPMT activity on 6-TGN accounting for confounders. Relationships between TPMT activity, drug dose, and 6-TGN levels were defined via average causal mediation effects.

**Results:** One hundred patients were included. No correlation was observed between TPMT activity, azathioprine dosing, and metabolite concentrations. Overall, 39% of the cohort had a therapeutic 6-TGN level of >230 pmol/8 × $10^8$ red blood cells (RBCs). No patient under 1 mg/kg achieved a therapeutic 6-TGN level, whereas 42% of patients taking 2.5 mg/kg did. The median 6-TGN concentration was higher for those in remission (254 pmol/8 × $10^8$ RBCs, interquartile range: 174, 309) versus those not in remission (177 pmol/8 × $10^8$ RBCs, interquartile range: 94.3, 287.8), though not significantly ($P = 0.08$). Smoking was the only clinical factor associated with 6-TGN level. On multivariate linear regression, only age, azathioprine dose, and obese body mass index were predictive of metabolite concentration.

**Conclusions:** Variations within the normal range of TPMT activity do not affect 6-TGN concentration.

### Lay Summary

Thiopurine methyltransferase (TPMT) activity in the normal or high range does not affect 6-thioguanine nucleotide concentration in patients with inflammatory bowel disease treated with azathioprine. Providers should not make assumptions about 6-thioguanine nucleotide concentration based on normal TPMT activity.

**Key Words:** therapeutic drug monitoring, azathioprine, inflammatory bowel disease, thiopurine methyltransferase, 6-thioguanine nucleotide

## INTRODUCTION

Azathioprine (AZA), a thiopurine prodrug, is utilized in the treatment of inflammatory bowel disease (IBD), both in Crohn disease (CD) and in ulcerative colitis (UC). Its effects are multifactorial and include inhibition of DNA synthesis, T-cell proliferation, and induction of apoptosis in dividing cells to suppress the inflammatory response.[1–4] 6-Thioguanine nucleotide (6-TGN) is the active metabolite of AZA responsible for its therapeutic and cytotoxic effects (hematopoietic toxicity). Clinical response has been found to occur with serum

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6-TGN levels of 230–450 pmol/8 × $10^8$ red blood cells (RBCs).[5–7] More recently, higher 6-TGN concentrations were associated with mucosal healing in CD patients, highlighting the potential importance of targeting a specific serum concentration.[8–9]

Although therapeutic cutoff concentrations of 6-TGN exist for both clinical and endoscopic remission, there is substantial individual variation in metabolite concentration related to numerous metabolic pathways. Upon absorption, AZA spontaneously converts to 6-mercaptopurine (6-MP), which is further metabolized into therapeutic and cytotoxic purine analog 6-TGN via hypoxanthine-guanine phosphoribosyltransferase and inosine-5′-monophosphate dehydrogenase. Alternatively, 2 competitive catabolic pathways for 6-MP also exist; the conversion into 6-thiouric acid by xanthine oxidase (XO) or into hepatotoxic 6-methylmercaptopurine (6-MMP) via thiopurine methyltransferase (TPMT).[10] The former catabolic pathway has clinical implications as the simultaneous use of allopurinol (a XO inhibitor) can cause high concentrations of 6-TGN and lead to profound bone marrow suppression. The latter catabolic pathway also has an important role in AZA therapeutic dosing and patient response.

TPMT is responsible for the methylation of active 6-TGN to inactivated 6-MMP. Heritable defective *TPMT* single-nucleotide polymorphisms exist, which affect TPMT enzymatic activity, 6-TGN levels, and the risk for adverse events in patients on thiopurine treatment. Approximately 0.3–1% of the population are homozygous for these defective variants, whereas an estimated 10–11% of the population are heterozygous for them.[11–14] TPMT genotyping helps to predict response to AZA and establish the risk for severe myelosuppression by identifying if a patient has these inactivating single-nucleotide polymorphisms.

Alternatively, TPMT phenotype (enzymatic activity level) can be directly measured to guide thiopurine dosing. In addition to identifying TPMT deficiencies, phenotyping may have the added benefit of capturing TPMT hypermetabolizers (potential drug nonresponders).[15] Enzyme activity is classified as low (presumptively homozygous for defective *TPMT* alleles), intermediate (presumptively heterozygous for one defective allele and one wild-type), or normal (2 normal alleles) based on predetermined cutoff levels. Low and intermediate activities are associated with higher 6-TGN levels and increased risk for severe myelosuppression. In these settings, thiopurines are dose reduced or avoided altogether.[13–14, 16] Conversely, normal-to-high TPMT activity may correspond with lower 6-TGN levels overall (due to increased metabolism and shunting of 6-MP toward the 6-MMP metabolite) resulting in subtherapeutic response to standardized weight-based dosing of AZA and increased risk for hepatotoxicity (due to high levels of hepatotoxic 6-MMP); however, this relationship is mostly based on inferences from decreased clinical responses to AZA with high TPMT activity.[17–18] The aim of this study was to determine whether normal-to-high TPMT enzyme activity is associated with 6-TGN concentration and/or if it mediates an effect between AZA dose and 6-TGN concentration. We hypothesized that high TPMT enzyme activity would lead to lower 6-TGN concentrations.

## MATERIALS AND METHODS

Subjects were identified using ICD 9 codes 556.X (UC) and 555.X (CD), and ICD 10 codes K51.X (UC) and K50.X (CD), who had TPMT testing between 2010 and 2019 from the Academic Health Center Information Exchange (AHC-IE) held in the clinical data repository, which houses electronic health records of >2 million Fairview and University of Minnesota Physicians' patients. Participants included were 18 years or older with diagnosed IBD confirmed by typical clinical, endoscopic, and histologic changes, who had normal or high TPMT enzyme activity, thiopurine metabolite results, AZA dosing information, and weight available for review. Those on concurrent allopurinol were excluded from the study due to the impact of XO inhibition on the metabolic pathway. Cases were reviewed in reverse chronological order based on metabolite testing date until 100 subjects who met the criteria were identified. This ensured a systematic way to manually review charts for the completion of thiopurine metabolite data. Additional data were collected regarding demographic information, disease-specific history, concomitant use of biologic agents, laboratory data and clinical status at the time of metabolite testing, and smoking status (current or not). Clinical status was determined by physician documented impression and, when available, serologic, radiographic, or endoscopic data. AZA drug adherence was evaluated by calculating the medication possession ratio (MPR) for the cohort; the sum of the days supplied for all fills over a given time period divided by the total days. The proportion of days covered (PDC) was also calculated for adherence, defined as the ratio of days covered in a given time period to the total number of days in that period. Adherence was classified ordinally as missing, low (<0.8), or adherent (≥0.8) and added to the final regression model as a sensitivity analysis.

To account for possible variations in TPMT activity assay measurement, TPMT was normalized to the low end of normal for the given assay based on the range provided in the report. TPMT was assessed continuously and also as 1.5 times and 2 times the lower limit of normal. 6-TGN concentration was also assessed continuously and as clinically relevant cutoffs of 230 and 400 pmol/8 × $10^8$ RBCs.

Correlation of continuous data was analyzed with the Spearman rho correlation coefficient. Differences between the groups were assessed with the Student *t* test or Wilcoxon test as appropriate. Multivariate linear regression was used to assess for the independent effects of various factors on 6-TGN concentration. Age, sex, TPMT activity, AZA dose, and body mass index (BMI) were included in the regression model a priori given their presumed influence on 6-TGN concentration, along with factors that were potentially associated with 6-TGN concentration (at $P < 0.1$) on univariate analysis. BMI was defined ordinally

as normal (<25 kg/m$^2$), overweight (25–29 kg/m$^2$), or obese (≥30 kg/m$^2$). Causal mediation analysis was used to identify the effect of TPMT enzyme activity on the relationship of AZA dose and 6-TGN concentration (R v3.6.0, mediation package).

A sample size of 85 was required to have an 80% chance of detecting a significance at the 5% level and a medium correlation (rho = 0.3) between TPMT enzyme activity and 6-TGN concentration (null hypothesis, rho = 0). Therefore, a total of 100 charts were reviewed to ensure adequate sample size and allow exploratory testing for additional factors affecting 6-TGN concentration.

## Ethical Considerations

This study was approved by the University of Minnesota Institutional Review Board.

# RESULTS

Of the 100 patients identified, 50% were female and the majority (89%) were white. The population reflected a typical IBD population in terms of disease distribution and other demographics (Table 1). TPMT enzyme activity varied based on the commercial assay utilized, though a majority of testing (67%) was performed by the Prometheus assay (Supplementary Table 1). The median time from TPMT testing to metabolite testing was 53 weeks (interquartile range [IQR]: 17.5, 134.5). The median time from AZA start date to metabolite testing was 31 weeks (IQR: 14, 90).

There was no correlation between TPMT enzyme activity and 6-TGN (rho = −0.15, $P = 0.15$) or 6-MMPN (rho = −0.097, $P = 0.34$) metabolite concentration (Fig. 1a, b). Additionally, there was no correlation between AZA dose (mg/kg) and metabolite concentration (Fig. 1c, d). Creating cutoffs for TPMT activity of 1.5 times and 2 times the lower limit of normal did not identify any trend between high TPMT activity and metabolite levels (Supplementary Fig. 1). When categorized by AZA dose (mg/kg), there was no significant change in 6-TGN concentration as dose increased (Table 2), nor were there any significant differences between dose-based groups (Supplementary Fig. 2). 6-TGN and 6-MMPN concentrations were positively correlated with each other (rho: 0.3, $P = 0.004$). A sensitivity analysis using only the most common TPMT assay (Prometheus) did not change any of the aforementioned results (data not shown).

**TABLE 1.** Demographics of the IBD Cohort

| Cohort Characteristics | N = 100 |
|---|---|
| Female | 50 |
| White | 89 |
| Median age at metabolite testing (IQR) | 32 (25, 46) |
| Median BMI (IQR) | 25 (22, 29) |
| Current smoker | 12 |
| IBD type | |
| Crohn disease | 47 |
| Ulcerative colitis | 49 |
| Indeterminate colitis | 4 |
| Median age at IBD diagnosis (IQR) | 26 (19, 40) |
| Concurrent mesalamine therapy | 29 |
| Concurrent biologic therapy | 50 |
| Concurrent steroid use | 37 |
| Clinical remission at time of testing | 16 |

On univariate assessment of clinical factors, only smoking status had an association (negative) with 6-TGN concentration, albeit borderline statistically significant ($P = 0.09$). IBD type, serum creatinine, serum albumin concentration, concurrent biologic therapy, and mesalamine use were not associated with 6-TGN concentration. Multivariate linear regression, assessing for factors significantly associated with 6-TGN concentration while controlling for a priori hypothesized confounders and smoking status, identified age, AZA dose, and obese BMI as predictive of 6-TGN concentration, although the effect size was small (Table 3). For example, a 10-year increase in age would be expected to increase the 6-TGN concentration by 20 pmol/8 × $10^8$ RBCs, whereas increasing the dose of AZA by 50 mg would cause an expected increase in 6-TGN concentration by ~30 pmol/8 × $10^8$ RBCs. Causal mediation did not find a significant effect of normal-to-high TPMT enzyme activity on the relationship between AZA dose and 6-TGN concentration (average causal mediation effects = −3.3, $P = 0.36$), no direct effect on dose (average direct effect = 44.7, $P = 0.09$), and no total significant effect ($P = 0.12$). As expected, when including low TPMT activity in this analysis, TPMT enzyme activity had a significant effect on the relationship of AZA dose and 6-TGN concentration (data not shown).

Sensitivity analyses were performed based on available AZA adherence data. MPR data were available for 30% (n = 30) of the cohort with 77% defined as adherent. PDC was available for 27% (n = 27) of the cohort with 67% defined as adherent. After adjusting for adherence, prior predictors of 6-TGN concentration including age, AZA dose, and obese BMI, remained significant with relatively no change on the effect estimates (Table 4, data shown for MPR only as PDC was identical). Adherence itself was significantly positively associated with 6-TGN concentration, and current smoking was borderline negatively associated with 6-TGN concentration.

Using the recommended cutoff of 230 pmol/8 × $10^8$ RBCs, only 39% of our cohort had therapeutic concentrations of 6-TGN; 37% on AZA monotherapy; and 41% on combination therapy with a biologic. Even at doses of 2.5 mg/kg, only 42% had a therapeutic concentration (Table 2). Among those with an MPR ≥ 0.8, 70% had a 6-TGN concentration over 230 pmol/8 × $10^8$ RBCs. However, no relationship was seen between dose (mg/kg) and therapeutic metabolite concentrations within those with an MPR ≥ 0.8. Similarly, TPMT activity was not a significant predictor of a therapeutic 6-TGN concentration

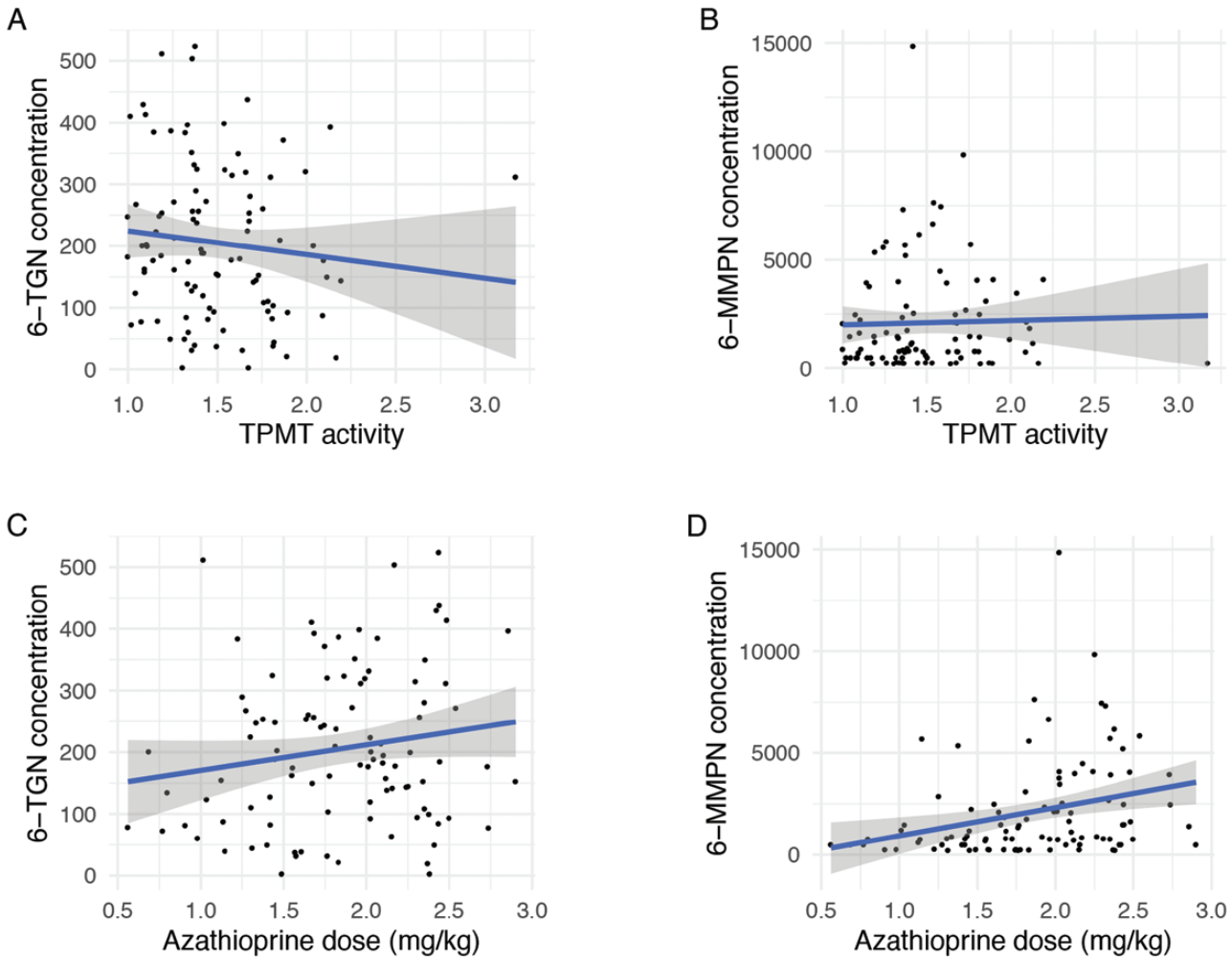


FIGURE 1. Correlation of thiopurine metabolite concentration to AZA dose and TPMT activity. A, 6-TGN concentration by TPMT activity. B, 6-MMPN concentration by TPMT activity. C, 6-TGN concentration by AZA dose. D, 6-MMPN concentration by AZA dose. All thiopurine metabolite concentrations expressed in (pmol/8 × $10^8$ RBCs). TPMT enzyme activity was normalized to the lower limit of normal reported per given assay.

**TABLE 2.** Metabolite Testing Based on Azathioprine Dose

| Dose (mg/kg) | Mean 6-TGN Level (pmol/8 × $10^8$ RBCs), (SD) | Proportion with therapeutic 6-TGN[a] (%) | Mean 6-MMPN level (pmol/8 × $10^8$ RBCs), (SD) |
|---|---|---|---|
| 0.5 | 140 (86.2) | 0 | 500, (—) |
| 1.0 | 165 (156) | 20 | 1188, (1637) |
| 1.5 | 197 (113) | 48 | 1111, (1102) |
| 2.0 | 220 (113) | 36 | 2629, (2925) |
| 2.5 | 216 (144) | 42 | 3021, (2697) |

[a]Therapeutic 6-TGN defined as ≥ 230 pmol/8 × $10^8$ RBCs.

among those defined as adherent by MPR or PDC (data not shown).

The median 6-TGN concentration was higher for those in remission (254 pmol/8 × $10^8$, IQR: 174, 309) versus those not in remission (177 pmol/8 × $10^8$, IQR: 94.3, 287.8), although not statistically significant ($P$ = 0.08) (Fig. 2a). Receiver-operating characteristic curve analysis identified a cutoff of 224 pmol/8 × $10^8$ RBCs for remission (area under curve = 0.64, Fig. 2b). Receiver-operating characteristic curve analysis of TPMT activity on clinical remission demonstrated a cutoff of 1.4 times the lower limit of normal (LLN). Exploratory multivariate regression to identify predictors of clinical remission identified a TPMT of <1.4 times LLN to be of borderline significance (odds ratio [OR] = 3.3, 95% confidence interval: 0.96, 12.6,

**TABLE 3.** Multivariate Linear Regression for 6-TGN Concentration

| Parameter | β Estimate | *P* |
|---|---|---|
| Male sex | −22 | 0.4 |
| Age | 2 | 0.009* |
| TPMT activity | −59 | 0.1 |
| AZA dose | 0.64 | 0.03* |
| BMI (ref: normal) | | |
| Overweight | −22 | 0.5 |
| Obese | −81 | 0.02* |
| Current smoker | −60 | 0.1 |

*Statistically significant: $P < 0.05$.

**TABLE 4.** Sensitivity Analysis of Multivariate Linear Regression for 6-TGN Concentration With Medication Possession Ratio

| Parameter | β Estimate | *P* |
|---|---|---|
| Male sex | −28.6 | 0.2 |
| Age | 1.9 | 0.01* |
| TPMT activity | −57.8 | 0.1 |
| AZA dose | 0.65 | 0.02* |
| BMI (ref: normal) | | |
| Overweight | −19.2 | 0.5 |
| Obese | −82.1 | 0.02* |
| Current smoking | −62.6 | 0.08 |
| MPR | 39.8 | 0.005* |

*Statistically significant: $P < 0.05$.

$P = 0.06$), whereas a 6-TGN > 230 pmol/8 × $10^8$ RBCs was a significant predictor (OR = 3.6, 95% confidence interval: 1.1, 12.2, $P = 0.04$), when controlling for sex, age, IBD type, and smoking status.

Laboratory abnormalities were uncommon. Only 11% of individuals had leukopenia (white blood cell [WBC] count < 4 × $10^9$/L), and 6% had elevations in alanine transaminase (ALT > 70 U/L). Neither 6-TGN nor 6-MMPN levels correlated with WBC, ALT, mean corpuscular volume, albumin, or creatinine alterations.

## DISCUSSION

Our study demonstrates that variations within normal TPMT enzyme activity do not affect 6-TGN metabolite concentration. It is well recognized that low TPMT activity alters the drug metabolism to increase 6-TGN concentrations.[14,16] As such, we hypothesized that normal or high TPMT activity would lead to lower 6-TGN concentrations due to increased metabolism and shunting towards the 6-MMP metabolite. Our results did not find a correlation between normal-to-high TPMT activity and thiopurine metabolite levels. Only age, AZA dose, and obese BMI were statistically significant predictors of 6-TGN concentration.

In general, our findings are consistent with prior published literature. BMI has previously been associated with 6-TGN concentrations.[19] Poon et al reported an 8% decrease in 6-TGN concentration for every 5 kg/$m^2$ increase in BMI. We chose to assess BMI ordinally and found that obese BMI significantly decreased 6-TGN concentration by an average of 80 pmol/8 × $10^8$ RBCs. In the same analysis, Poon et al found that active smokers had higher 6-TGN concentrations. However, we did not identify active smoking as a significant predictor and conversely noticed a consistent trend toward lower 6-TGN concentrations. TPMT activity may be higher in smokers compared to nonsmokers, although the effect of this clinically is uncertain.[20] Although age is not known to affect TPMT enzyme activity, it is associated with lower 6-TGN concentrations in children, although it is unclear if this is related to a metabolic affect or an absorption or adherence issue.[21–22] Finally, as expected, AZA dose was positively associated with 6-TGN concentration.

Although variations in normal TPMT activity were not associated with 6-TGN concentration, low-normal TPMT activity (<1.4 times the LLN) demonstrated a borderline statistically significant association with clinical remission (OR = 3.3, $P = 0.06$). In a pediatric population, patients with low TPMT enzyme activity were 6.2 times more likely to have a clinical response to AZA.[18] Given the complex relationship between AZA dose, TPMT enzyme activity and 6-TGN concentration, we performed a mediation analysis, which did not identify normal TPMT enzyme activity to mediate dosing and 6-TGN concentration. Although we cannot rule out a mild effect of TPMT activity on clinical response, it is more likely that dosing and subsequent 6-TGN concentration are more important for clinical outcomes.

We identified a 6-TGN cutoff of 224 pmol/8 × $10^8$ RBCs as the threshold for remission, which is similar to previously published data reporting a threshold of 230 pmol/8 × $10^8$ RBCs.[5–6] In our cohort, less than half of individuals achieved a therapeutic 6-TGN concentration (>230 pmol/8 × $10^8$ RBCs), including those on 2.5 mg/kg of AZA monotherapy; however, these results were strongly affected by adherence, as 70% of patients defined as adherent by MPR did have a therapeutic 6-TGN concentration (similar results for PDC).

The current American Gastroenterological Association (AGA) guidelines on therapeutic drug monitoring recommend reactive assessment of thiopurine metabolites in the setting of ongoing inflammation.[7] Our study highlights the importance of this recommendation as many individuals will have subtherapeutic metabolite concentrations, although a

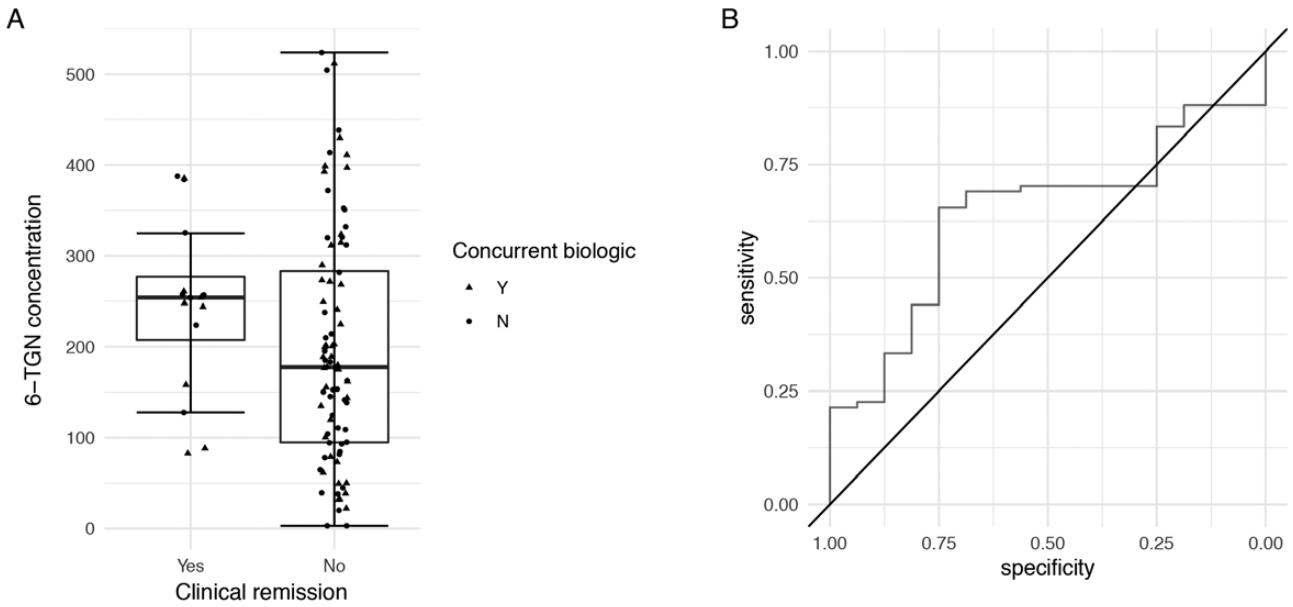


FIGURE 2. A, 6-TGN concentrations and clinical remission. B, Receiver-operating characteristic curve for remission based on 6-TGN concentration. An optimal cutoff was identified at 224 pmol/8 × $10^8$ RBCs; area under curve = 0.64.

quarter to a third of this could be tied to medication adherence. While a given patient's therapeutic window may vary, if there is any need to achieve a specific cutoff or if there is concern of ongoing inflammation or potential thiopurine side effects, providers should not make assumptions about metabolite concentration based on the drug dose prescribed or TPMT activity level. In general, given the unpredictable nature of thiopurine metabolites, we advocate for routine drug concentration monitoring to maximize treatment efficacy, assess for adherence, and minimize any dose-related risks.

We did not identify any correlation with WBC, mean corpuscular volume, ALT, or creatinine and 6-TGN or 6-MMPN metabolite levels. This may be related to selection bias as those who developed leukopenia or elevated ALT early on in treatment likely underwent dose-reduction or discontinuation prior to metabolite testing. Overall, the rate of leukopenia and elevated transaminases in our cohort was low, limiting our ability to detect a correlation.

Our study is limited by residual bias due to the nature of a retrospective cohort. However, the primary outcome of our study was laboratory data, which limit some forms of bias in a retrospective study. Factors such as clinical remission were abstracted from physician reporting and subject to bias, although ultimately, we were able to reproduce similar 6-TGN thresholds for clinical remission as previously reported in the literature. Our TPMT activity measurements were from various assays, which may influence results. However, to control for this, we normalized the documented enzyme value to the low end of normal listed on each assay report. Although there is a chance that we could not see a correlation due to variations in assays, this is unlikely as the results were similar in sensitivity analysis using only the most common assay type. We could not reproduce prior correlations between thiopurine metabolites and laboratory abnormalities, although this was probably related to low number of laboratory abnormalities. Last, measures of adherence were only available in a small subset of the population. In general, adherence was high when available; however, limiting the population to confirmed adherence resulted in a small sample size that was insufficiently powered to detect meaningful differences.

## CONCLUSION

Our findings suggest that normal-to-high range TPMT enzyme activity does not affect thiopurine metabolite concentrations. Obese BMI was negatively associated with 6-TGN concentration, whereas age and AZA dose (mg) were positively associated with 6-TGN concentration. Adherence strongly affected the ability to achieve a therapeutic 6-TGN concentration. Physicians should have a low threshold to directly measure metabolite concentrations when using AZA as primary or adjunct therapy for IBD.

## SUPPLEMENTARY MATERIAL

Supplementary data are available at *Crohn's & Colitis 360* online.

## DATA SHARING STATEMENT

As consent for research was waived due to the retrospective nature of this study, and the data set contains indirect identifies, sharing will only be performed on a case-by-case basis.

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