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# TPMT GENE POLYMORPHISMS AND DRUG INTERACTIONS: THE INFLUENCE ON THE AZATHIOPRINE THERAPEUTIC RESPONSE IN IBD

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**ABSTRACT**

Crohn's disease (CD) and ulcerative colitis (UC) are the most proeminent forms of inflammatory bowel diseases (IBDs). Azathioprine (AZA) is the most common drug used to maintain clinical remission in IBDs. However, the treatment with AZA leads to some drug side effects. Gene polymorphism of thiopurine S-methyltransferase (TPMT) correlates with decreased enzyme activity, which causes an increase in the incidence of these side effects. In addition, the association of AZA with some drugs might modify the final levels of active metabolites. The aim of this study was to investigate the impact of polymorphisms in *TPMT gene* as well as the interactions by the association of AZA with other drugs in IBD treatment. We studied 90 patients (CD = 53 and UC = 37). Quantitative analysis of AZA metabolites has performed by High Performance Liquid Chromatography-UV. The *TPMT gene* polymorphisms have evaluated by Polymerase Chain Reaction/Fragment Length Polymorphism. AZA+ infliximab group has shown the highest percentage of patients with 6-TGNt toxic levels (13.3%) and AZA+ mesalazine group has presented the highest percentage of patients with therapeutic levels (16%). The presence for each genotypes variation was demonstrated as follows: *TPMT*1* (87.8%), *TPMT*2* (1.1%), *TPMT*3A* (1.1%), *TPMT*3B* (5.6%) and *TPMT*3C* (4.4%). In our study, the metabolism of azathioprine was influenced by non-genetic factors. These factors were resulted of drug interactions caused by associations in IBD treatment. Polymorphisms detected in this study did not demonstrate negative effect on levels of metabolites.

**Keywords:** Azathioprine metabolites; Inflammatory Bowel Disease; Thiopurine S-methyltransferase *gene*; polymorphisms.

## INTRODUCTION

Inflammatory bowel disease (IBD) is a common chronic gastrointestinal disorder characterized by alternating periods of remission and active intestinal inflammation. Crohn's disease (CD) and ulcerative colitis (UC) are the most prominent forms of these diseases. The etiology in both diseases remains unknown. However, it has suggested that genetic, infectious and immunological factors play a role in the inflammatory process as well as tissue damage [1].

The therapeutic pharmacological approaches used for treatment include the aminosalicylates, corticosteroids as well as immunosuppressors such as azathioprine (AZA). AZA, the most common drug used to maintain clinical remission in IBDs, was developed from the structure of 6-MP (6-mercaptopurine) in order to protect the molecule of 6-MP self-oxidation *in vivo*. However, the treatment with azathioprine leads to some side effects [2].

The metabolism of AZA is complex, initially, at a non-enzymatic reaction about 88% of AZA. This drug is converted to 6-MP in the liver through the cleavage of the imidazole grouping in a dependent reaction of glutathione or from other proteins that contain auxiliary sulfhydryl groups. The 6-MP can has been inactivated by xanthine

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oxidase (XO) and Thiopurine S-methyltransferase (TPMT) or it been converted to cytotoxic 6-thioguanine nucleotides (6-TGN) *via* HPRT (Hypoxanthine phosphoribosyl transferase) in a multi-enzymatic process [3].

TPMT or XO deficiency result in greater conversion of 6-MP to 6-TGNt, which are the predominant active metabolites related to drugs efficacy. Moreover, this conversion also induces toxicity. Patients with low TPMT activity display higher 6-TGNt levels when treated with standards doses of AZA as well as an increased risk of myelosuppression. On the other hand, patients with high TPMT activity are usually resistant to thiopurines or require a higher dose to achieve efficacy, which increases the risk of hepatotoxicity [4].

Gene polymorphism of TPMT correlates with decreased enzyme activity, which causes an increase in the incidence of these side effects in patients treated with thiopurines. Most prevalent allelic variants are *TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C* [5].

Schaeffeler *et al*., [6] showed a correlation between genotype and phenotype for TPMT which is 98.4% with an estimate of sensitivity and specificity >90%.

In addition, the association of AZA with aminosalicylates and biological anti-inflammatory drugs might alter the final levels of active metabolites of this drug, and the mechanisms by which this occurs are not clearly defined [7].

In this study, we investigated the influence of the TPMT gene polymorphisms and drug interactions on the azathioprine therapeutic response in IBD patients.

## MATERIALS AND METHODS

### Patient Selection

We investigated 90 patients (53 women and 37 men; mean age of 43 years, range 16-79 years) with IBD followed in clinic of inflammatory bowel diseases of Hospital São Paulo - Universidade Federal de São Paulo – Escola Paulista de Medicina - Brazil (UNIFESP/EPM).
Diagnoses of Crohn´s disease (CD) and ulcerative colitis (UC) were established by standard clinical, radiological, histological and endoscopic parameters. The confirmation of IBD required a throrough history, a physical examination and laboratory tests.

These patients has used AZA monotherapy or in combination with other drugs to treat IBD as a follow: eighteen (CD 14, UC 4) in monotherapy, fourteen (CD 4, UC 10) in combination AZA+sulfasalazine, twenty-two (CD 7, UC 15) AZA+mesalazine, twenty-one (CD 17, UC 4) AZA+infliximab. Fifteen patients (CD 11, UC 4) have suspended the treatment with AZA.

Patients in use with allopurinol [8], furosemide [9] and warfarin [10] were excluded of the study. After collection, the samples have transported immediately to the laboratory for processing and storing to further quantitative analysis of metabolites and molecular polymorphisms.

Clinical data as Truelove & Witts index for UC activity [11] and Harvey-Bradshaw Index (HBI) [12] for CD activity, were included.

### Quantitative Analysis of 6-TGNt and 6-MMPr by HPLC-UV

The quantitative analysis of AZA metabolites: 6-thioguanine totals (6-TGNt) and 6-methylmercaptopurine ribonucleotides (6-MMPr) have performed by High Performance Liquid Chromatography-UV (HPLC-UV) in 75 samples of whole blood of patients with IBD under treatment with AZA.

Analitical curves were performed for calibrators in seven different concentrations: 0.30, 2.99, 14.95, 29.90, 44.56, 59.81 e 89.71 µmol/L for 6-TGNt (6-Thioguanine ≥ 98%, Sigma-Aldrich). For the 6-MMPr (6-Methylmercaptopurine riboside ≥ 99%, Sigma-Aldrich) were performed the calibrators in the following concentrations: 0.31, 3.13, 15.64, 31.29, 46.93, 62.58 and 93.86 µmol/L.

For the quality control assay, was performed the mixed control in the concentration of 2.42 µmol/L (acceptance range of 2.19 - 2.65 µmol/L) for 6-TGNt and the 50.46 µmol/L (acceptance range of 45.45 - 55.67 µmol/L) for 6-MMPr.

We used a reverse phase column Inertsil ODS-3 (150x4.6 mm, 5 um) Sigma-Aldrich (St. Louis, USA), in high pressure liquid chromatography system (HPLC Shimadzu Corporation, LC-20AT - Kyoto/Japan) with UV detection (Shimadzu Corporation, SPDM20A - Kyoto/Japan) from method developed and validated by Pacheco Neto [13].

For metabolites elution, the mobile phase was composed of two solutions: methanol (Methanol, Merck, Valencia, CA/USA). The flow of mobile phase was set to 1 ml/minute. For the satisfactory separation of compounds, we defined as gradient: 2.5% mobile phase B until the 14 minutes, rising up from 2.5% to 15% for the following 11 minutes. Subsequently, it decreased from 15% to 2.5%, and thus maintained the last 6 minutes. The injection volume was 100 µL and the quantification of both metabolites has performed at 342 nm in a total time of 32 minutes of running. The active metabolite 6-TGNt is the amount of 6-thioguanine monophosphate (6-TGN-MP), 6-thioguanine diphosphate (6-TGN-DP) and 6-Thioguanine triphosphate (6-TGN-TP). The second active metabolite 6-MMPr (6-Methylmercaptopurine ribonucleotides) corresponds the amount of 6-methylthioinosine monophosphate (6-MTIMP), 6-methylthioinosine diphosphate (6-MITDP) and 6-methylthioinosine triphosphate (6-MTITP).

After samples processing by HPLC-UV, the presence of 6-TGNt and 6-MMPr in the erythrocytes was

shown by the chromatographic peaks. The peaks of interest were evaluated by comparison and overlay of their retention time regarding an external pattern from these analytical data.

In according with the methodology performed, the quantitative mathematic analysis show the results in µmol/L and for these metabolites levels analysis, the results has converted in ρmol/8x$10^8$ RBC. Based in another studies [14, 15], the 6-TGNt levels has considered: > 235 ρmol/8x$10^8$ without therapeutic response; 235-450 ρmol/8x$10^8$RBC with good therapeutic response; and < 450 ρmol/8x$10^8$ toxic response. For the 6-MMPr levels has considered > 5700 ρmol/8x$10^8$RBC hepatotoxic response.

**Analysis of the TPMT Genotype**

The samples were analyzed by Polymerase Reaction Chain Reaction (PCR) and subsequently genotyped for Polymorphism Length Restriction Fragment (RFLP) obtained by restriction enzymes (PCR/RFLP) according to methodology of Gastal[16].

**DNA extraction and PCR assay**

Whole blood specimens were collected into EDTA tubes, and genomic DNA was isolated from peripheral blood leukocytes using the QIAmp Blood Mini kit according to the manufacturer's instructions (Qiagen Valencia, CA/USA). The *TPMT gene* polymorphisms, *TPMT*2* (G238C), *TPMT*3A* (G460A/A719G), *TPMT*3B* (G460A) and *TPMT*3C* (A719G) genotypes, were detected by PCR/RFLP. For all the studied variants were used primers sequences described by Gastal [16].

Briefly, PCR were performed using a 50-100ng of genomic DNA, 12µL of MasterMix PCR (Qiagen Valencia, CA/USA), 10pmol of each primer and sterile ultrapure water to a final volume of 25µL. For amplification, the DNA was primarily denatured for 3 min at 94℃, followed by 40 cycles of denaturation for 1 min at 94℃, annealing for 2 min at 55℃ for A719G, 54.2℃ for G460A and 64℃ for G238C; and extension for 1 minute at 72℃. At the end of the 40 cycles, an additional 7 min cycle at 72℃ was performed.

**Detection of TPMT*2 - G238C**

In order to detect the G238C polymorphism, a PCR assay was performed as previously described. Unpurified PCR products with a length of 256 base pairs were analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. A DNA fragment was amplified with primers P2M and P2C when G238C (mutant) was present, whereas a DNA fragment was amplified with primers P2W and P2C when (wild type).

**Detection of TPMT*3B - G460A**

To detect the G460A polymorphism, a PCR assay was performed as previously described. The PCR product ware digested with *MwoI* (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions. The digested products analyzed by gel electrophoresis. *MwoI* digestion of wild-type DNA yielded fragments of 267 and 98 base pairs, whereas DNA containing the G460A polymorphism was not digested, resulting in an uncleaved fragment of 365 base pairs.

**Detection of TPMT*3C - A719G**

To detect the A719G polymorphism, the PCR assay was performed as previously described. The PCR product was digested with *AccI* (New England Biolabs, Ipswich, MA, USA) According to manufacturer's instructions and analyzed by electrophoresis. The A179G polymorphism introduces a restriction site for the endonuclease *AccI*, and therefore the modified version was detected by observing two fragments (150 bp and 86), while the wild variant remained intact.

**Data Analysis**

For the correlation between AZA dose (mg/day or mg/kg/day) and the 6-TGNt and 6-MMPr levels we used non-parametric Spearman and Pearson correlation tests. The Chi-square or Fisher's exact test was used for the associations between the studied groups with 6-TGNt and 6-MMPr levels.

The association of TPMT genotype, activity indices for CD and UC as well as 6-TGNt and 6-MMPr levels, we used the normality test Shapiro-Wilk, Mann-Whitney, Kruskal-Wallis and t-Student tests for independent samples. Data has reported as minimum, maximum, mean, median and standard deviation.

The analysis has performed using the Excel 2010 software and SPSS 19.0 - Statistical Package for Social Sciences 19.0 with a significance level of 5%.

**Ethical Considerations**

The Ethical Committee of Universidade Federal de São Paulo, UNIFESP, approved this study (N°430.127). The study protocol was in accordance with the ethical principles for medical research involving human subjects' statement of Helsinki Declaration.

**RESULTS**

Ninety patients were recruited (37 UC, 41.1% and 53 CD, 58.9%). The mean doses of AZA in mg/kg/day as a follow: 125 mg/kg/day for UC patients and 2.14 mg/kg/day for CD patients (range: 50-200 mg/day; 0.84-2.97 mg/kg/day; respectively).

**Quantitative analysis of 6-TGNt and 6-MMPr by HPLC-UV**

In all analyses, we follow the assay performance by the precision of quality control: 2.4 µmol/L for 6-TGNt (range: 2.19-2.65 µmol/L) and 50.46 µmol/L for 6-MMPr (range: 45.45-55.67 µmol/L). The

average of the five tests carried out on different datelines for the 6-TGNt was 2:51 µmol/L with standard deviation of 0.143 and coefficient of variation of 5.7% and for the 6-MMPr, the average concentration values was 52.91 µmol/L with standard deviation of 1.998 and 3.8% coefficient of variation. The linearity of the calibration curves was $R^2$ greater than 0.98 for both metabolites with an average of 0.99165. The external standard 6-MMPr concentration of 40.20 µmol/L showed in the five determinations evaluated, average areas of 33592 with standard deviation of 357.9 and 1.1% coefficient of variation. Regarding external standard of 6-TGNt with a concentration of 35.90 µmol/L average, the area was 1428.910 with standard deviation of 31787.3 and 2.2% variation coefficient.

The results of the 6-MMPr and 6-TGNt measurement showed minimum levels of 403 ρmol/8x10$^8$RBC and 105 ρmol/8x10$^8$RBC and maximum 9821 ρmol/8x10$^8$RBC and 1875 ρmol/8x10$^8$RBC the mean concentrations of 3350 ρmol/8x10$^8$RBC and 383 ρmol/8x10$^8$RBC respectively. The results of 6-MMPr and 6-TGNt quantification in different subgroups studied are shown in Table 1.

The correlation between the daily AZA dose (mg/day) and the 6-MMPr levels, showed marked dispersion by Pearson correlation test ($P$>0.05) and Spearman test ($P$>0.05). Similar results were observed in correlation between the daily AZA dose (mg/kg/day) and the 6-MMPr levels. Similarly, there was a marked dispersion (Pearson: $P$>0.05), Spearman: $P$>0.05).

This behavior has been observed for 6-TGNt metabolite for daily dose in mg/day (Pearson: $P$>0.05 and Spearman $P$>0.05, respectively). Similarly, results were observed between the AZA dose (mg/kg/day) and the 6-TGNt levels. Similarly, there was a marked dispersion (Pearson: $P$>0.05, Spearman: $P$>0.05).

**6-MMPr and 6-TGNt measurement**

Regarding 6-MMPr measurement, 74.7% of the patients presented the levels below 5700 ρmol/8x10$^8$RBC and 25.3% presented values above 5700 ρmol/8x10$^8$RBC. Significant statistical differences ($0.04 < P < 0.05$) were observed in AZA+infliximab group when compared with other groups.

Most patients presented therapeutic levels (44%), 20% of the patients were out of the therapeutic threshold and 36% was related to toxicity levels. An important result was observed, 16% of the AZA+mesalazine group patients showed therapeutic levels, in contrast, 13.33% of the AZA+infliximab group patients presented toxic levels**.**

According to descriptive analysis of 33 patients with UC, 26 presented mild disease and 7 moderate diseases, according to the classification of Truelove&Witts[11]. Patients with mild disease had median of 6-TGNt levels (403 ρmol/8x10$^8$RBC). Patients with moderate disease had 270 ρmol/8x10$^8$RBC median levels ($t$-Student test, $P$>0.05)

Regarding to CD, of 42 patients, 12 patients presented clinical remission, 22 patients mild disease and eight patients moderate disease, according to the classification Harvey Bradshaw Index (HBI)[12]. Our results of 6-TGNt have demonstrated that patients in clinical remission showed median levels of 368 ρmol/8x10$^8$RBC, mild disease median of 410 ρmol/8x10$^8$RBC and patients with moderate disease median levels of 507 ρmol/8x10$^8$RBC (Kruskal-Wallis test, $P$>0.05)**.**

**TPMT polymorphisms analysis**

Our results showed prevalence of heterozygous genotype in 11.1% of the population study. An individual presented genotype homozygous for allelic variant *TPMT*2*.Results of allelic variations in the types of disease (CD or UC) and association between 6-MMPr and 6-TGNt levels are shown in Table 2.

**Table 1. Results of metabolites measurement in subgroups of patients studied in Percentile (%).**

| Subgroups | N | Measurement in ρmol/8x10$^8$ RBC | | | | | Azathioprine Median Daily dose | | *P*-Value |
|---|---|---|---|---|---|---|---|---|---|
| | | 6-TGNt | | | 6-MMPr | | | | |
| | | < 235 | 235-450 | >450 | < 5700 | >5700 | mg/day | mg/kg/day | |
| AZA Monoterapy | 18 | 8.0 | 9.33 | 6.67 | 17.33 | 6.67 | 125 | 2.38 | n.s |
| AZA Sulfasalazine | 14 | 5.33 | 8.0 | 5.33 | 12.0 | 6.67 | 125 | 2.20 | n.s |
| AZA Mesalazine | 22 | 2.67 | 16.0 | 10.67 | 18.67 | 10.67 | 125 | 1.89 | n.s |
| AZA Infliximab | 21 | 4.0 | 10.67 | 13.33 | [A]26.67 | 1.33 | 125 | 2.14 | 0.049 |
| Without treatment AZA (ST) * | 15 | | | | | | | | |
| Ulcerative colitis | 37 | 9.33 | 20.0 | 14.67 | 18.67 | 25.33 | 125 | 1.93 | n.s |
| Crohn´s disease | 53 | 10.67 | 24.0 | 21.33 | 49.33 | [A]6.67 | 125 | 2.14 | 0.003 |

Note. *ST: Suspended the treatment with AZA; n.s (non significant); [A]*P <0.05.*

**Table 2. Results of allelic variations**

| Polymorphism | Allelic Frequency n = 90 | Allelic Frequency in subgroups of patients studied | | | | | Median levels of 6-TGNt (pmol/8 x$10^8$RBC) | Median levels of 6-MMPr (pmol/8 x$10^8$RBC) | Allelic Frequency in type of diseases | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Aza Mono n = 18 | Aza Sulfa n =14 | Aza Mesa n = 22 | Aza IFX n = 21 | *ST n = 15 | | | UC n = 37 | CD n = 53 |
| Wild type *TPMT*1/1* | 79(87.8%) | 16(88.8%) | 12(85.8%) | 20(90.9%) | 20(95.2%) | 11(73.2%) | 382 | 3350 | 32(86.5%) | 47(88.6%) |
| Heterozygous *TPMT*1/*3A* | 1(1.1%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 1(6.7%) | * | * | 0(0.0%) | 1(1.9%) |
| Heterozygous *TPMT*1/*3B* | 5(5.6%) | 1(5.6%) | 1(7.1%) | 2(9.1%) | 0 (0.0%) | 1(6.7%) | 516 | 2983 | 4(10.8%) | 1(1.9%) |
| Heterozygous *TPMT*1/*3C* | 4(4.4%) | 1(5.6%) | 1(7.1%) | 0 (0.0%) | 1(4.8%) | 1(6.7%) | 344 | 1651 | 1(2.7%) | 3(5.7%) |
| Homozygous *TPMT*2* | 1(1.1%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 1(6.7%) | * | * | 0(0.0%) | 1(1.9%) |

Note: *ST: Suspended Treatment with AZA.

## DISCUSSION

The thiopurines immunosuppressants have used for the treatment of various diseases, which the immune system is present as a triggering mechanism, including the IBD. 6-MP and AZA are the main compounds of this drug family. The metabolism of AZA is complex and involves many enzymatic pathways, which is produced active, inactive and potentially toxic metabolites. Genetic (polymorphisms) and non-genetic factors may interfere with the metabolism of thiopurines, therefore it might influence the treatment response of IBD [17].

Regarding the daily dose of AZA, our findings were similar to de Graaf [18].It has found no correlation between the dose in mg/day or mg/kg/day to 6-MMPr and 6-TGNt levels. This fact has described by other studies[19], which reinforces the need for thiopurines metabolites therapeutic monitoring to prevent side effects.

In this study, 25.3% of patients showed 6-MMPr levels above 5700 ρmol/8x$10^8$RBC. Analyzing these results by disease type, patients with UC showed a higher percentage linked to liver toxicity values (18.67%) when compared to CD patients (6.67%). This fact may be associated with the treatment regimen used to treat both diseases. The combination of AZA with aminosalicylates is suitable in maintaining remission of IBD, particularly in moderate or severe UC. However, this may interfere in AZA metabolism[3]. Patients undergoing treatment of fixed AZA doses in association with 2g of 5-ASA/day (main serum metabolite of mesalazine) have shown no change in 6-MMPr levels, but an increasing level for 4g of aminosalicylate, thereby, decreasing the metabolite levels. This suggests a possible link with TPMT activity inhibition. Similarly, de Boer [19] and Hande [20] have demonstrated no change in 6-MMPr levels in patients under treatment with AZA and 6-MP through the action of 5-ASA with daily doses of 2 g.

The patients from AZA+mesalazine group have presented the highest percentage (10.67%) of 6-MMPr levels above 5700 ρmol/8x$10^8$RBC and patients of AZA+sulfasalazine group have shown only 6.67%. A possible explanation for these findings may be the prescribed dose of sulfasalazine and mesalazine. According to the records of our patients, we have found the mean daily dose used was 2.5 g of sulfasalazine and 2.7g of mesalazine, which is consistent with the literature[18]. In a similar manner, we have found that the patients in AZA monotherapy group reached the same percentage of hepatotoxic levels when compared to patients from AZA+sulfasalazine group; however, the possibility of drug interaction has ruled excluded.

Regarding the 6-TGNt quantification, 20% of studied patients presented levels considered out of the therapeutic threshold, especially in the AZA monotherapy group (8%). On the other hand, the AZA+mesalazine group had a higher rate of patients at therapeutic levels (16%). Our findings are in accordance with the study performed by Actis [21]. This indicates the synergistic effect of the interaction between AZA and mesalazine, with high 6-TGNt levels. Furthermore, Dewit [22] has demonstrated a significant decrease in metabolite levels when the use of aminosalicylates with AZA has discontinued. Therefore, this interaction is able to promote the maintenance of remission of diseases such as UC; however, it can also lead to an increasing in 6-TGNt levels. Thereby, there are a possibility of myelotoxicity. It is known that this side effect may occur by inhibition of TPMT, resulting in greater availability of 6-MP for

Hypoxanthine phosphoribosyl transferase (HPRT) which leads to formation of 6-TGNt metabolites [23].

In contrast, the AZA+infliximab group has showed the highest rate of patients with 6-TGNt toxic levels (13.33%). Roblin [7] also reported high levels of this metabolite. They showed a significant increase of 6-TGNt in the period between 1 and 3 weeks after the first infusion of biological drug with its subsequent stabilization after 3 months. This shows values exceeding 400 ρmol/8x$10^8$RBC, and they has considered good tolerance and favorable response. Yarur [24] showed strong correlation between the 6-TGNt levels of 125 ρmol/8x$10^8$RBC or greater, as more appropriate to achieve favorable therapeutic response. The divergence of 6-TGNt levels present in this interaction, require further studies in order to characterize better this association.

When we compare the clinical response and the 6-TGNt levels in both diseases, we found results consistent with other studies that obtained 6-TGNt levels of between 235-450 ρmol/8x$10^8$RBC as good therapeutic response [25]. Patients with mild disease showed median levels of 403 ρmol/8x$10^8$RBC and moderate disease 271 ρmol/8x$10^8$RBC. CD patients in both clinical remission and mild diseasepresented median values of 398 ρmol/8x$10^8$RBC and 410 ρmol/8x$10^8$RBC, respectively. However, patients with moderate disease presented higher 6-TGNt levels (507 ρmol/8x$10^8$RBC).

In order to identify polymorphisms in the *TPMT gene* in patients participating in this study, it was used an analysis performed on 90 patients. We observed the prevalence of 11.1% for the heterozygote genotypes: *TPMT*3A* (1.1%), **3B* (5.6%) and **3C* (4.4%). The *TPMT*3B* variant was the most prevalent in our study, diverging from some Brazilian studies. Reis [26] observed prevalence for *TPMT*3C* variant. In a study reported by Silva [27] and Gastal [16] the most prevalent variant was *TPMT*3A* and Boson [28] was *TPMT*2*. One possible explanation for this is the large miscegenation of the Brazilian population, as well as the South American continent.

Medical records of 15 patients demonstrated the interruption with AZA treatment by the side effects such as leukopenia, thrombocytopenia or pancytopenia. This fact motivated us to investigate the reasons whereby the treatment had suspended. Once it was not possible to quantify the 6-TGNt and 6-MMPr metabolites due their half-life in erythrocytes (13 days), we performed only the genetic profile. Interestingly, in this study, we observed only 4.4% of these patients have presented polymorphisms.

Of the 15 patients who discontinued the treatment, 13 had made use of AZA in combination with sulfasalazine, mesalazine, or infliximab for more than 8 weeks, after the appearance of complications was suspended treatment with AZA, and made a substitution of another immunosuppressant. About the two patients, they used AZA for two weeks and thereafter, they developed leukopenia. One of these patients had *TPMT*3A* variant which determines a low TPMT activity phenotype. The other patient of this group showed homozygous genotype for *TPMT*2* variant, which also contributed for low activity of TPMT. This may lead to increasing 6-TGNt formation, triggering leukopenia [29].

In the patients where the polymorphisms has not detected, the cause may not have been only the genetic factor but also a sum of factors that may interfere with the AZA pharmacokinetics when combined with other drugs [24].

In the comparison between the polymorphism and 6-MMPr levels, we observed median levels of 2984 ρmol/8x$10^8$RBC and 1652 ρmol/8x$10^8$RBC for the *TPMT*3B* and *TPMT*3C* variants, respectively. These genotypes have related with the intermediate TPMT activity that leads to an increasing of 6-TGNt production by the interference in their ability to balance the metabolites (6-TGNt and 6-MMPr) formation [30].

As the influence of these polymorphisms on 6-TGNt levels, we found median levels of the 431 ρmol/8x$10^8$RBC in seven patients with positivity for allelic variants studied, therefore, levels considered favorable for a good therapeutic response. Our group has noted that four of these patients presented the *TPMT*3B* variant, with median of 516 ρmol/8x$10^8$RBC, two of those with toxic levels. Median levels of 6-TGNt for the three patients with positivity for *TPMT*3C* variant, was 345 ρmol/8x$10^8$RBC and only one patient found levels considered toxic. Our results are in agreement with other studies that link the interference of these polymorphisms in 6-TGNt levels [31].

The genotyping and/or metabolite assays for monitoring of thiopurines metabolites for prevent, reduced the side effects and presented better therapeutic response was reported in the literature, especially regarding the dosage of active metabolites. This is a controversy fact, once this quantification has not performed in leukocytes but in erythrocytes and these do not produce some enzymes that are included in the metabolic thiopurines pathways [32]. In contrast, the most clinicians are in favor of observing these metabolites in order to check failure of therapy detect sub-dosage and identify non-responders [29].

Assay of TPMT activity and monitoring of metabolites may be useful when genetic tests are not available [33]. However, these three parameters has needed to achieve a good response, while minimizing the side effects [34]. Further studies are necessary with each drug combination used in the IBD treatment, to determine a consensus of these metabolites concentration, which it has correlated with a favorable clinical response, endoscopic activity and laboratory test.

## CONCLUSION

In our study, the metabolism of azathioprine was influenced by non-genetic factors such as interactions due to the combination of drugs used in the treatment of inflammatory bowel disease or another not included in this study. Polymorphisms detected did not demonstrate negative effect on metabolites levels. This study suggests the follow up of prior genetic screening, periodically monitoring of TPMT activity and levels of active metabolites demonstrated to be relevant on the contribution of selecting the appropriate therapeutic regimen as well as to monitor patients using azathioprine to obtain benefits of treatment and consequent reduction of side adverse effects.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

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