Plasma thiopurine S-methyltransferase levels and azathioprine-related adverse events in patients with Behçet's disease

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ABSTRACT

Objective. *Thiopurine S-methyltransferase (TPMT) is the key enzyme inactivating azathioprine (AZA), an immunosuppressive agent commonly used for treating inflammatory diseases including Behçet's disease (BD), systemic lupus erythematosus (SLE) and systemic vasculitis. Low TPMT levels facilitate occurrence of AZA-related adverse effects. We investigated TPMT levels in patients with BD, compared to healthy controls and patients with SLE or systemic vasculitis.*

Methods. *This cross-sectional study included 101 BD (77 using AZA), 74 SLE (35 using AZA), and 44 vasculitis (18 using AZA) patients and 101 healthy controls. Plasma TPMT levels were measured using ELISA. Student's t- and Kruskal-Wallis tests were used to compare TPMT levels according to possible risk factors. Receiver operating characteristic (ROC) analysis was used to determine whether a cut-off TPMT level could be found to predict AZA-related adverse effects.*

Results. *Plasma TPMT levels (mean± SD ng/mL) in BD (22.80±13.81) were comparable with healthy controls (22.71±13.49), but significantly lower than in SLE group (29.37±11.39) ($p<0.001$). TPMT levels in 130 patients receiving AZA were similar to the rest of the group. AZA-related adverse effects were identified in only 8 patients (5 with BD and 3 with SLE). TPMT levels were significantly lower in those 8 patients (14.08±9.49 vs. 25.62±12.68) ($p=0.013$), besides a cut-off value for predicting adverse effects was determined for the BD group with ROC analysis (area under the curve: 0.813).*

Conclusion. *This is the first study to evaluate TPMT activity in a Turkish adult population. Although low plasma TPMT level is not the only factor determining AZA toxicity, a TPMT cut-off value may help to predict AZA-related adverse effects in BD.*

Introduction

Thiopurine S-methyltransferase (TPMT) is a genetically moderated key enzyme involved in the metabolism of thiopurine-based drugs including azathioprine (AZA) and 6-mercaptopurine. TPMT catalyses S-methylation and inactivation of these drugs (1-3). AZA is widely used for the treatment of many manifestations of Behçet's disease (BD), and its efficacy in the treatment of Behçet's uveitis was previously shown in a controlled trial (4). Other than BD, AZA is also used for the treatment of many systemic inflammatory diseases including systemic lupus erythematosus (SLE), various systemic vasculitis and inflammatory bowel diseases.

It is well known that both clinical efficacy and adverse effects of AZA demonstrate wide inter-individual variability, which mainly results from thiopurine metabolism, largely affected by TPMT status (5-8). The various adverse effects of AZA include myelosuppression, hepatotoxicity, pancreatitis, and flu-like symptoms. Since reduced or absent TPMT activity may place patients at increased risk for AZA-related adverse effects, determining TPMT levels and/or activity before starting AZA therapy may be important. Possible toxic effects can be anticipated and high doses of AZA can be avoided in individuals with low TPMT activity (8).

TPMT status of a patient can be assessed either by genotyping for variant alleles or by phenotyping TPMT enzymatic activity (8). TPMT enzyme activity is clearly decreased in the presence of certain variant TPMT alleles (9). In

general, individuals heterozygous for a variant TPMT allele have intermediate enzymatic activity, while those with homozygous variant TPMT alleles have very low or absent enzymatic activity (9). For phenotyping enzymatic activity, red blood cell lysate or whole blood may be used (10). Measurement of intra-erythrocyte TPMT enzymatic activity is generally the method of choice and can be accomplished by a number of different ways, including radiochemical assay, mass spectrometry, and high-performance liquid chromatography with absorbance or fluorescence. Recently, there have been attempts to measure TPMT enzyme levels in whole blood, plasma or serum, mostly using Enzyme-Linked Immunosorbent Assay (ELISA) (10, 11).

Only a few studies have investigated TPMT activity in Turkish patients, conducted in a paediatric population with haematological disorders (12, 13). To our knowledge, no study has evaluated TPMT activity in a Turkish adult population. The aim of the present study was to assess plasma TPMT levels in patients with BD, both in subgroups who have used or have never used AZA, and to compare our findings with a healthy control group, and disease control groups including SLE and systemic vasculitis. Furthermore, in patients receiving AZA treatment, we tried to determine whether a cut-off TPMT level could be found to predict AZA-related adverse effects, using receiver operating characteristic (ROC) analysis.

Methods

In the present cross-sectional study, 101 patients with BD (mean age 42.15±10.33 years; M/F: 61/40) who fulfilled the International Study Group for BD criteria (14), 74 patients with SLE (M/F: 11/63; mean age 39.86±11.69 years) fulfilling the American College of Rheumatology (ACR) criteria (15), 44 patients with various systemic vasculitis (mean age 47.09±13.36 years; M/F: 21/23) and 101 healthy controls, age- and sex-matched with the BD group (mean age 41.87±9.91; M/F: 61/40) were included. Patients with SLE and systemic vasculitis were selected as positive disease controls, because similar to BD, AZA was also among the therapeutic agents which may be used for treating these diseases. Systemic vasculitis group was not homogeneous, and included patients with granulomatous polyangiitis (n=14), microscopic polyangiitis (n=3), eosinophilic granulomatosis with polyangiitis (n=4), polyarteritis nodosa (n:1), primary angitiis of central nervous system (n=1), Takayasu arteritis (n=18) and temporal arteritis (n=3). In all patient groups, some of the patients have been receiving or had previously used AZA, while some had never used AZA. Detailed past medical data including age, sex, diagnosis, laboratory results, and current and previous medications were obtained from the patients' records. Among patients with AZA experience, occurrence of AZA-related adverse events was also carefully noted. Informed consent was obtained from all subjects, and the study protocol was approved by the Human Research Ethics Committee of Ege University in Izmir.

Plasma TPMT level was measured using a commercial ELISA kit (USCN, E92821Hu). The kit was a sandwich enzyme immunoassay for *in vitro* quantitative measurement of TPMT in human plasma. Ten mL of blood was collected in tubes coated with EDTA and centrifuged for 15 minutes at 1000xg within 30 minutes after collection. Since haemolysis would influence the results, haemolytic specimens were excluded. Plasma samples were stored in aliquot at -80°C until measurements were made. In order to avoid loss of bioactivity and contamination, measurements were performed in two-months time. All of the frozen plasma samples were kept at room temperature (18–25°C) before use. There was no history of red blood cell transfusion in study participants during the last three months. None of the study participants were receiving any of the drugs including naproxen, ibuprofen, ketoprofen, furosemide, sulfasalazine, mesalamine, mefenamic acid, thiazide diuretics, and benzoic acid inhibitors that inhibit TPMT enzyme activity. In patients using low dose aspirin, this treatment was stopped one week before collection of blood.

Plasma TPMT levels of study groups were compared with each other. Among patients using AZA, plasma TPMT levels of those who experienced AZA-related adverse events were compared with those without AZA toxicity.

Statistical analysis

The continuous variables were described as mean ± standard deviation (mean±SD). The categorical variables were described as numbers and percents. For the comparison of continuous variables, p-value <0.05 was considered statistically significant. Kolmogorov-Smirnov test was used to evaluate whether plasma TPMT activity followed a normal distribution.

Mean plasma TPMT level was the dependent variable. Student's t-test was used to compare mean plasma TPMT levels according to age (categorised as higher or lower than 40 years), gender (male/female), serum creatinine level (categorised as ≥ or <1.2 mg/dl) and haemoglobin (Hb) value (categorised as ≥ or <12 gr/dl). Kruskal-Wallis test was used to compare mean TPMT among patient groups (BD, SLE, systemic vasculitis) and drugs currently being used were analysed.

Receiver operating characteristic (ROC) analysis was used for AZA users to determine whether a cut-off value for plasma TPMT level could be used to predict AZA-related toxicity.

We calculated area under the curve (AUC), adjusting that lower plasma TPMT levels indicate occurrence of more AZA-related adverse events. We investigated the coordinates of AUC to determine a cut-off value for predicting AZA-related toxicity.

On the other hand, based upon the literature data reporting that nearly 11% of the population had low/intermediate level of TPMT activity (16, 17), we stratified plasma TPMT levels from the highest to lowest value in 130 AZA users. Using chi-square analysis, we compared those at or below the 11th percentile with the rest of the group above the 11th percentile, in regard to occurrence of AZA-related adverse events.

Results

The demographic features of the study groups were given in Table I. Plasma

TPMT levels of the study groups from the highest to lower values were as follows: 29.37±11.39 ng/mL in SLE group, 26.24±11.4 ng/mL in systemic vasculitis group, 22.80±13.81 ng/mL in BD group, and 22.71±12.4 ng/mL in healthy control group (Table I). Plasma TPMT levels in BD group was significantly lower than in SLE group (p<0.001), and comparable with healthy control group (p=0.951). There was no significant difference between BD group and the systemic vasculitis group (p=0.156).

At the time of enrolment in this study, 140 patients (63.9%) have been on corticosteroid treatment and 91 patients (41.6%) on AZA treatment. Including those patients who had previously used AZA, altogether there were 130 patients, (77 with BD, 35 with SLE, and 18 with systemic vasculitis) who had used AZA in the past or have been currently using AZA. Plasma TPMT levels in 130 patients receiving AZA were similar to the rest of the group. Likewise, in each patient group, plasma TPMT levels were not significantly different between AZA users and those who have never used AZA. Among various defined parameters, only Hb and serum creatinine levels were found to influence plasma TPMT levels. Plasma TPMT levels were significantly lower in patients having Hb≥12 gr/dl compared to patients having Hb<12 gr/dl (p=0.01), but significantly higher in patients having creatinine ≥1.2 mg/dl compared to patients having creatinine <1.2 mg/dl (p=0.026). On the other hand, age (p=0.772), sex (p=0.252) and current use of AZA (p=0.782) did not significantly influence plasma TPMT levels.

Among 130 AZA users, AZA-related adverse effects were identified in only eight patients (five with BD and three with SLE). The reported adverse effects were bone marrow toxicity (n=7) and hepatotoxicity (n=1). The characteristics of those eight patients who developed AZA-related adverse effects were demonstrated in the Table II. Plasma TPMT levels were significantly lower in those 8 patients, compared to rest of the group (14.08±9.49 *vs*. 25.62±12.68, p=0.013). Besides, using ROC analysis, cut-off values for predicting adverse effects were determined both in patients with BD and in the whole group of patients; areas under the curve were 0.813 and 0.751, respectively (Fig. 1A-B). When we determined the cut-off value of plasma TPMT levels for BD group as 30 ng/mL, this showed a sensitivity of 100% and specificity of 66%. Similarly, when the cut-off value of plasma TPMT levels for the whole group of patients was accepted as 28 ng/mL, this showed a sensitivity of 100% and specificity of 43.4%.

Among 130 AZA users, following stratification of plasma TPMT levels from the highest to lowest value, we found out that 3 out of 8 patients with AZA-related adverse events were located in the group below the 11th percentile (3/15; 20%), while 5 out of 8 patients were located in the group above the 11th percentile (5/115; 4.3%). Using chi-square analysis, frequency of AZA-related adverse events was significantly higher in the first group with plasma TPMT levels below the 11th percentile (Fisher's exact p=0.049).

Table I. The demographic features and plasma TPMT levels of study groups.

Study group	n	Age (mean ± SD)	Gender (Male/female)	TPMT activity (mean ± SD ng/mL)
Behçet's disease	101	42.15 ± 10.33	61/40	22.80 ± 13.81
SLE	74	39.86 ± 11.69	11/63	29.37 ± 11.39
Systemıc vasculitis	44	47.09 ± 13.36	21/23	26.24 ± 11.4
Healthy controls	101	41.87 ± 9.91	61/40	22.71 ± 13.49

TPMT: Thiopurine S-methyltransferase; SLE: Systemıc lupus erythematosus.

Table II. Characteristics of patients who developed azathioprine-related adverse events.

Age/Sex	Disease	AZA dose (mg/kg)	Side effect	TPMT Activity (ng/mL)
49/Female	SLE	1.04	Myelosuppression	13.3
37/Female	SLE	1.61	Myelosuppression	16.4
28/Female	SLE	0.90	Myelosuppression	27.9
31/Male	BD	1.21	Myelosuppression	4.2
36/Female	BD	1.41	Myelosuppression	6.44
49/Male	BD	0.61	Hepatotoxicity	6.35
49/Male	BD	0.58	Myelosuppression	9.84
28/Female	BD	0.54	Myelosuppression	28.2

AZA: Azathioprine; TPMT: Thiopurine S-methyltransferase; SLE: Systemıc lupus erythematosus; BD: Behçet's disease.

Discussion

To our knowledge this is the first study evaluating TPMT activity in Turkish adult population. We found out that plasma TPMT levels in BD were comparable with healthy controls, but significantly lower than in SLE group. We confirmed that lower plasma TPMT levels were significantly associated with occurrence of AZA-related adverse events. Furthermore, using ROC analysis we could determine cut-off values for predicting AZA-related adverse events both in patients with BD and in the whole group of patients (Fig. 1A-B). In the literature, there is an ongoing debate about whether TPMT testing should be performed to a patient before commencement of AZA treatment to reduce AZA-related adverse events, or not (5, 18, 19, 20). However it should be kept in mind that TPMT status is not the only factor predicting AZA-related adverse events. It has been reported that up to 70% of patients with adverse events may have normal TPMT activity, and other factors such as viral infections, concomitant drug treatments and other disturbances in the thiopurine metabolic pathway probably also play a role (21). Similarly, some patients may not experience AZA-related adverse events, despite presence of low plasma TPMT levels, as also seen in some patients in our study. Such observations, further confirm the information that plasma TPMT level is not the only factor determining AZA toxicity. Although, a plasma TPMT cut-off value may help

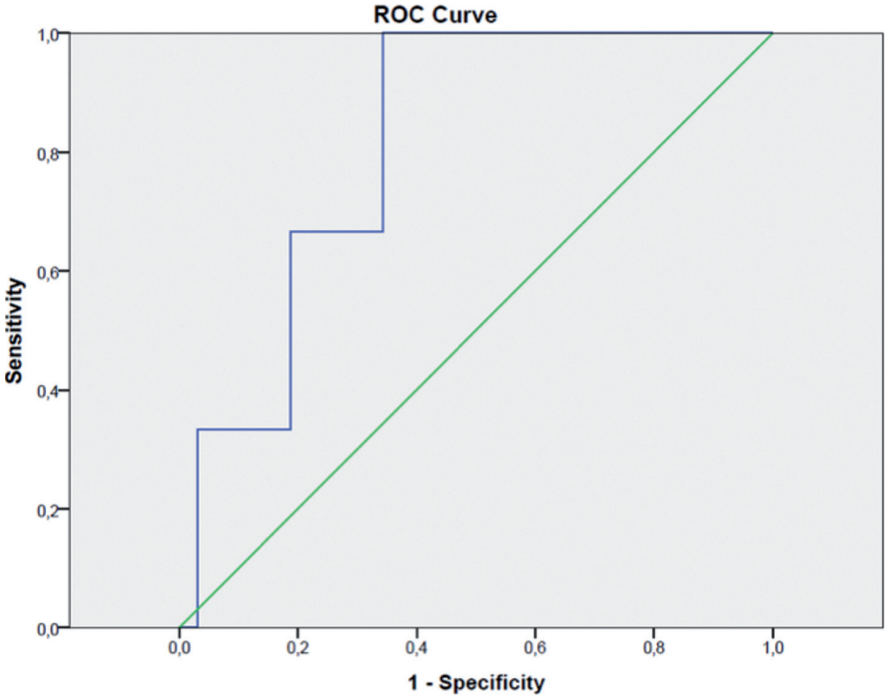

Fig. 1A. ROC curve representing sensitivity and specificity of TPMT testing among patients with Behçet's disease. AUC=0.813 (n=77).

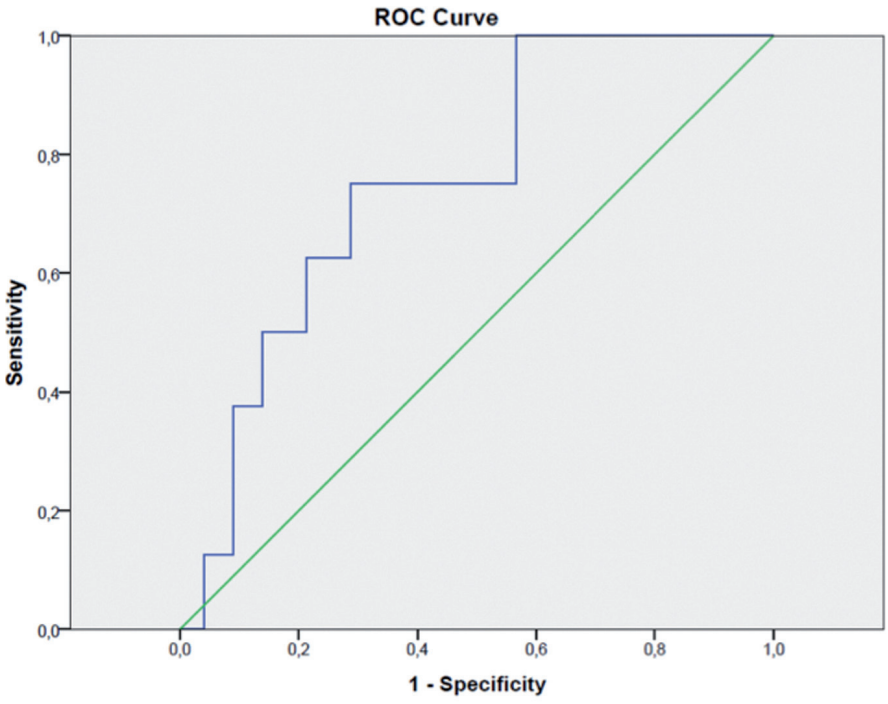

Fig. 1B. ROC curve representing sensitivity and specificity of TPMT testing among all AZA-receiving patients. AUC=0.751 (n=130).

to predict AZA-related adverse effects, as shown in the present study, currently we have no evidence to suggest TPMT testing before commencement of AZA for patients with BD.

We cannot exactly explain our finding of significantly higher plasma TPMT levels in SLE group compared to BD group; however lower mean age, higher "female to male" ratio and more importantly higher frequency of renal dysfunction in SLE group might have possibly contributed to this finding. Since, plasma TPMT values were found to be significantly higher in patients with serum creatinine levels ≥1.2 mg/dl than those with serum creatinine levels <1.2 mg/dl, this might have led to higher plasma TPMT levels in SLE group and possibly in vasculitis group, having more frequent renal involvement than BD group.

A study from Spain also reported significantly different TPMT activities in different groups of diseases including inflammatory bowel disease, autoimmune hepatitis, multiple sclerosis, myasthenia gravis, pemphigus and chronic renal failure (22). Ethnicity, presence of chronic diseases, concurrent drug treatments, red cell kinetics and transfusions clearly affect TPMT activity (18). The effect of gender on TPMT activity is controversial (23-25). However, age seems to be more important, and children are reported to have higher TPMT activities than adults (26). The age profiles of the erythrocytes are also important; younger erythrocytes have higher TPMT activities than older cells (27).

Although TPMT gene expression may be decreased during or after AZA treatment, no change in enzyme activity was reported during or after AZA treatment (28, 29). In the present study, we also could not find significant difference of plasma TPMT levels between subgroups of BD patients with and without AZA.

It has been reported that nearly 89% of individuals in a certain population have high/normal TPMT activity. Nearly 11% of the population is heterozygous for a variant TPMT allele and have intermediate enzymatic activity, while roughly 0.3% of the population is homozygous for a variant TPMT allele and have very low or absent enzymatic activity (30-33). However, TPMT enzyme activity is highly variable and the frequencies may obviously change depending upon ethnicity (32). Besides, sometimes genotype-phenotype discrepancies may also be observed and this may be partially explained with epigenetic factors such as TPMT inhibition by co-administered medications.

One of the limitations of the present study is the lack of TPMT genotyping analysis for technical reasons. However, the concordance between genotyping

and phenotyping has been reported to be approximately 60–70% for patients with low TPMT activity and higher than 90% overall (34, 35). Therefore, some authors have suggested that phenotyping enzymatic analysis may be preferred for detecting TPMT activity for practical purposes. Interestingly, sometimes phenotypic analysis may be more important than genotyping. Among heterozygous individuals, there may be a two- to three-fold variation in TPMT activity, which may be detected only by measuring enzyme levels. Furthermore, a number of homozygous "wild-type" individuals reveal very high TPMT activity, and they might need treatment with higher than standard doses of AZA (10).

Another weak point in the present study may be the choice of the method for phenotyping analysis of TPMT. Although many studies measured intra-erythrocyte TPMT enzymatic activity for phenotyping analysis, we measured plasma TPMT levels using ELISA. However, measuring TPMT levels in the whole blood, plasma or serum, mostly using ELISA method is a recently reported alternative approach, which may have some advantages over red blood cell (RBC) analysis (10, 11). Firstly, it is easier and quicker, and does not require preparation of washed RBC. Less sample handling errors, more reproducibility and increased accuracy were reported with whole blood analysis. The original whole blood sample may be more homogeneous than a pellet of washed RBC and may better represent general TPMT status, rather than intra-erythrocyte levels. Besides, plasma TPMT levels are not affected from Hb levels and recent blood transfusions (10). Notably, Ford *et al*. reported that the results of new whole blood and standard RBC lysate methods were comparable and overall concordance with genotypic analysis was 97% for both phenotypic methods (10).

A further limitation of the present study is the low numbers of the patient groups. Finally, the patient group of systemic vasculitis was not homogeneous and consisted of many different types of vasculitis.

When AZA-related adverse events occur in patients with BD having low TPMT levels, AZA dose may be reduced or discontinued. In such patients, and in general, in patients with treatment-resistant BD, anti-cytokine biologic agents may be effectively used. Other than anti-TNF agents, anti-IL-1 agents such as anakinra and canakinumab, the anti-IL-1β antibody gevokizumab and the anti-IL-6 agent tocilizumab seem promising in such patients, as discussed in two recently published reviews (36, 37).

In conclusion, this is the first study to evaluate TPMT activity in a Turkish adult population. TPMT levels in BD were comparable with healthy controls, but significantly lower than in patients with SLE. We observed more frequent AZA-related adverse effects in the presence of low TPMT levels, and using ROC analysis we could determine a cut-off value which may help to predict AZA-related adverse effects. However, we accept that TPMT levels may not be the only factor determining AZA toxicity, and currently we have no evidence to suggest TPMT testing before commencement of AZA for patients with BD.

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