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Original paper

Predictors of azathioprine toxicity in children with autoimmune hepatitis

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Abstract

Aim of the study: Azathioprine (AZA) is an important steroid-sparing drug in the management of autoimmune hepatitis (AIH). Avoidance of its adverse events that could be severe and carry a risk of mortality in a few cases is important, preferably with cheap and easy assessments that could be feasible in developing countries with the unavailability of molecular assays. Assessment of thiopurine methyltransferase (TPMT), the key enzyme for the inactivation of AZA, as a predictor of AZA toxicity had been a matter of conflict. This work aimed to study the role of TPMT serum level assessment and other host-, disease-, and treatment-related factors in predicting AZA toxicity.

Material and methods: Sixty-six children with AIH, divided into two groups, were recruited. Group 1 included twelve children with AZA toxicity and group 2 included fifty-four children without AZA toxicities. Both groups were compared for demographic, clinical, laboratory, histopathological, and treatment-related factors, and serum TPMT level, measured by ELISA.

Results: TPMT serum level was comparable in both groups (p = 0.363). Duration of treatment until enzyme normalization and duration of AZA therapy were significantly associated with AZA toxicity (p = 0.007 and p = 0.01, respectively). At the first follow-up treatment with AZA, total leucocyte count (TLC) and neutrophil counts were significantly lower in group 1 (p = 0.005 and p = 0.002, respectively). Moreover, the percentage reduction of TLC and neutrophil counts were significantly higher in group 1 (p < 0.001, for both).

Conclusions: Monitoring for AZA adverse events in those with the defined predictors of AZA-related adverse events is more important than TPMT assessment.

Key words: autoimmune hepatitis, adverse events, azathioprine, myelosuppression, thiopurine methyltransferase.

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Introduction

Autoimmune hepatitis (AIH) is an aggressive liver disease that affects children and adults and constitutes a significant proportion of liver diseases in children. If untreated, it carries a high rate of complications that end in mortality. Fortunately, most of these cases will respond to medical treatment, especially if it is started early and well monitored. However, steroids, as the first-line immunosuppressive therapy, have many adverse events [1].

Azathioprine (AZA) is a keystone drug in the treatment of AIH; it is of proven benefit as a steroid-sparing agent. Despite high efficacy, adverse reactions occur in a proportion of patients, including gastrointestinal intolerance, pancreatitis, hypersensitivity, and myelosuppression, that could be rarely fatal or result in treatment withdrawal. Predicting patients who have a high

propensity for these adverse events, especially with cheap parameters that could be available in developing countries, is warranted [2].

Thiopurine methyltransferase (TPMT) is the key enzyme for the inactivation of AZA. The TPMT enzyme is encoded by a highly polymorphic gene, thus leading to varying levels of enzyme activity in individuals. Many previous studies have targeted TPMT assessment or AZA metabolites for predicting AZA toxicity. However, these studies were conflicting with some supporting the utility of its assessment [3, 4] while others discourage this practice [5-7]. These different results could be attributed to the different methodologies of this assessment. Some studies relied on the genotyping or phenotyping assay of the TPMT. Others relied on measurement of the AZA metabolites. All of these previous assessments have one or more defects in the actual assessment of the TPMT and prediction of AZA toxicity [1, 6, 8-10], besides their costs. Moreover, despite many such kinds of research in adults, pediatric studies are still scarce [2, 7].

Notably, insufficient research has concerned other factors that could modify AZA toxicities, such as host-, disease-, and treatment-related factors. So, in the present study, we aimed to assess these factors together with the measurement of the TPMT enzyme level in relation to AZA toxicities.

Material and methods

Study population

This observational retrospective cohort study included sixty-six children with AIH. They were recruited from the Department of Pediatric Hepatology, Gastroenterology, and Nutrition.

In this study, all children diagnosed with AIH within the last ten years (from June 2010 to June 2020) and who received AZA as a steroid-sparing drug were recruited. They were divided into two groups. Group 1 constituted twelve children who developed AZA-related adverse events within the study duration. Group 2 constituted fifty-four children who did not develop any AZA-related adverse events within the same duration.

Forty-nine cases were excluded from the study due to the following exclusion criteria: the presence of associated liver disease (such as viral hepatitis), receiving concomitant drugs that affect AZA metabolism, recent blood transfusion (within three months [11]), those who are non-compliant or skipped follow up, and cases with overlap with sclerosing cholangitis.

Drugs that could affect the AZA metabolism and were checked in this study were xanthine oxidase inhibitors (e.g., allopurinol [12], aminosalicylates [13]), liver microsomal enzyme inducers (e.g. phenobarbitone, rifampicin, and carbamazepine), or inhibitors (e.g. fluconazole and erythromycin).

The treatment regimen they received was prednisolone 1-2 mg/kg/day up to a daily dose of 60 mg with a reduction of the dose for 6-8 weeks according to the decrease of aminotransferase levels targeting a maintenance dose of 5 mg/day. AZA was added for all recruited cases as a steroid-sparing drug after the 2nd week of steroid therapy, starting with a dose of 0.5 mg/kg/day and increasing gradually according to the absence of adverse events targeting a daily dose of 2 mg/kg/day. Follow-up duration of recruited cases ranged from a minimum of 8 months to a maximum of 8 years.

The study was approved by the Research Ethics Committee and conforms to the 1975 Declaration of Helsinki and its later amendments. No informed consent was required due to the retrospective nature of the study.

Etiological diagnosis

Diagnosis of AIH relied on suggestive clinical, laboratory, and pathological criteria for AIH [14] with the exclusion of other possible etiologies such as viral hepatitis, drug-induced liver injury, and metabolic liver disorders, e.g. Wilson's disease. All history data, clinical examination, and investigations at the time of presentation were registered from the patient files together with all follow-up data as regards treatment response and adverse events. AIH modes of presentation, types, and different treatment responses were defined as reported by the American Association for the Study of Liver Diseases [15]. The treatment responses were: complete remission – defined as normalization of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and immunoglobulin G (IgG) levels and/or absence of inflammation in liver tissue after treatment; treatment failure – worsening laboratory or histological findings despite compliance with standard therapy; incomplete response – improvement of laboratory and histological findings that are insufficient to satisfy criteria for complete remission; and relapse after remission – exacerbation of disease activity after induction of remission and drug withdrawal (or nonadherence).

AZA-related adverse events

Azathioprine toxicity was defined as the development of any AZA-related adverse events after the start

of AZA treatment. The reported AZA-related adverse events in the study group were myelosuppression (neutropenia, thrombocytopenia, and anemia) and hepatotoxicity. Neutropenia was defined as an absolute neutrophil count of less than 1000/µl. Thrombocytopenia was defined as a platelet count of less than 100,000/µl. Anemia was defined as hemoglobin levels < 10 g/dl and hematocrit values < 30% [16, 17]. Occult or overt gastrointestinal bleeding was excluded in those with new-onset anemia. The confirmation that anemia is attributed to AZA was when hemoglobin levels rise after the reduction of the AZA dose. For those who do not respond to dose reduction, iron status was evaluated followed by a trial of iron therapy accordingly [18]. Hepatotoxicity was defined in our cases as cholestasis or elevation in aminotransferases that resolved after AZA dose reduction or discontinuation [19].

TPMT serum level measurement

Using an enzyme-linked immunosorbent assay (ELISA) methodology, TPMT serum level was measured at presentation for all cases by a TPMT ELISA Kit, catalog No.: E10435h (Wuhan EIAab Science Co., Ltd, Wuhan, China).

Serum autoantibodies

All children were tested for serum autoantibodies and γ globulins at presentation. Antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA), liver-kidney microsomal antibody-1 (LKM1), and antimitochondrial antibody (AMA) were tested by an indirect immunofluorescence technique using a Fluoro-Kit Combo Pak (DiaSorin, Stillwater, Minnesota, USA).

Liver biopsy

Ultrasonography (US)-guided liver biopsy was performed for all children, at presentation, with an acceptable coagulation profile using a Tru-Cut needle, size 16G. Histological evaluation of chronic hepatitis was performed using the Ishak scoring system [20]. Inflammatory activity, fibrosis, and steatosis were graded as we reported before [21].

Statistical analysis

Quantitative variables were expressed as mean ± standard deviation (SD) or a median (minimum-maximum) according to the nature of the data, while qualitative variables were expressed as number (percentage) of individuals with a condition. For quantitative data, statistical significance was tested by either the independent samples *t*-test or by the non-parametric Mann-Whitney *U* test according to the nature of the data. For qualitative data, significance was tested by the chi-square test or Fisher's exact test. The cut-off for optimal clinical performance was determined from the receiver operator characteristic (ROC) curve. The diagnostic performance was measured as sensitivity, specificity, and accuracy. Results were considered significant if the *p*-value was < 0.05. Statistical analysis was performed using SPSS, version 16 (SPSS Inc., Chicago, IL, USA).

Results

Demographic and clinical characteristics of the studied groups at presentation

The groups were comparable as regards all demographic and clinical data at presentation, as shown in Table 1.

Laboratory data of the studied groups at baseline and two weeks after therapy with AZA

Baseline laboratory parameters of the two groups, including liver function tests, international normalized ratio, gamma globulins, hemoglobin level, total leucocyte count (TLC), neutrophils, and platelet count, were comparable with *p*-values > 0.05 for all. On the other hand, the laboratory results of the first follow-up treatment with AZA showed a significantly lower TLC and neutrophil count in group 1. The percentage TLC reduction and percentage neutrophil reduction from the previous laboratory data were significantly higher in group 1 (Table 2).

Bilirubin level at the timing of AZA introduction

There was no statistically significant difference between the two groups as regards the level of total bilirubin at the timing of AZA introduction ($p = 0.087$). Levels ranged from 0.7-2.0 mg/dl with a median of 1.3 mg/dl for group 1. On the other hand, the median value for group 2 was 1.8 mg/dl with a range of 0.4-7.0 mg/dl.

Histopathological findings of the studied groups at presentation

Liver biopsy data of ten cases were missing. Analysis of the histopathological findings of the available

Table 1. Demographic and clinical data of the studied groups at presentation

Variable	Group 1 (AIH children with AZA toxicity) (*n* = 12)	Group 2 (AIH children without AZA toxicity) (*n* = 54)	*P* value
Age (years)	6.7 (1.5-11)	6.3 (0.75-16)	0.993
Sex (female)	8 (66.7%)	32 (59.3%)	0.635
Presentation mode			
Acute	3 (25%)	19 (35.2%)	0.264
Insidious	3 (25%)	21 (38.9%)	
Asymptomatic elevation of transaminases	2 (16.7%)	8 (14.8%)	
Complications of cirrhosis	4 (33.3%)	6 (11.1%)	
AIH type			
Type 1	8 (66.7%)	40 (74.0%)	0.533
Type 2	3 (25%)	7 (13.0%)	
Seronegative	1 (8.3%)	7 (13.0%)	
Clinical presentation			
Jaundice	9 (75%)	43 (79.6%)	0.723
Abdominal pain	3 (25%)	19 (35.2%)	0.498
Melena	1 (8.3%)	1 (1.9%)	0.333
Hematemesis	1 (8.3%)	0 (0%)	0.182
Vomiting	3 (25%)	11 (20.4%)	0.723
Diarrhea	2 (16.7%)	7 (13%)	0.735
Anorexia	4 (33.3%)	12 (22.2%)	0.417
Arthralgia	0 (0%)	2 (3.7%)	1.0
Generalized body ache	0 (0%)	3 (5.6%)	1.0
Hepatomegaly	11 (91.7%)	43 (79.6%)	0.328
Splenomegaly	12 (100%)	48 (88.9%)	0.582
Ascites	1 (8.3%)	5 (9.3%)	1.0
LL edema	0 (0%)	5 (9.3%)	0.575

AIH – autoimmune hepatitis, AZA – azathioprine, LL – lower limb

fifty-six cases showed no significant difference between the groups, as shown in Table 3.

Treatment data and AZA-related adverse events of the studied groups

Azathioprine-related adverse events appeared after a minimum of 6 months duration of AZA treatment (Fig. 1B). The most frequently reported AZA-related toxicity was anemia (10/12; 83.3%), followed by thrombocytopenia (9/12; 75%) and neutropenia (8/12; 66.7%). On the other hand, hepatotoxicity occurred in only 3 cases (25%). During hepatotoxicity, the peak bilirubin was 5 mg/dl and the peak AST/ALT was 213/167 U/l. The three cases were resolved within 15-30 days with a reduction of AZA dose to 0.5 mg/kg/day.

The longer the duration of AZA treatment, the more likely it was to have AZA toxicity. Cut-off value and its diagnostic performance are shown in Figure 1A and Table 4. Moreover, the longer the duration of immunosuppressive treatment to achieve normal liver enzymes is, the greater is the likelihood to have AZA toxicity. The cut-off value and its diagnostic performance are shown in Figure 1C and Table 4.

The AZA dose at the time of the development of its related adverse events was ~2 mg/kg/day for all cases. There was no difference between the two groups regarding the starting and maximum AZA dose. Treatment responses were comparable in both groups (p-value > 0.05, for all). Complete remission, relapse after remission, and incomplete response occurred in group 1 vs. group 2 in 11 (91.7%) vs. 52 (96.3%), 10 (83.3%) vs. 28 (52.8%), and 1 (8.3%) vs. 2 (3.7%), respectively.

Table 2. Laboratory data of the studied groups at baseline and two weeks after therapy with azathioprine

Variable	Group 1 (*n* = 12)	Group 2 (*n* = 54)	*P* value
Baseline laboratory parameters			
Total bilirubin (mg/dl)	2.85 (0.8-7.3)	3.1 (0.5-31)	0.444
Direct bilirubin (mg/dl)	1.5 (0.2-5.4)	2.3 (0.1-15)	0.396
Total proteins (g/dl)	8.2 ±1.5	7.9 ±1.2	0.580
Albumin (g/dl)	3.2 ±0.6	3.2 ±0.6	0.955
ALT (U/l)	134 (52-445)	318 (40-1435)	0.109
AST (U/l)	245 (46-705)	464 (53-6388)	0.054
ALP (U/l)	326 (26-565)	294 (45-1040)	0.750
GGT (U/l)	46 (23-206)	59 (8-568)	0.606
INR	1.5 ±0.41	1.5 ±0.44	0.917
γ globulins (g/dl)	3.9 (1.8-6.8)	3.2 (1.4-6.1)	0.202
Hemoglobin (g/dl)	11.2 ±1.0	11 ±1.1	0.695
TLC (×10³/µl)	6.8 (4.4-16)	6.6 (4-13)	0.861
Neutrophils (×10³/µl)	3.2 (1.5-10.4)	3.6 (1.1-7.9)	0.907
Platelets (×10³/µl)	159 (115-336)	165 (101-592)	0.809
Laboratory parameters 2 weeks after therapy with azathioprine			
Hemoglobin (g/dl)	11.2 ±1.1	11.1 ±1.2	0.777
TLC (×10³/µl)	5.6 (3.7-11.8)	9.7 (4-16.5)	**0.005**
TLC reduction (%)	12 (0-38)	0 (0-13)	**< 0.001**
Neutrophils (×10³/µl)	2.7 (1.0-6.1)	4.8 (1.5-8.6)	**0.002**
Neutrophil reduction (%)	26 (0-45)	0 (0-30)	**< 0.001**
Platelets (×10³/µl)	159 (112-420)	164 (102-524)	0.677

Bolding indicates statistical significance. AZA – azathioprine, ALP – alkaline phosphatase, ALT – alanine transaminase, AST – aspartate transaminase, GGT – γ-glutamyltransferase, INR – international normalized ratio, PT – prothrombin time, TLC – total leucocyte count

Table 3. Hepatic histopathological findings of the studied groups at presentation

Variable	Group 1 (*n* = 10)	Group 2 (*n* = 46)	*P* value
Architecture			
Preserved	2 (20%)	16 (34.8%)	0.364
Distorted	8 (80%)	30 (65.2%)	
Activity grade			
No	0 (0%)	0 (0%)	0.713
Mild	4 (40%)	14 (30.4%)	
Moderate	4 (40%)	25 (54.3%)	
Severe	2 (20%)	7 (15.3%)	
Fibrosis stage			
No	0 (0%)	2 (4.3%)	1.0
Mild	1 (10%)	7 (15.2%)	
Moderate	6 (60%)	24 (52.2%)	
Severe	3 (30%)	13 (28.3%)	
Interface hepatitis			
No	0 (0%)	0 (0%)	0.728
Mild	2 (20%)	13 (28.3%)	
Moderate	5 (50%)	24 (52.2%)	
Severe	3 (30%)	9 (19.6%)	
Steatosis grade			
No	10 (100%)	42 (91.3%)	1.0
< 10%	0 (0%)	3 (6.5%)	
11-30%	0 (0%)	1 (2.2%)	
Eosinophilic infiltration	10 (100%)	35 (76.1%)	0.085
Plasma cell infiltration	10 (100%)	36 (78.3%)	0.104
Rosetting	5 (50%)	22 (47.8%)	0.901
Lymphocytic infiltration	10 (100%)	40 (87%)	0.578

TPMT serum level and predictors of AZA-related adverse events

The median TPMT was higher in group 1 than group 2 (Fig. 1D). AZA-related toxicities were significantly associated with different parameters that do not include the TPMT serum level, as shown in Table 4.

Discussion

For many years, researchers were concerned about reducing the AZA-related toxicities. Many of these studies targeted the assessment of the AZA metabolizing enzyme, TPMT, while others relied on the assessment of the AZA active metabolites. Despite extensive studies, results were contradictory [6, 22].

The reasons for this contradiction were multiple. The frequency of severe TPMT deficiency is low, reaching approximately up to 0.5% in the general population [23], and its presence does not universally result in AZA-induced bone marrow toxicity [8, 9]. Moreover, AZA toxicities have been reported to occur in the absence of TPMT deficiency [8, 9]. So, TPMT testing does not predict all cases of leucopenia; moreover, it cannot predict hypersensitivity adverse effects [24]. Furthermore, most studies missed the assessment of other disease-related factors. All of these issues make the reliance on TPMT assessment to avoid fatal AZA-related toxicities questionable.

In this study, we assessed the utility of TPMT serum level measurement in detecting AZA-related toxicities and other host-, disease-, and treatment-related factors, in children with AIH.

In the present study, the TPMT serum level was not significantly different between the 2 studied groups

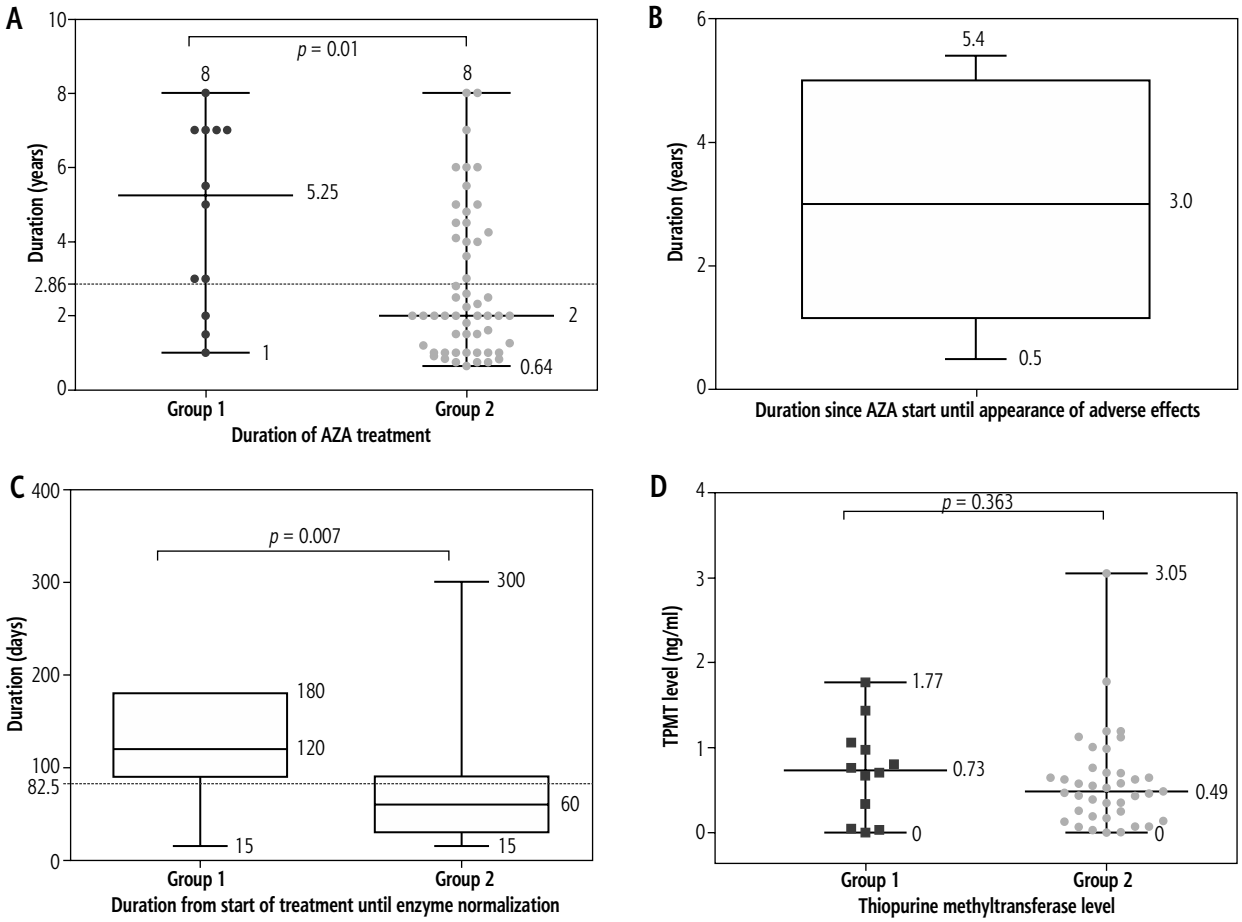

Fig. 1. Azathioprine treatment criteria and thiopurine methyltransferase serum level. **A**) Group 1 had a significantly longer duration of AZA treatment. **B**) All cases developed AZA-related toxicities after at least 6 months duration of treatment with a median of three years duration. **C**) The median duration from the start of treatment until enzyme normalization was significantly higher in group 1. The dotted line presents the cut-off value for differentiation between the groups. **D**) No significant difference was found between the groups regarding thiopurine methyltransferase (TPMT) serum levels

(p = 0.363). Czaja and Carpenter [8] found that AZA intolerance occurs in AIH patients with normal TPMT activity as in patients with deficient activity (p = 0.7). Moreover, they found no difference between the rate of AZA complications in TPMT screened and non-screened patients (p = 0.5). Heneghan *et al.* [9] found that neither TPMT genotype nor activity predicts AZA toxicity in patients with AIH. Also, Ferucci *et al.* [25] found that patients with leucopenia due to AZA were no more likely to have abnormal TPMT enzyme levels than those without leucopenia (p = 1.0) and no specific level of AZA metabolites was associated with remission or leucopenia.

On the other hand, Fuggle *et al.* [26] found that TPMT activity screening reduces adverse reactions in patients having low TPMT activity. Also, Lee *et al.* [27] demonstrated that a normal TPMT enzyme activity does not preclude AZA toxicity.

So, it is apparent that the utility of testing for TPMT status before initiating AZA therapy is a matter of controversy. These conflicting study results and different recommendations could be due to not considering other parameters involved in the development of these adverse events. Accordingly, we extensively analyzed both groups for all possible host-, disease-, and treatment-related factors in AZA toxicity development.

Only a few studies were concerned with some factors other than TPMT or AZA metabolites assessment to predict the likelihood of AZA adverse events. Heneghan *et al.* [9] found that advanced fibrosis predicts AZA toxicity in patients with AIH. Also, Czaja and Carpenter [8] found that cytopenia occurred more frequently in those with cirrhosis and even normal or high TPMT levels than in those without cirrhosis and a low TPMT level (p = 0.04).

In the present study, it was apparent that AZA adverse events do not depend on TPMT but result from

Table 4. Diagnostic performance of factors associated with AZA-related adverse events

Parameter	AUROC	*P* value	Cutoff	Sensitivity (%)	Specificity (%)	Accuracy (%)
TLC after therapy (×10³/μl)	0.77	0.005	8.8	83.3	58.1	70.7
TLC reduction after therapy (%)	0.846	< 0.001	14	50	100	75
Neutrophils after therapy (×10³/μl)	0.793	0.002	3.16	75	81.4	78.2
Neutrophil reduction after therapy (%)	0.788	0.002	15	58.3	93	75.7
Duration of treatment until enzyme normalization (days)	0.769	0.007	82.5	90	73	81.5
Duration of AZA treatment (years)	0.738	0.01	2.86	75	67	71

AUROC – area under ROC curve, AZA – azathioprine, TLC – total leucocyte count

a multifactorial process that depends mainly on some disease- and treatment-related factors. In the present study, while the stage of fibrosis was not related to AZA adverse events ($p = 1.0$), other disease- and treatment-related factors were found to be significant predictors of AZA toxicity, namely duration of immunosuppressive treatment until enzyme normalization and duration of AZA treatment. More interestingly, some laboratory parameters 2 weeks after the start of AZA treatment were significantly different between the groups. It was found that on first AZA treatment, TLC was significantly lower in group 1 ($p = 0.005$). At a cut-off value of 8.8×10^3/μl and lower, it can predict the occurrence of AZA-related myelosuppression with a sensitivity of 83.3%. Also, a percent decrease of 14% and more from the pre-AZA-treatment for TLC can predict myelosuppression with a specificity of 100%. Also, a neutrophil count of 3.16×10^3/μl and less can predict myelosuppression with a sensitivity of 75%. A percent decrease of 15% and more from the pre-AZA-treatment for neutrophils can predict myelosuppression with a specificity of 93%. Lewis *et al.* [28] noted a greater reduction in TLC from pretreatment to first on-treatment assessment in those developing myelosuppression.

It was reported in previous studies that AZA-related toxicities concerning treatment duration are highly variable, ranging from the 1st week after initiation of therapy until 17 years after the start of therapy [29]. In the present study, AZA-related adverse events had been reported as early as 6 months after the start of therapy until 5.4 years duration with a median of 3 years. It was found that the longer the duration of AZA therapy was, the greater was the risk for developing toxicity. So, follow-up for AZA-related adverse effects should be maintained all through the treatment duration.

Azathioprine hepatotoxicity could be in the form of asymptomatic elevation of transaminases or cholestatic hepatitis, which commonly occurs within the first year of therapy. The other chronic forms of hepatotoxicity usually occur after the first year of therapy [30]. In the present study, three of the children within group 1 developed cholestatic hepatitis that resolved within one month of AZA dose reduction from 2 mg/kg/day to 0.5 mg/kg/day. These children were maintained on this low dose of AZA.

Despite the retrospective nature of the study, it could be considered a limitation on one hand while it could be viewed as a point of strength on the other hand, as it permitted a long duration of follow-up that could be difficult in prospective studies.

The strength of the present study is the analysis of the different host-, disease-, and treatment-related factors regarding the development of AZA toxicity for a long followup duration. More importantly, it is the implementation of how to avoid adverse events of this important and commonly used drug with cheap and simple assessments in developing countries where there is no availability of the expensive genotyping and phenotyping assays for TPMT and AZA metabolites.

Conclusions

In conclusion, TPMT measurement is not a reliable predictor of AZA toxicity in children with AIH. Nevertheless, some treatment-related factors could predict its occurrence. A prospective study on a large cohort is warranted to assess their predictive value.

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Disclosure

The authors declare no conflict of interest.

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