CME

Lower 6-MMP/6-TG Ratio May Be a Therapeutic Target in Pediatric Autoimmune Hepatitis

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ABSTRACT

Background: Azathioprine (AZA) is the mainstay of maintenance therapy in pediatric autoimmune hepatitis (AIH). The use of thiopurines metabolites to individualize therapy and avoid toxicity has not, however, been clearly defined.

Methods: Retrospective analysis of children ≤18 years diagnosed with AIH between January 2001 and 2016. Standard definitions were used for treatment response and disease flare. Thiopurine metabolite levels were correlated with the corresponding liver function test.

Results: A total of 56 children (32 girls) were diagnosed with AIH at a median age of 11 years (interquartile range [IQR] 9). No difference in 6-thioguanine-nucleotide (6-TG) levels (271[IQR 251] pmol/8 × 10^8 red blood cell vs 224 [IQR 147] pmol/8 × 10^8 red blood cell, $P = 0.06$) was observed in children in remission when compared with those who were not in remission. No correlation was observed between the 6-TG and alanine aminotransferase levels ($r = -0.179$, $P = 0.109$) or between 6-methyl-mercaptopurine (6-MMP) and alanine aminotransferase levels ($r = 0.139$, $P = 0.213$). The 6-MMP/6-TG ratio was significantly lower in patients who were in remission (2[7] vs 5 (10), $P = 0.04$). Using a quartile analysis, we found that having a ratio of <4 was significantly associated with being in remission with OR 2.50 (95% confidence interval 1.02–6.10), $P = 0.047$. Use of allopurinol with low-dose AZA in 6 children with preferential 6-MMP production brought about remission in 5/6 (83.3%).

Conclusions: Thiopurine metabolite levels should be measured in patients with AIH who have experienced a loss of remission. A 6-MMP/6-TG ratio of <4 with the addition of allopurinol could be considered in these patients.

Key Words: autoimmune hepatitis, remission, response, thiopurines

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What Is Known

- Data on the use of thiopurine metabolites measurements in pediatric autoimmune hepatitis are limited and contradictory.
- The role of the measurement of thiopurines metabolites has not been clearly established and therapeutic metabolite levels have not been clearly defined.

What Is New

- Thiopurine metabolite levels should be measured in patients of autoimmune hepatitis with loss of remission.
- Targeting a 6-methyl mercaptopurine/6-thioguanine-nucleotide ratio of <4 with the addition of allopurinol could help in these patients.

With appropriate treatment 80% of patients with autoimmune hepatitis (AIH) achieve remission and long-term survival (1). Unfortunately, despite a good initial response to immunosuppression, long-term mortality of patients with AIH still continues to be greater than that of the general population (2), and the development of end-stage liver disease requiring liver transplantation may occur despite treatment in approximately 10% of children with AIH (3). There is a need for further optimization and individualization of immunosuppression in the management of AIH so that outcomes are further improved.

Azathioprine (AZA) was introduced for the treatment of AIH in the 1970s and has been the mainstay of maintenance treatment over the last 5 decades (4–6). About 85% to 90% of the prodrug AZA is converted into 6-mercaptopurine (MP), which undergoes metabolism by 3 competing pathways to form the active metabolites, 6-thioguanine-nucleotides (6-TG) which disrupt the DNA replication of activated T-cell lymphocytes and suppresses the Rac1 protein, which participates in T-cell maturation and proliferation (7) (Supplemental Figure 1, *http://links.lww.com/MPG/B485*). This is the mechanism for the immunosuppressive and anti-inflammatory properties of AZA; however, it can also cause myelosuppressive toxicity. 6-Methyl mercaptopurine (6-MMP) is another by-product of AZA metabolism. Elevated 6-MMP levels have been associated with elevated transaminases and cholestasis (8). A proportion of patients on AZA have preferential generation of 6-MMPs instead of 6-TG (so-called ''shunters''). This leads to a deleterious situation of high 6-MMP levels that are associated with hepatotoxicity and also poor immunosuppressive efficacy due to low levels of 6-TG. In addition, patients may shift their metabolism, that is, develop shunting while on long-term AZA therapy, which is again potentially resulting in toxicity.

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The need for therapeutic drug monitoring in AIH is vital, as derangement of liver function tests on therapy, perceived as a flare of disease or nonresponse to AZA may be in fact AZA hepatotoxicity, contributing to progressive liver disease. In addition, there is no consensus regarding second and third lines of therapies, each of which have their own toxicities, increasing the need to utilize AZA optimally (9).

Measurement of the AZA metabolites 6-TG and 6-MMP has been validated as a clinical tool to identify therapeutic levels, drug toxicity, underdosing and nonadherence in inflammatory bowel disease (IBD) (10,11).

Data on the use of thiopurine metabolites measurements in pediatric AIH are limited and contradictory (12,13). The role of the measurement of thiopurines metabolites has not been clearly established and therapeutic metabolite levels have not been clearly defined.

The aim of our study was to assess the relationship between AZA metabolite concentrations and therapeutic response in children with AIH with a view to identifying either a therapeutic range or toxicity profile.

METHODS

Patient Population

We carried out a retrospective analysis of children aged ≤18 years diagnosed with AIH in our unit between January 2001 and 2016, who have been on maintenance treatment with AZA after induction of remission. The diagnosis of AIH had been based on the revised criteria from the International Autoimmune Hepatitis Group (14). The period of treatment from the time of diagnosis till the time to remission (normalization of alanine aminotransferase [ALT] [<40 U/L]) was the induction period after which children were continued on maintenance treatment. Only children on maintenance treatment were enrolled in the study.

Treatment Protocol of the Unit

All the children after diagnosis had been commenced on prednisolone therapy ($2\,mg \cdot kg^{-1} \cdot day^{-1}$, gradually decreased for a period of 4–8 weeks) for the induction of remission. AZA was introduced 1 to 4 weeks later ($0.5–2\,mg \cdot kg^{-1} \cdot day^{-1}$). Patients with heterozygous thiopurine methyl transferase (TPMT) genotype were started at a lower dose. In children who did not show an initial response to steroid therapy or did not go into remission with the combination therapy of steroids and AZA, tacrolimus (trough level of 5–8 ng/mL) was added. Patients with an overlap with primary sclerosing cholangitis (PSC) were also started on ursodeoxycholic acid ($20\,mg \cdot kg^{-1} \cdot day^{-1}$ in 2 divided doses).

Thiopurine Methyl Transferase Genotyping and Thiopurine Metabolite Assay

TPMT genotyping was done in a plasma sample by genesFX Health (Melbourne, Australia) (15) at the time of diagnosis. The assay used polymerase chain reaction (PCR) amplification followed by single nucleotide primer extension to detect the following alleles—*1, *2, *3A, *3B, and *3C. Among these *1 is the functional TPMT allele, whereas the rest are nonfunctional. Hence, a child with a *1/*1 diplotype was considered to have a homozygous ''wild-type'' or normal genotype. A child who had 1 functional *1 allele in combination with the others had a heterozygous genotype with intermediate enzyme activity. Those with a combination of 2 nonfunctional alleles had no enzyme activity.

Thiopurine metabolites were measured at different time points by individual clinicians at their own discretion. Measurement of AZA metabolites used high-performance liquid chromatography and results were reported in pmol/8×10^8 red blood cells (RBCs). It was performed by Eastern Health pathology (Melbourne, Australia) (16) Only tests performed at least 4 weeks after a stable AZA dosage were included in the analysis, allowing for the metabolite levels to reach a steady state. Poor compliance to treatment was defined as—6-TG + 6-MMP below 150 pmol/8×10^8 RBC) (12).

Data Collection

We recorded the demographic and baseline characteristics, TPMT genotyping, thiopurine metabolites and their corresponding liver function tests, and the clinical course and outcome of all our patients. The corresponding dose of AZA (in $mg \cdot kg^{-1} \cdot day^{-1}$ using the daily dose of AZA and the recorded weight in the medical records) and the ALT level was recorded.

Thiopurine levels obtained while on other concomitant immunosuppresive agents (apart from low-dose [≤5 mg/day] prednisolone), 5-ASA preparations, an intercurrent illness or disease that could have contributed to deranged ALT were excluded while analyzing the correlation between thiopurine metabolites and ALT. For a secondary analysis, patients with overlap with PSC were excluded.

Outcome Definitions

Children were stratified according to the therapeutic response. Complete response (CR) of AIH was defined as an ALT level of less than the upper limit of normal (≤40 IU/L) (14). In patients in whom some degree of improvement in ALT was seen, but CR was not achieved were said to have an incomplete response (IR). An occurrence of relapse was defined in accord with the International Autoimmune Hepatitis Group criteria (14), as elevation of ALT to more than twice the upper limit of normal.

Side-effects of AZA therapy that were recorded were as follows: leucopenia (white cell count of $<3.5 \times 10^9$/L), nausea (severe enough to cause stoppage of therapy), and pancreatitis (17).

The study was approved by the Human Research Ethics Committee (HREC/16/RCH/167), The Royal Children's hospital Melbourne.

Statistical Analysis

All results are presented as median (interquartile range [IQR]). Statistical comparisons were performed using Mann-Whitney *U* test for 2 unpaired continuous variables and Fisher's exact test for dichotomous variables. Correlations were assessed by Spearman's rank-correlation coefficient. A receiver operating curve (ROC) was made between 6-TG and 6-MMP levels and ALT values, and the best cutoff was obtained as $(\text{specificity} + \text{sensitivity})_{max}$. All tests were 2-tailed, and *P* value was significant at 0.05. Statistical analysis was performed using the IBM Statistical Package for the Social Sciences v. 20.0 (SPSS, Armonk, NY; IBM Corp).

RESULTS

Patient Characteristics

A total of 56 children with AIH (32 girls), median age of diagnosis of 11 (IQR 9) years were identified. (Supplemental Figure 2, *http://links.lww.com/MPG/B485*)

Thirteen (23.2%) children had another concomitant autoimmune diseases (celiac disease—3, ulcerative colitis—4, Crohn disease—1, autoimmune hemolytic anemia—2, Graves disease—1, microscopic polyangitis1, chronic recurrent multifocal

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osteomyelitis—1). Nineteen (34%) children were found to have an overlap with PSC. Data for TPMT genotyping were available in 46 patients. Forty-one patients had the wild-type genotype and 5 were heterozygous.

Overview of Treatment and Therapeutic Response

After initiation of treatment 52 (93%) patients achieved CR after a median duration of 6 (IQR 7.25) months of treatment, while an IR (Supplemental Figure 2, *http://links.lww.com/MPG/B485*) was seen in 4 (7%) patients. One patient after attaining CR, developed progressive PSC and eventually underwent a liver transplant.

Among the patients who achieved CR, 37/52 remained in sustained CR, whereas 15 (29%) patients had a loss of response with 2 (1–7) relapses in a median follow-up of 49 (6–182) months. In 5 patients poor compliance to AZA was identified as the cause of the relapse.

Drug Toxicity

Side-effects of AZA were experienced in 4 patients. All these patients had normal TPMT enzyme activity and none of them had cirrhosis. Side-effects seen were—nausea (n = 2), pancreatitis (n = 1), and leucopenia (n = 1). In both patients with nausea, AZA was discontinued and switched over to 6-MP which was well tolerated. In the child with pancreatitis, AZA was discontinued and switched over to tacrolimus. In the child with leucopenia, decreasing the dose of AZA improved and normalized the white cell count.

Thipourine Metabolite Concentrations and Biochemical Response

Thiopurine metabolites (120 values [median 1 (1–4) per patient]) were available for analysis; however, 31 were excluded (Supplemental Figure 2, *http://links.lww.com/MPG/B485*), 59 of these were performed at the time of remission, and 30 were done when not in remission.

There was no difference in the median value of 6-TG between those in remission and those not in remission. Even after the patients with an overlap with PSC were excluded from the analysis; there was no difference between both the groups (Table 1).

Even though individual values of 6-MMP and 6-TG were not different between the groups, we observed that the ratio between 6-MMP and 6-TG was significantly lower in patients who were in remission. We carried out a quartile analysis (Fig. 1) and found that a ratio of <4 had a significantly higher chance of being in remission with an Odd's Ratio (OR) of 2.50 (95% confidence interval 1.02–6.10), $P = 0.047$.

The correlation between the dose of AZA (mg/kg) and the corresponding 6-TG values was poor (r = 0.140, $P = 0.208$). There was a poor correlation between the 6-TG and ALT levels (r = –0.179, $P = 0.109$) and between MMP and ALT levels (r = 0.139, $P = 0.213$) (Supplemental Figure 3A, 3B, *http://links.lww.com/MPG/B485*).

On plotting an ROC curve, with an AUC of 0.61, we found that a 6-TG value of 238 pmol/8×10^8 RBC gave the best cutoff with sensitivity 66.1% and specificity 56.7% (Fig. 2A). For 6-MMP, we derived a cutoff of <1335 pmol/8×10^8, sensitivity 50% and specificity 67.8% (Fig. 2B).

After deriving the best 6-TG cutoff, we analyzed the subgroup in which the 6-TG values were above this level (6-TG > 238, n = 52 values) and found that the patients who were not in remission had had a significantly higher 6-MMP value [2828 (IQR 5631) vs 634 (IQR 1302) $P = 0.03$] as compared to those who were in remission.

Use of Allopurinol

Allopurinol was added in 6 patients with loss of response and shunting, that is, preferential generation of 6-MMP identified on 2 consecutive thiopurine metabolite profiles (Table 2). This was identified 9 (range 6–15) months after the diagnosis. In all these patients, allopurinol was started in a dose of 50 mg and the dose of AZA was reduced to 25% to 30% of the current dose. After 2 (range 1–4) months the median 6-TG levels increased from 170 (IQR 67) pmol/8×10^8 RBC to 244 (IQR 129) pmol/8×10^8 RBC and the 6-MMP/6-TG ratio decreased from 21.5 (IQR 10) to 1 (IQR 0). No side-effects of allopurinol were seen and 5 patients went into remission. No relapses have occurred in these 5 patients for a follow-up period of 21 (IQR 7) months.

In the only patient who did not respond to allopurinol, the 6-TG increased from 212 to 654, the 6-MMP level decreased from 4882 to 195 and the ratio decreased from 23 to 1, but the ALT (IU/L) increased from 149 to 191. This child also has an overlap with PSC.

TABLE 1. Treatment, metabolite concentrations, and alanine aminotransferase for patient with autoimmune hepatitis in ''remission'' versus ''not in remission''

All patients	Remission (n = 59)	Not in remission (n = 30)	*P*
AZA dose, mg/kg	1.8 (0.63)	2.0 (1.4)	0.39
6-TG	271 (251)	224 (147)	0.06
6-MMP	559 (1378.5)	1339 (3373.50)	0.24
6-MMP/6-TG ratio	2 (7)	5 (10)	0.04
ALT	28 (10.5)	58 (30)	0.001
Only AIH (excluding PSC)	Remission (n = 22)	Not in remission (n = 16)	
AZA dose, mg/kg	1.53 (0.9)	1.73 (1.6)	0.34
6-TG	279 (305)	277 (136)	0.41
MMP	887 (1302)	1883 (2023)	0.52
6-MMP/6-TG ratio	2 (2.7)	10 (9)	0.02
ALT	30.5 (10.7)	57 (22)	0.000

Data are represented as median (interquartile range).
AIH = autoimmune hepatitis; PSC = primary sclerosing cholangitis; 6-TG = 6-thioguanine; 6-MMP = 6-methyl mercaptopurine.

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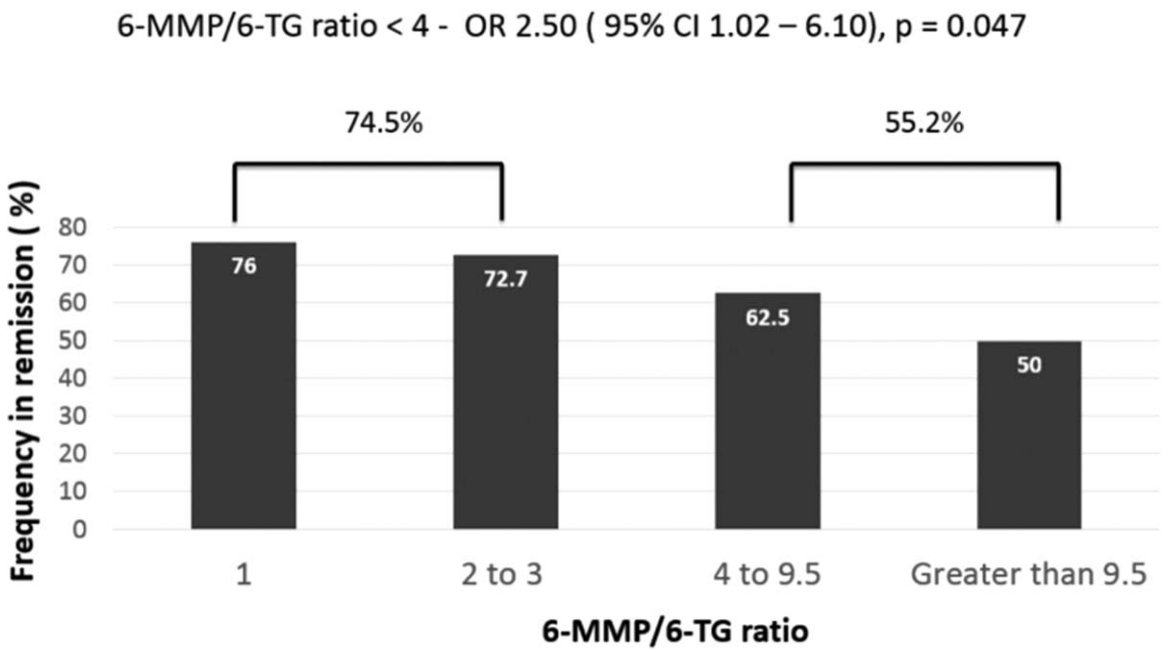

FIGURE 1. 6-Methyl mercaptopurine/6-thioguanine ratio and biochemical remission.

DISCUSSION

AIH is a lifelong disease requiring ongoing therapy for maintenance of remission (18). AZA is the mainstay of the maintenance therapy in children with AIH. It is therefore vital to optimize the use of this medication by individualizing therapy based on response and potential toxicity. Treatment-related side-effects (13%), treatment failure (9%), and an IR (13%) have been observed in these patients (19–21). The toxicity of thiopurines have been shown to be mediated by the levels of their principal intracellular metabolites and dose-dependent adverse reactions and inadequate

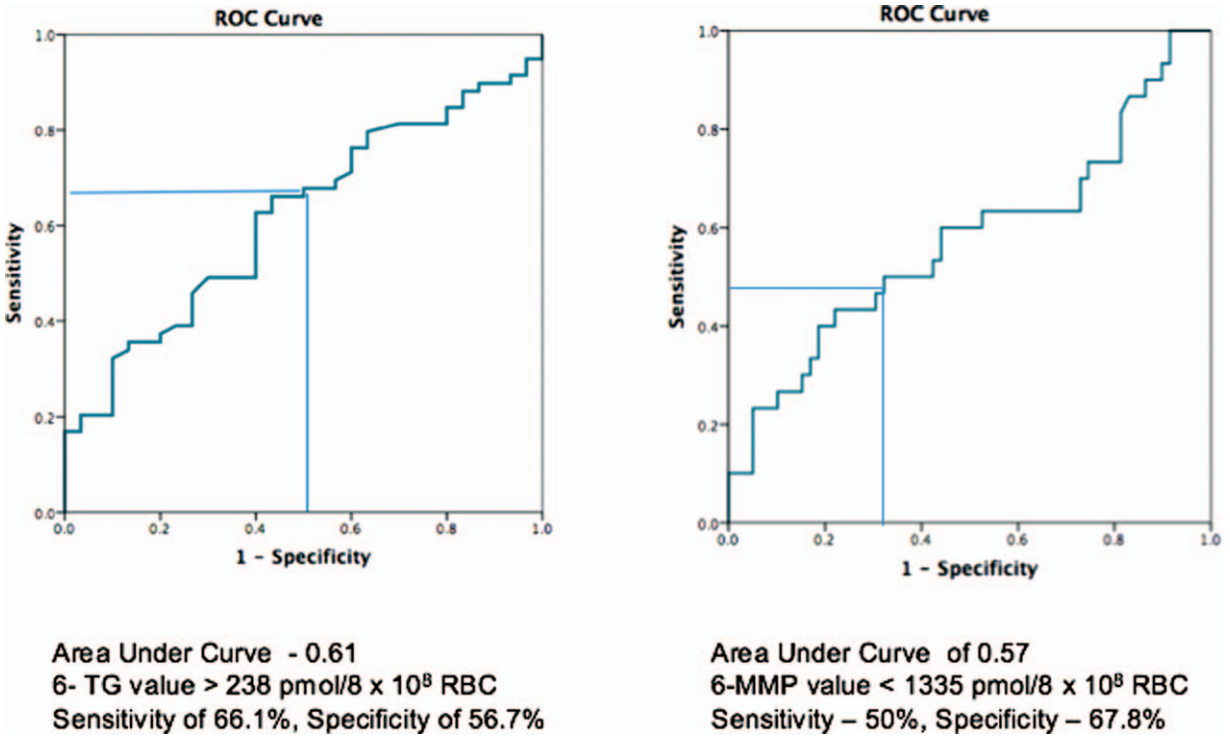

FIGURE 2. Determination of a 6-thioguanine and 6-methymercaptopurine cutoff.

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TABLE 2. Outcome of allopurinol-thiopurine combination therapy

				Pre-therapy			Post-therapy			
Patients	Age*	Sex	Overlap	6-TG	6-MMP	Ratio	6-TG	6-MMP	Ratio	Biochemical remission#
1	15	M	Yes	212	4876	23	654	195	1	No
2	3	M	No	211	4431	21	340	235	1	Yes
3	12	M	Yes	237	2370	10	371	224	1	Yes
4	3	M	Yes	162	2268	14	177	78	1	Yes
5	9	F	Yes	329	9870	30	224	682	3	Yes
6	7	F	No	134	2412	18	241	199	1	Yes

*Age at diagnosis.
#Biochemical response—alanine aminotransferase (ALT) <40 IU/L.6-TG = 6-thioguanine; 6-MMP = 6-methyl mercaptopurine.

dose are the most likely explanations for AZA failure. The role of the measurement of thiopurine metabolites in children with AIH has, however, not been defined (12,13,22). In adults the literature is conflicting (23–26) and cannot be extrapolated to children because there are differences in the disease phenotype and AZA metabolism between children and adults (27).

In IBD, a 6-TG value of >235 pmol/8×10^8 RBC has been associated with a therapeutic response (10). We found that there was a weak correlation between 6-TG levels and ALT levels. This is in concordance with other recent pediatric studies that have also found no correlation between individual 6-TG levels with ALT levels. Sheiko et al found that a wide range of 6-TG values (ranging from 50 to 250 pmol/8 × 108 RBC) were associated with biochemical remission and Nguyen et al found no difference in metabolite concentrations between children in remission and those with active disease (12,13). The 6-TG cutoff of 238 that we determined is similar to the cutoff of >220 determined by Dhaliwal et al (26) in adults but had a poor sensitivity and specificity. We do not recommend routinely targeting that level as a large proportion of patients will achieve a response at lower 6-TG levels.

We found that 29% of our patients had relapses, which is similar to the 35% to 40% relapse rate reported in literature (13,28). We found that in this subgroup after exclusion of identifiable causes for a relapse, a high 6-MMP/6-TG ratio was the likely explanation for the high ALT levels and a ratio of <4 was optimal for sustained remission. These patients developed a loss of response after attaining CR in spite of being compliant with AZA therapy and without any obvious precipitating factor. This is most likely due to individual variations in drug metabolism (29). These individuals are preferential producers of 6-MMP and hence have a higher 6-MMP/6-TG ratio than those who were in remission. In patients with IBD who fail AZA therapy around 70% patients exhibit this phenotype of preferential 6-MMP production (28).

Interestingly, apart from these ''natural'' shunters, it has been shown that in some patients treated with AZA, TPMT activity slowly increases during treatment, presumably as a result of enzyme induction. This may increase the TPMT-catalyzed methylation rate in preference to 6-TG formation and would shift the metabolism toward 6-MMP leading to an elevation in ALT (30,31).

Splitting the AZA dose or using allopurinol, a xanthine oxidase inhibitor can be used in such patients to shift the metabolism away from 6-MMP back toward 6-TG (32–34). We used allopurinol in 5 patients with success. There is limited literature on the use of allopurinol in AIH. Only 13 patients (3 children) have been reported in published literature to date (35,36). The exact mechanism of action of allopurinol is not clear. There is some suggestion that allopurinol mediated increase in thioxanthine levels inhibits the activity of TPMT (37). Using allopurinol with low-dose AZA may help in improving clinical outcomes in a subset of patients with AIH, as has been demonstrated in individuals with IBD (38).

The fact that we found significantly high 6-MMP levels in children who lost remission when we controlled 1 arm of the ratio (ie, in our subgroup of patients with ''adequate'' TG levels) explains why the ratio and not individual levels correlate with ALT. Interestingly, the median 6-MMP value in these patients [2828 (IQR 5631)] was below the hepatotoxic level of >5700 pmol/8×10^8 RBC described in patients with IBD (10). We hypothesize that in children with AIH, perhaps baseline liver dysfunction with a lower hepatocyte reserve renders increased susceptibility to AZA hepatotoxicity, hence the need to target a lower ratio.

Overall, the frequency of side-effects of AZA observed was low in our population of patients. A possible explanation for this is the low number of children with cirrhosis in our cohort who tend to have an increased risk of developing AZA related side-effects. Children were diagnosed early in the course of their disease and only 5/56 (8.9%) had cirrhosis on their biopsy. Another possibility is that patients were followed closely and changes in AZA were made preemptively before clinically significant leucopenia could occur.

The strength of our study is that for our analysis we excluded children who had factors that could have contributed to a rise in ALT, such as an intercurrent infection or concomitant disorder like hemolytic anemia, celiac disease, hypothyroidism, and others or an overlap with PSC. We focused only on children who were in the maintenance phase of therapy. Not only have we determined the ideal 6-MMP/ 6-TG ratio of <4 for sustained remission in AIH but also have demonstrated that lowering the 6-MMP/6-TG ratio below 4 with the use of allopurinol helps in achieving sustained remission. An important limitation of our study is its retrospective nature. There were no fixed timelines at which the thiopurine metabolites were measured. Due to a limited number of measurements per patient we could not assess intra-patient variations in levels.

To conclude, our data suggest that there is a poor correlation between 6-TG levels and remission in children with AIH treated with AZA and a proportion can achieve remission with levels of 6-TG lower than those traditionally recommended for children with IBD. In children with AIH who loose response at conventional doses of AZA, regular monitoring of thiopurine metabolites helps in identifying those who have developed a shifted metabolism (Supplementary Table 1, *http://links.lww.com/MPG/B485*). Targeting a 6-MMP/6-TG ratio of <4 with the addition of allopurinol could help in achieving remission in these patients. Further prospective studies are required to validate these findings.

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