**Association of Inosine Triphosphatase 94C>A and Thiopurine S-Methyltransferase Deficiency with Adverse Events and Study Drop-Outs under Azathioprine Therapy in a Prospective Crohn Disease Study**

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**Background:** Azathioprine (aza) therapy is beneficial in the treatment of inflammatory bowel disease, but 10%–30% of patients cannot tolerate aza therapy because of adverse drug reactions. Thiopurine S-methyltransferase (TPMT) deficiency predisposes to myelotoxicity, but its association with other side effects is less clear. Inosine triphosphatase (ITPA) mutations are other pharmacogenetic polymorphisms possibly involved in thiopurine metabolism and tolerance.

**Methods:** We analyzed data from a 6-month prospective study including 71 patients with Crohn disease undergoing first-time aza treatment with respect to aza intolerance. Patients were genotyped for common TPMT and ITPA mutations and had pretherapy TPMT activity measured.

**Results:** Early drop-out (within 2 weeks) from aza therapy was associated with ITPA 94C>A [P = 0.020; odds ratio (OR), 4.6; 95% confidence interval (95% CI), 1.2–17.4] and low TPMT activity [<10 nmol/(mL erythrocytes · h); P = 0.007; OR = 5.5; 95% CI, 1.6–19.2]. A high-risk group defined by ITPA 94C>A or TPMT <10 nmol/(mL erythrocytes · h) showed significant association with early drop-out (P = 0.001; OR = 11.3; 95% CI, 2.5–50.0) and all drop-outs (P = 0.002; OR = 4.8; 95% CI, 1.8–13.3). For only drop-outs attributable to aza-related side effects (n = 16), there was a significant association with ITPA 94C>A (P = 0.002; OR = 7.8; 95% CI, 2.1–29.1). Time-to-event analysis over the 24-week study period revealed a significant association (P = 0.031) between the time to drop-out and ITPA 94C>A mutant allele carrier status.

**Conclusions:** Patients with ITPA 94C>A mutations or low TPMT activity constitute a pharmacogenetic high-risk group for drop-out from aza therapy. ITPA 94C>A appears to be a promising marker indicating predisposition to aza intolerance.

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Thiopurine drugs, which include 6-mercaptopurine (6-MP) and azathioprine (aza), were introduced into clinical practice more than 50 years ago (1). They are used in the chemotherapy of acute leukemia, for immunosuppres-
sion after solid-organ transplantation, and increasingly for immunomodulation in autoimmune disease. Currently, aza is standard treatment in patients with chronically active inflammatory bowel disease (IBD) (2–4). Polymorphisms in thiopurine S-methyltransferase (TPMT; EC 2.1.1.67) can lead to diminished TPMT enzyme activity, which could in turn lead to toxic concentrations of 6-thiopurine nucleotides and severe leukopenia at standard aza doses (5, 6). TPMT catalyzes the conversion of thiopurines to nontoxic or less toxic methylated compounds, as opposed to their metabolic activation by hypoxanthine phosphoribosyltransferase (EC 2.4.2.8), ultimately leading to active 6-thiopurine nucleotides (7). Pretherapeutic TPMT genotyping is cost-effective (8) because it reduces the incidence of leukopenia and its potentially life-threatening complications (9). The genotype–phenotype correlation is generally excellent because of the small number of relevant TPMT deficiency alleles (10–12). Although a compelling connection exists (9, 13) between complete deficiency and myelosuppression, there is still controversy regarding the association of (partial) TPMT deficiency with other types of thiopurine intolerance, such as hepatotoxicity, nausea and vomiting, pancreatitis, and influenza-like illness (14). Recently it was postulated that a deficiency in the polymorphic enzyme inosine triphosphatase (ITPA; EC 3.6.1.19), which would potentially lead to increased concentrations of thioinosine triphosphate metabolites, could represent a new pathomechanism for thiopurine side effects in addition to TPMT (15). Three retrospective studies investigated the association of ITPA and TPMT polymorphisms on the adverse effects of thiopurine. Whereas one case-control study found the ITPA 94C>A polymorphism significantly associated with adverse drug reactions (ADRs), flu-like symptoms, rash, and pancreatitis (16), another case-control study could not confirm these findings (17). There was no association between thiopurine-induced myelosuppression and ITPA 94C>A or IVS2+21A>C mutations (18).

Retrospective studies often tend to overestimate risks and are critically dependent on the case-control matching. To overcome these drawbacks, we analyzed data from a prospective study of patients undergoing first treatment with aza for IBD with respect to aza tolerance in relation to ITPA and TPMT mutation status and TPMT activity.

Materials and Methods

Study Design

The Zytrim study was originally designed to compare 6-thioguanosine nucleotide (6-TGN) concentration-guided aza dosing with standard dosing according to body weight. Aza tablets (Zytrim™; 25 mg or 50 mg) were provided by Merckle GmbH (Ulm, Germany). The results of this randomized, double-blind, open trial at 11 German centers will be published separately. Patients (n = 71) were all of Caucasian origin; they were recruited between 2000 and 2002 and gave written, informed consent for participation in the study. The study was approved by the University of Ulm ethics committee as well as by the ethics committees of the participating study centers. Inclusion criteria were age >18 years, active Crohn disease (defined as a score of 150–450 on the Crohn disease activity index), and prednisone treatment >300 mg during the last 4 weeks or a relapse within 6 months after steroid pulse therapy. All patients were initially on steroids. Prednisolone was tapered according to protocol from the patient’s current dose to 30 mg/day from week 3 on and further tapered to zero until week 15 if the patient’s clinical condition permitted. Only mesalazine was allowed, per protocol, for additional antiinflammatory treatment. Patients with a history of cancer or preexisting renal or hepatic disease, or who were pregnant or breast-feeding were excluded.

All patients initially received a standard aza dose of 2.5 mg·kg⁻¹·day⁻¹ for the first 2 weeks. Thirteen patients dropped out of the study during this period; thus, a total of 58 patients were randomized to either continue receiving a standard aza dose of 2.5 mg·kg⁻¹·day⁻¹ (n = 33) or to receive an aza dose that was adapted to achieve 6-TGN concentrations between 250 and 450 pmol/8 × 10⁸ erythrocytes, determined by a published HPLC procedure (19) based on the original method described by Lennard (20).

For the analysis of ADRs to aza, myelosuppression was defined as leukocyte counts <2.5 × 10⁹/L or platelet counts <100 × 10⁹/L, hepatotoxicity as aspartate or alanine aminotransferase ≥2 times the upper limit of reference interval, and pancreatitis as upper abdominal pain with pancreatic amylase or lipase >2 times the upper limit of the reference interval. Influenza-like illness was defined as an arthralgia and/or myalgia and/or fever.

The Crohn disease activity index, IBD questionnaire scores, and TPMT activity were evaluated 2 weeks before initiation of aza therapy. Safety data (blood count, aspartate aminotransferase, alanine aminotransferase, pancreatic amylase, and lipase) were collected during regular visits at 1, 4, 8, 12, 16, and 24 weeks after the start of aza therapy. Adverse events were recorded throughout the study.

Laboratory Methods

TPMT activity was measured by a common radiochemical assay (21) with minor modifications as described previously (22). This assay measures the TPMT-catalyzed transfer of the ³H-labeled methyl group from 5-adenosylmethionine to 6-MP in lysed packed erythrocytes. Typical between-day imprecision (as CV) for the assay in our laboratory was 12% at a TPMT activity of 12.9 nmol/(mL erythrocytes·h) and 7.0% at 4.9 nmol/(mL erythrocytes·h).

TPMT and ITPA genotyping was performed with inhouse assays described elsewhere (11, 23). TPMT genotyping included mutations *2 and *3A/B/C; ITPA genotyping included mutations 94C>A and IVS2+21A>C.
Appropriate control samples with sequence-verified genotypes were assayed in every run.

STATISTICAL ANALYSIS

Descriptive statistics were calculated with SPSS 12 for MS-Windows. Independent group comparisons were performed with the Mann–Whitney U-test. Time-to-event data were compared by the log-rank test. Inference for 2 × 2 tables was calculated with the Barnard exact test. The unconditional complement of the Fisher exact conditional test (25). A 2-sided \( P < 0.05 \) was considered significant. Because this is a secondary analysis of prospective data, we chose to report the odds ratio (OR) instead of the relative risk. The 2-sided 95% confidence interval (CI) for the OR was calculated according to Miettinen and Nurminen (26). The 95% CI for the single binomial parameter was calculated according to Wilson (27).

Results

The demographic data for the study patients are presented in Table 1.

Mesalazine intake (total \( n = 23 \) patients) in our study was not associated with early drop-out; of the 13 drop-outs, 4 were on mesalazine (\( P = 0.975 \)). Nor was there an association with drop-out during the study period: 10 of 32 patients who were drop-outs were on mesalazine (\( P = 0.795 \)). Mesalazine medication did not alter the observed TPMT activities (\( P = 0.466 \)) or 6-TGN concentrations (\( P = 0.123 \)) compared with those values found in patients not on mesalazine. Two patients occasionally took acetylsalicylate, but both completed the study. Two patients received hydrochlorothiazide therapy, and again both completed the study. One patient was on phenprocoumon and completed the study.

Mean (SD) TPMT activities were significantly lower than in our reference group [11.7 (3.3) vs 14.2 (3.2) nmol/(mL erythrocytes · h); \( P < 0.001 \)] measured with a comparable method (28). We also found that 21 of 71 study patients (30%; 95% CI, 20%–41%) but only 19 of 243 healthy controls (8%; 95% CI, 5%–12%) fell below our established TPMT low-activity cutoff [\(< 10 \text{ nmol}/(\text{mL erythrocytes} \cdot \text{h})\)]. The 5 patients carrying heterozygous TPMT gene mutations had the lowest TPMT activities in the study group [6.4 (1.1) nmol/(mL erythrocytes · h)]. The frequency of heterozygous TPMT gene mutations was 0.07 (95% CI, 0.03–0.15). The allele frequencies for ITPA 94C>A and IVS2 + 21A>C polymorphisms were 0.09 (95% CI, 0.05–0.15) and 0.15 (0.10–0.22), respectively.

Patients were first analyzed on an intent-to-treat basis, in which we globally tested for an association between TPMT and/or ITPA and early (within 2 weeks) drop-outs or all drop-outs under aza therapy.

There were 13 drop-outs from the study during the first 2 weeks of aza treatment. The reasons were nausea and vomiting (\( n = 5 \)), subileus/abdominal pain (\( n = 3 \)), and others (\( n = 5 \)). Nineteen other patients dropped out during the further course of the study. Ten of these patients were from the standard dose group, and 9 patients were from the group in which the aza dose was adapted according to erythrocyte 6-TGN concentrations. Despite adaptation, the standard and adapted treatment groups did not differ significantly throughout the study with respect to aza dose, 6-TGN concentrations, or 6-methylmercaptopurine nucleotide concentrations (data not shown). This was partly attributable to the fact that in accordance with the package insert, aza doses were not increased above 3.0 mg · kg⁻¹ · day⁻¹. Mean aza doses at week 16 were 2.7 (0.1) mg · kg⁻¹ · day⁻¹ in the standard group and 3.0 (0.7) mg · kg⁻¹ · day⁻¹ in the adapted group. The reasons for drop-out were nausea (total, \( n = 3 \)); standard group, \( n = 2 \), hepatotoxicity (total, \( n = 3 \)); standard group, \( n = 2 \), patient’s request (total, \( n = 3 \)); standard group, \( n = 1 \), pancreatitis (total, \( n = 2 \)); standard group, \( n = 1 \), abdominal pain (total, \( n = 2 \)); standard group, \( n = 0 \), lost to follow-up (total, \( n = 2 \)); all standard group) and others (total, \( n = 4 \)); standard group, \( n = 2 \)).

All 5 patients carrying a TPMT gene mutation dropped out during the course of the study (Table 2). Reasons for early drop-out were nausea (the patient was also heterozygous for the ITPA 94C>A allele) and subileus (the patient was also homozygous for the ITPA IVS2 + 21A>C polymorphism).

Table 1. Demographic data of Crohn disease study patients undergoing aza therapy.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total patients, n</th>
<th>M/F, n</th>
<th>Mean (SD) age, years</th>
<th>Mean (SD) height, cm</th>
<th>Mean (SD) weight, kg</th>
<th>Mean (SD) baseline IBD questionnaire</th>
<th>Mean (SD) baseline Crohn disease activity index</th>
<th>Mean (SD) baseline IBD questionnaire</th>
<th>Mean (SD) baseline TPMT, nmol/(mL erythrocytes · h)</th>
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* Baseline refers to before aza treatment.
* Data available for 64 patients.
* mut, mutant; wt, wild type.
* One patient was compound heterozygous for ITPA 94C>A and IVS2 + 21A>C mutations.
was also heterozygous for the mutant allele, lost to follow-up, and patient’s request (this patient allele), respectively. Reasons for late drop-out were pancreatitis, lost to follow-up, and patient’s request (this patient was also heterozygous for the ITPA IVS2 + 21A>C allele). In our study, there were 3 TPMT gene mutation carriers with additional mutations in ITPA. A single case with a homozygous ITPA 94C>A mutation dropped out early because of abdominal pain. He concomitantly had increased liver enzymes, fulfilling the criteria for hepatotoxicity.

Overall, the investigated combinations of ITPA and/or TPMT mutations or low TPMT activity phenotype were more significantly associated with early drop-out from aza therapy than with all drop-outs during the 6-month study duration (Table 2). There were no significant associations (only one borderline significance for TPMT mutation carriers) with drop-out during the late study phase (weeks 2–24; data not shown).

The ITPA 94C>A mutation alone was associated (Table 2) with early drop-out (OR = 4.6; 95% CI, 1.2–17.4), as was the combination of either ITPA 94C>A or IVS2 + 21CC (OR = 4.7; 95% CI, 1.3–16.8). The latter combination reached borderline significance ($P = 0.060$) for the variable “all drop-outs” during the study. Although no significant association was observed between ITPA 94C>A mutant allele carrier status and all drop-outs, the time to drop-out from aza therapy during the course of the study was significantly different ($P = 0.031$) for ITPA 94 wild-type vs mutant allele carriers (Fig. 1). Of the 8 patients with an ITPA 94C>A mutant allele who dropped out of the study, 7 had aza-related side effects. When only drop-outs attributable to aza-related side effects ($n = 16$) were considered, there was a significant association ($P = 0.002$; OR = 7.8; 95% CI, 2.1–29.1) with ITPA 94C>A mutant allele carrier status. ITPA IVS2 + 21A>C allele carrier status (15 heterozygotes and 3 homozygotes) alone was not significantly associated with drop-out from the study. The combination conferring the highest risk for early drop-out was ITPA 94C>A mutant allele carrier combined with TPMT activity <10 nmol/(mL erythrocytes ⋅ h) (OR = 11.3; 95% CI, 2.5–50) and possibly additional ITPA IVS2 + 21A>C mutant allele carrier status (OR = 9.8; 95% CI, 1.5–62; Table 2).

We next tested for individual associations between adverse events that were previously classified as ITPA-associated aza-related ADRs (16), but which did not necessarily cause the patient to withdraw from the study. In particular, the association was tested between ITPA 94C>A and influenza-like symptoms ($n = 14$; 1 mutation carrier; $P = 0.366$), nausea and vomiting ($n = 15$; 3 mutation carriers; $P = 0.449$), and hepatotoxicity ($n = 18$; 4 mutation carriers; $P = 0.449$). The were also no significant associations between TPMT activity <10 nmol/(mL erythrocytes ⋅ h) and influenza-like symptoms ($n = 2$ low activities; $P = 0.178$).

### Table 2. Association between drop-out from aza therapy and mutations in ITPA or TPMT or low TPMT activity.

<table>
<thead>
<tr>
<th></th>
<th>Drop-out within 2 weeks of aza therapy ($n = 13$)</th>
<th>All drop-outs within 6 months ($n = 32$)</th>
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<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>$P$</td>
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<tr>
<td>ITPA 94C&gt;A ($n = 12$)</td>
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<td>0.020</td>
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<td>ITPA IVS2 + 21A&gt;C ($n = 18$)</td>
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<td>ITPA 94C&gt;A or IVS2 + 21CC ($n = 15$)</td>
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<td>0.015</td>
</tr>
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<td>ITPA 94C&gt;A or IVS2 + 21A&gt;C ($n = 29$)$^c$</td>
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<td>0.305</td>
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<tr>
<td>TPMT &lt;2 or &gt;3$^a$ ($n = 5$)</td>
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<td>0.125</td>
</tr>
<tr>
<td>TPMT &lt;10 nmol/(mL erythrocytes ⋅ h) ($n = 21$)</td>
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<td>0.007</td>
</tr>
<tr>
<td>ITPA 94C&gt;A or TPMT &lt;10 nmol/(mL erythrocytes ⋅ h) ($n = 30$)</td>
<td>11</td>
<td>0.001</td>
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<tr>
<td>ITPA 94C&gt;A or IVS2 + 21A&gt;C or TPMT &lt;10 nmol/(mL erythrocytes ⋅ h) ($n = 44$)</td>
<td>12</td>
<td>0.010</td>
</tr>
</tbody>
</table>

$^a$Low TPMT activity was defined as <10 nmol/(mL erythrocytes ⋅ h). A total of 71 patients were studied.

$^b$Confidence interval was calculated only if $P < 0.05$.

$^c$One patient was compound heterozygous for ITPA 94C>A and IVS2 + 21A>C mutations.

$^d$All patients were heterozygous.

Fig. 1. Time-to-event (survival) curve showing probability of remaining on aza therapy stratified according to ITPA 94C>A status. CC, wild type; CA, heterozygous mutation; AA, homozygous mutation.
nausea and vomiting (n = 7 low activities; P = 0.096), and hepatotoxicity (n = 4 low activities; P = 0.433).

The numbers of patients who developed a rash (n = 3) or pancreatitis (n = 2) were small, and myelosuppression was not observed during the course of the study and could not be analyzed.

**Discussion**

Despite the clearly beneficial effects of aza in the setting of IBD (29), therapy is often limited by common aza-related side effects. A long-known polymorphism in the drug-metabolizing enzyme TPMT (21) is responsible for the dose-dependent myelosuppression that ultimately leads to severe leukopenia in those completely lacking TPMT activity (9). In addition, an estimated 10%–30% of patients cannot tolerate aza therapy because of other adverse effects, such as influenza-like symptoms, nausea and vomiting, hepatotoxicity, pancreatitis, fever, rash, and other side effects (14, 30–32). In this study of previously aza-naive patients, we observed 45% (95% CI, 34%–57%) drop-outs, one half of which were classified as aza-related (23 (14–34)%). This is well within the expected range and illustrates the moderate tolerability of this drug.

The TPMT activities of our study patients were surprisingly low, with ~30% having a TPMT activity <10 nmol/(mL erythrocytes · h) before initiation of treatment with aza. It is currently not known whether this low-activity TPMT phenotype is typical for the underlying disease. We have previously shown that the radiochemical TPMT activity assay that was routinely carried out during the study (22) has an excellent genotype–phenotype correlation with a cutoff activity of 10 nmol/(mL erythrocytes · h) (11) between persons who are heterozygous for a TPMT-deficiency allele and those who possess 2 wild-type alleles. TPMT assay methodology is poorly standardized, but our results should apply equally to the respective cutoff activities of other assays; for example, when 6-thioguanine is used as substrate. Furthermore, the TPMT activity in IBD populations seems poorly defined. In our study, the patients’ mean TPMT activity was 82% of that of a control group of healthy individuals studied by the same method (27). This is exactly what Campbell et al. (33) found in aza-naive patients compared with controls. Ansari et al. (34) reported that TPMT activities in IBD are not different from the reference interval, but their patients were apparently already under treatment with aza, a medication known to increase TPMT activity (35). Lowry et al. (36) also studied TPMT activity in IBD. They found that pretreatment activities for most of their patients (89%) were within the reference interval; however, the TPMT activity surprisingly decreased after treatment with thiopurines.

Several drugs have been suspected to interfere with TPMT activity (6, 37, 38). Medication with sulfasalazine and mesalamine (5-aminosalicylic acid) may influence the measured TPMT activity, although this does not properly reflect the putative in vivo activity because the inhibitor is stochastically removed during the assay washing steps (39). We found no difference in our analysis of patients with or without mesalazine comedication with regard to TPMT activity, 6-TGN concentrations, or 6-methylmercaptopurine nucleotide concentrations. This is in line with the findings of others that mesalazine, the only aminosalicylate administered during the study, has low interfering potential (40–42). Other known or suspected TPMT inhibitors were taken by only a very few patients and could not have influenced our conclusions (see the Results).

Patients carrying TPMT mutant alleles had the lowest observed TPMT activities. None of these patients completed the study. Of those with low TPMT activity [<10 nmol/(mL erythrocytes · h)], a significant number dropped out within the first 2 weeks of aza treatment (Table 2). In addition, heterozygous TPMT mutations and low TPMT activity were overall significantly associated with drop-out during 24 weeks of aza treatment. There was no association with aza-related toxicity regarding specific symptoms such as influenza-like symptoms, nausea and vomiting, or hepatotoxicity. These findings add to the ongoing debate concerning whether these nonmyelosuppression (often called non–dose-dependent) adverse effects are related to the TPMT polymorphism (14, 16, 34, 43, 44).

We believe that in light of the initial findings by Marinaki et al. (16, 45), ITPA deficiency caused by mutations in the ITPA gene is responsible for the nonmyelosuppression adverse effects that can accompany aza therapy in IBD patients. Myelosuppression during aza therapy seems not to be related to ITPA gene mutations (18). ITPA catalyzes the hydrolysis of inosine triphosphate to inosine monophosphate (and the 6-thio derivative thereof), thereby recycling (6-thio)purines that might otherwise be trapped in the form of (6-thio)inosine triphosphate (15, 46). The ITPA 94C>A mutation codes for a Pro32Thr exchange. In heterozygous mutation carriers, ITPA activity is decreased to ~25%, which is compatible with impaired enzyme dimer formation (15). Another mutation in ITPA, IVS2 + 21A>C (15), leads to inefficient splicing as we demonstrated previously (23). This polymorphism has a higher allele frequency (15% (95% CI, 10%–22%) in this study) than the 94C>A polymorphism [9 (5–15%) in this study], and we observed 3 IVS2 + 21A>C homozygotes in the study population compared with only one 94C>A homozygote. The ITPA IVS2 + 21A>C heterozygotes in this study had a mean ITPA activity that was 60% of the activity in wild-type individuals; accordingly, we expect IVS2 + 21A>C homozygotes to have an activity comparable to that of 94C>A heterozygotes. There was a significant association between ITPA 94C>A heterozygosity or IVS2 + 21A>C homozygosity and drop-out from aza therapy within 2 weeks (Table 2). ITPA 94 mutant allele carriers were significantly more likely to experience drop-out from aza therapy (Fig. 1), primarily because of aza-related side effects.

There was no association of diminished TPMT activity
with specific aza-related toxicity that did not necessarily cause withdrawal from the study, such as influenza-like symptoms, nausea and vomiting, or hepatotoxicity. This is in contrast to the findings of Marinaki et al. (16, 45) but in line with the report of Gearry et al. (17). It is not completely clear in the latter study whether all patients were on aza or whether some took 6-MP. In addition to these retrospective case-control studies, which were sensitive to patient and control selection, we found a global association with drop-out from aza therapy. This may be of even more interest for clinicians as it has the potential to generally identify patients at increased risk for drug intolerance.

To our knowledge, reports evaluating potential differences between aza and 6-MP with regard to ITPA-related thiopurine intolerance have not been published. Both disease and medication should be considered independently. Early aza-induced ADRs seem particularly common in the IBD population compared with patients with autoimmune hepatitis (47). Interestingly, the findings of Marinaki et al. (16, 45) of an ITPA effect on aza tolerance in IBD patients could not be reproduced by the same group when they investigated liver transplant recipients (48). However, an influence of donor genetics on study outcome cannot be excluded in this setting. Aza is the 1-methyl-4-nitro-5-imidazolyl derivative of 6-MP, and it was speculated that the imidazole released during metabolic activation might be responsible for early aza intolerance. McGovern et al. (49) and Domènech et al. (50) successfully converted three fourths of their intolerant patients from aza to 6-MP. Others found that rechallenge with a lower aza dose was an alternative when nausea and vomiting occurred (51). In summary, the profile of ADRs to 6-MP is similar to that of aza (52, 53), and we expect our findings to be equally relevant to 6-MP therapy in IBD although this remains to be demonstrated.

In conclusion, this prospective study in patients undergoing first-time aza treatment for Crohn disease showed important pharmacogenetic associations. The presence of ITPA or TPMT mutations could alert the physician that certain patients have a significantly higher risk of intolerance during aza therapy and may require closer follow-up and counseling with regard to aza-related side effects.

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