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ORIGINAL ARTICLE

The glutathione transferase Mu null genotype leads to lower 6-MMPR levels in patients treated with azathioprine but not with mercaptopurine

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The conversion of azathioprine (AZA) to mercaptopurine (MP) is mediated by glutathione transferase Mu1 (GSTM1), alpha1 (GSTA1) and alpha2 (GSTA2). We designed a case-control study with data from the TOPIC trial to explore the effects of genetic variation on steady state 6-methylmercaptopurine ribonucleotide (6-MMPR) and 6-thioguanine nucleotide (6-TGN) metabolite levels. We included 199 patients with inflammatory bowel disease (126 on AZA and 73 on MP). *GSTM1-null* genotype carriers on AZA had two-fold lower 6-MMPR levels than AZA users carrying one or two copies of *GSTM1* (2239 (1006–4587) versus 4371 (1897–7369) pmol/8×10^8 RBCs; $P < 0.01$). In patients on MP (control group) 6-MMPR levels were comparable (6195 (1551–10712) versus 6544 (1717–11600) pmol/8×10^8 RBCs; $P = 0.84$). The 6-TGN levels were not affected by the GSTM1 genotype. The presence of genetic variants in *GSTA1* and *GSTA2* was not related to the 6-MMPR and 6-TGN levels.

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INTRODUCTION

Thiopurines exert cytotoxic and immunosuppressive effects and therefore are used in transplantation medicine and the treatment of cancer and a wide range of immune-mediated diseases, including Crohn's disease and ulcerative colitis.[1–3]

Azathioprine (AZA) is a nitro-imidazole analog of mercaptopurine (MP). AZA and MP are metabolized in several enzymatic steps to yield 6-thioguanine nucleotides (6-TGN), which are considered the main pharmacologically active compounds leading to therapeutic efficacy (Figure 1). The 6-methylmercaptopurine ribonucleotides (6-MMPR) are abundant side products formed during this process, and elevated levels have been linked to hepatotoxicity.[4] The formation of AZA from MP is considered to be primarily non-enzymatic; however, recent evidence surfaced that this process is mediated by the action of glutathione transferases.[5] In the presence of gluthatione transferases, the electrophilic carbon of AZA is conjugated by glutathione, which results in the formation of MP and the glutathione-nitroimidazole conjugate.[6]

Glutathione transferases belong to a family of enzymes that have crucial functions in the conjugation, detoxification and transport within the cell of potentially toxic or carcinogenic compounds.[7,8] In the wide range of glutathione transferase subclasses, only glutathione transferase alpha 1 (GSTA1), glutathione transferase alpha 2 (GSTA2) and glutathione transferase mu 1 (GSTM1) have a significant contribution in the conversion of AZA to MP.[5] Genetic variants in the corresponding genes may affect enzyme activity and thus influence the conversion rate.[7] Higher GSTA1, GSTA2 or GSTM1 activity theoretically leads to increased conversion of MP from AZA. In addition to higher thiopurine metabolites levels, this increase may also lead to the production of more reactive oxygen species due to glutathione depletion.[9]

GSTM1 is located on chromosome 1. Notably, ±50% of the Caucasian and Asian populations carry a homozygous gene deletion in the *GSTM1* gene (the so called *GSTM1-null* genotype). This deletion results in the complete absence of GSTM1 enzyme activity.[10–12] Clinical studies that exclusively included patients on AZA revealed that patients with the *GSTM1-null* genotype formed lower thiopurine metabolite concentrations and had a lower rate of side effects compared to patients carrying one or two copies of the *GSTM1* gene.[13,14] However, no clinical studies have included patients on MP as a control group.[15]

GSTA1 and *GSTA2* are both positioned on chromosome 6p12 and have high structural similarities.[16,17] For *GSTA1,* several genetic variants have been described in the promoter region. These variants are all in linkage disequilibrium and lead to reduced *GSTA1* expression. The liaison between *GSTA1* and *GSTA2* is illustrated by a compensatory increase in *GSTA2* expression.[17] Genetic variants in *GSTA1* and *GSTA2* may also affect the protein structure, leading to a change in substrate specificity.[18–20] Although many combinations are possible, variants in *GSTA1* and *GSTA2* mainly occur in two haplotypes.[21] Untill now, no *in vivo*

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studies have been conducted to establish the effects of genetic variants in *GSTA1* and *GSTA2* on thiopurine metabolite formation.[6]

The aims of this study are two-fold: first, to provide further evidence of *GSTM1* involvement in AZA metabolism by investigating the effects of the *GSTM1-null* genotype on AZA metabolism using patients treated with MP as the control group, and second, to explore the effects of genetic variants in *GSTA1* and *GSTA2* on AZA metabolism.

METHODS

Patients

We designed a case-control study with data from the Thiopurine response Optimization by Pharmacogenetic testing in Inflammatory bowel disease Clinics (TOPIC) trial, which evaluated the efficacy and cost-effectiveness of pretreatment *thiopurine S-methyltransferase (TPMT)* genotyping on thiopurine-induced myelosuppresion.[22] In the TOPIC trial, thiopurine-naive patients with inflammatory bowel disease who had an indication for thiopurine treatment (as determined by the treating physician) were randomized 1:1 for thiopurine dosing based on their *TPMT* genotype versus the standard thiopurine AZA (2.0–2.5 mg kg^{-1}) or MP dose (1.0–1.5 mg kg^{-1}). Patients randomized to the before treat genotyping arm who carried a heterozygote variant in *2, *3A or *3C of the *TPMT* gene received 50% of the original dose, whereas carriers of a homozygote variant received 0–10% of the original dose. The main exclusion criteria were previous thiopurine treatment, a known *TPMT* genotype or activity, liver test abnormalities (alanine transaminase, aspartate transaminase, alkaline phosphatase, and/or gamma glutamate transpeptidase ≥ 2 times the normal upper limit), or a leukocyte count $< 3.0 \times 10^9$ per L. Physicians were free in their choice of AZA or MP and were advised to start the full dose immediately. The patients were followed for 20 weeks. In total, 852 patients were assessed for eligibility; of the 796 randomized patients, 768 started with a thiopurine. For a detailed description of the study design, patient selection and patient data, please refer to Coenen *et al.*[22]

Blood samples were collected from all patients for *TPMT* genotyping, although the genotyping was performed after execution of the clinical trial in the patients randomized for standard thiopurine dosing. The 6-TGN and 6-MMPR metabolite levels were assessed at week 8 in the first 301 patients included in the trial. The TOPIC trial was approved by the institutional ethics committee, and all patients provided written informed consent prior to participation (clinicaltrials.gov, NCT00521950).

For the current study, all patients who started with AZA or MP in whom the 6-TGN and 6-MMPR levels were assessed at week 8 were eligible. Two exclusion criteria were applied. First, patients with a thiopurine dose adjustment within the first eight weeks were excluded because these adjustments affect metabolite concentrations. Second, patients with a variant in *TPMT* were excluded due to the large interference of variants with thiopurine metabolism and because half of this group (patients randomized to the intervention arm) received a 50% dose reduction.

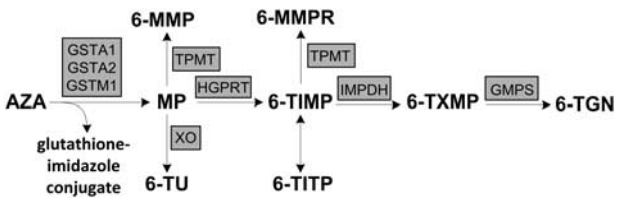

Figure 1. Overview of AZA and MP metabolism. The involved enzymes are indicated in gray boxes. AZA, azathioprine; MP, mercaptopurine; GSTA, glutathione transferase alpha; GSTM, glutathione transferase mu; 6-MMP, 6-methylmercaptopurine; 6-MMPR, 6-methylmercaptopurine ribonucleotides; TPMT, thiopurine S-methyltransferase; XO, xanthine oxidase; 6-TUA, 6-thiouric acid; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; 6-TIMP, 6-thioinosine monophosphate; 6-TITP, 6-thioinosine triphosphate; IMPDH, inosine monophosphate dehydrogenase; 6-TXMP, 6-thioxanthosine monophosphate; GMPS, guanosine monophosphate synthetase; 6-TGN, 6-thioguaninenucleotides.

Polymerase chain reactions

Genomic DNA was extracted automatically using the Chemagic DNA isolation kit special (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions and stored at 4 °C before use. The presence of the *GSTM1-null* genotype was analyzed as previously described.[12] Briefly, the primers 5'-CTC CTG ATT ATG ACA GAA GCC-3' and 5'-CTG GAT TGT AGC AGA TCA TGC-3' were used with a Mg^{2+} concentration of 2.0 mM and an annealing temperature of 58 °C. To detect the *GSTM1-null* genotype a real-time polymerase chain reaction (PCR) and a melt curve analysis were performed with Bio-Rad Precision Melt Analysis Software version 1.0 (Bio-Rad, Hercules, CA, USA). The absence of a 650 base pair (bp) product suggests a homozygous gene deletion. The PCR was performed twice on two separate occasions. This method distinguishes homozygous and heterozygous carriers of the gene from the *GSTM1-null* genotype.[23]

Genetic variants in *GSTA1* and *GSTA2* were identified through restriction fragment length polymorphism analysis as previously described.[17,19,20] The primers and PCR conditions are depicted in Supplementary Table 1. Briefly, four functional genetic variants in the *GSTA1* promoter region have been described, all of which are in linkage disequilibrium. The − 697G, − 633G, − 135 T, − 118 A combination (previously denoted with the base pairs − 631, − 567, − 69, − 52) is designated as *GST1*A* and the − 697 T, − 633 T, − 135C, − 118G combination is designated as *GSTA1*B*.[24] The PCR results in a 480-bp gene-specific product. The presence of a GSTA1*B variant leads to a cut at the − 135C position by the digestion enzyme EarI, resulting in 96-bp and 384-bp products.[17] The products were visualized by electrophoresis on a 2% agarose gel to identify patients who are heterozygous or homozygous carriers of the *GSTA1*B* variant.[17] The three most relevant genetic variants for *GSTA2* were identified in a similar fashion. The *GSTA2* variants were designated as *GSTA2*B*, *GSTA2*C* and *GSTA2*E* according to previous reports.[21] A strong association exists between *GSTA1* and *GSTA2,* and the variants primarily occur in two haplotypes. The homozygous *GSTA1*A* and homozygous *GSTA2*C* (haplotype 1) and heterozygous *GSTA1*B* and *GSTA2*C* (haplotype 2) combinations are most prevalent in Caucasians. The haplotypes were generated for *GSTA1* and *GSTA2* using Partition-Ligation-Expectation-Maximization (PL-EM) software.[25] The haplotypes were used as markers for the total GSTA1/GSTA2 activity and were correlated with the thiopurine metabolite levels. The most prevalent haplotypes were used for the analysis.

6-MMPR and 6-TGN metabolite measurements

The 6-TGN and 6-MMPR levels were determined in red blood cells (RBCs) by high-performance liquid chromatography according to the Lennard method.[26] The thiopurine metabolite concentrations are presented as the median pmol/8×10^8 RBCs with the interquartile range (IQR).

Statistical analysis

We assumed the presence of an effect due to genetic variants in *GSTA1, GSTA2* and *GSTM1* in patients treated with AZA but not in patients treated with MP. Therefore, we analyzed the effects of the genotypes separately in the AZA and MP users. First we compared the baseline characteristics of the AZA users with the MP users to ensure that potential differences in the metabolite levels between polymorphisms found only in the AZA or MP users contributed to the tested polymorphism. We used the chi-square test, Student's t test or Mann–Whitney U test as appropriate.

Next, we compared the baseline characteristics and week 8 6-MMPR and 6-TGN levels between patients on AZA with and without a *GSTM1-null* genotype. In addition, the 6-MMPR/6-TGN ratio, which provides an indication for toxicity and therapeutic efficacy, was compared between the *GSTM1* genotypes.[27] For these analyses, we used the chi-square test, Student's *t* test or Mann–Whitney U test as appropriate. Separate analyses were performed for the patients on MP. In the same way, differences in patient characteristics and metabolite levels were compared for the two most frequent GSTA1/GSTA2 haplotypes in the AZA and MP users.

Genetic variants leading to a significant change in the metabolite levels and known factors from the literature that might influence the metabolite levels were included in an analysis of covariance. General linear models were performed with the metabolite levels set as the dependent variable. The 6-MMPR levels were first log-transformed due to a skewed distribution. Gender and concomitant use of 5-aminosalicylic acid were included as fixed factors, and age and thiopurine dosage (mg/kg) were included as covariates.[28–30] The AZA dose was converted by 2.08 to compare the dose in mg/kg with MP.

For genotypes leading to a significant change in the metabolite levels, we evaluated the clinical relevance based on the treatment response and incidence of hepatotoxicity. The treatment response was compared using the Harvey-Bradshaw Index for Crohn's disease and the partial Mayo score for ulcerative colitis. A positive treatment response was defined as a reduction of three points or more at week 20 compared with week 0. Hepatotoxicity was defined as an increase in the serum concentration of alanine transaminase, aspartate transaminase or alkaline phosphatase $\geqslant 2$ times the upper normal limit. Both parameters were compared using Pearson's chi-square test. All statistical analyses were performed using SPSS version 20.0.0.1 (SPSS Inc., Chicago, IL, USA). Tests were two-sided and a *P*-value < 0.05 was considered statistically significant.

RESULTS

Patients

Metabolite levels from week 8 were available for 301 patients. Thirty-eight patients were excluded due to a variant in the *TPMT* gene, and an additional 64 patients were excluded due to dose adjustments before week 8, resulting in the inclusion of 199 patients (126 on AZA and 73 on MP) in this analysis. The baseline characteristics are provided for the patients treated with AZA and MP (Table 1). No differences were found between the two groups with the exception of a relatively higher thiopurine dose in mg/kg in the patients treated with MP. Table 2 shows the genotype frequencies of the *GSTM1*, *GSTA1* and *GSTA2* genetic variants. The genotype frequencies were in line with the population references.

GSTM1-null genotype influences AZA metabolism

No differences in the baseline characteristics were observed between patients with and without a *GSTM1-null* genotype in both the AZA and MP users (Table 3). The *GSTM1-null* genotype was reported in 56% of the patients on AZA and 47% of the patients on MP ($P = 0.22$). Patients treated with AZA carrying a *GSTM1-null* genotype had lower 6-MMPR levels than the hetero- or homozygous carriers of the *GSTM1* gene (2239 (1006–4587) versus 4371 (1897–369) pmol/8×10^8 RBCs, $P = 0.006$) (Figure 2). In the patients treated with MP, no differences in the 6-MMPR levels were found per *GSTM1* genotype (6543 (1717–11600) versus 6195 (1551–10712) pmol/8×10^8 RBCs) (Figure 2). The week 8 6-TGN levels were similar in the AZA and MP users (Table 3). In the patients on AZA, the reduced 6-MMPR levels in combination with the unaffected 6-TGN levels in the patients with a *GSTM1-null* genotype resulted in a significantly lower 6-MMPR/6-TGN ratio (Table 3). Despite the higher 6-MMPR levels, no increased rate of hepatotoxicty (8.6% versus 8.9%, $P = 0.94$) was observed in the patients on AZA with a *GSTM1-null* genotype compared with the patients carrying one or two copies of the *GSTM1* gene. Additionally, the clinical response did not differ between the groups (24% versus 26%, $P = 0.89$).

Variants in GSTA1 and GSTA2s do not affect the thiopurine metabolite levels

The *GSTA1* and *GSTA2* genotypes varied widely in frequency, with *GSTA1*B* and *GSTA2*C* showing the highest prevalence (Table 2). The generation of haplotypes showed two predominant haplotypes: the homozygous GSTA1*A and homozygous *GSTA2*C* (haplotype 1) and heterozygous *GSTA1*B* and *GSTA2*C* (haplotype 2) combinations. Together, these two combinations were found in approximately 60% of the patients. The patient characteristics were similar for both groups (Table 4). The 6-MMPR and 6-TGN levels and the 6-MMPR/6-TGN ratio did not differ between the two haplotypes in the patients on AZA and patients MP (Table 4).

Analysis of covariance with factors influencing thiopurine metabolism

The univariate analysis only showed a significant effect of the *GSTM1-null* genotype on the 6-MMPR levels in the patients on AZA. Therefore, a multivariate analysis was conducted in the patients on AZA to evaluate the effect of the *GSTM1-null* genotype on the log-transformed 6-MMPR levels after controlling for the following factors known to influence the 6-MMPR levels: age, gender, thiopurine dose and 5-aminosalicylic acid use. Multivariate analysis showed that the *GSTM1-null* genotype ($P = 0.04$) and gender ($P < 0.01$) have a significant effect on 6-MMPR levels in patients using AZA (Table 5).

DISCUSSION

In this study, we provide strong clinical evidence for the involvement of *GSTM1* in the conversion of AZA to MP. Patients on AZA with a *GSTM1-null* genotype have twofold lower 6-MMPR levels. As a control, the 6-MMPR levels remained unaffected by the *GSTM1* status in patients treated with MP. Taking potential confounders into account, the *GSTM1-null* variant remained significantly related to the 6-MMPR levels in patients on AZA. Genetic variants in *GSTA1* and *GSTA2* had no impact on the steady state week 8 thiopurine metabolite levels in both the AZA and MP users.

Despite robust biochemical evidence showing the involvement of *GSTM1*, *GSTA1* and *GSTA2* in the conversion of AZA to MP,[5] few studies have explored the clinical effects of these variants.[13,14,31] Our results corroborate the studies that demonstrated the

Table 1. Baseline characteristics of the patients on AZA and MP included in this study

	AZA users (n = *126)*	*MP users* (n = *73)*	P-*value*
Gender, male, *n* (%)	58 (46)	32 (44)	0.76
Age (years), median (IQR)	31 (24–48)	40 (26–50)	0.14
Weight, mean kg (SD)	72 (14)	71 (19)	0.72
Thiopurine dose in mg/kg, median (IQR)	2.20 (2.09–2.33)	1.21 (1.11–1.30)	N/A
Thiopurine dose in mg/kg, median (IQR) after conversion of AZA dose by 2.08	1.06 (1.00–1.12)	1.21 (1.11–1.30)	< 0.01
Disease type, CD, *n* (%)	76 (60)	41 (56)	0.68
TPMT activity in mg/mmol Hb.h, mean (SD)	96 (21)	95 (18)	0.58
Baseline disease activity			
CD (HBI), mean (SD)	3.1[a] (2.3)	3.5[b] (3.6)	0.54
UC (partial Mayo), mean (SD)	3.6[c] (1.9)	4.2[d] (2.0)	0.27

Abbreviations: AZA, azathioprine; CD, Crohn's disease; HBI, Harvey-Bradshaw Index; MP, mercaptopurine; N/A, not applicable; TPMT, thiopurine S-methyltransferase; UC, ulcerative colitis. [a]Available for 63 patients. [b]Available for 31 patients. [c]Available for 47 patients. [d]Available for 26 patients.

relevance of the frequently seen *GSTM1-null* genotype in AZA metabolism, although some points require further clarification. In a study with 72 patients with inflammatory bowel disease on AZA the 6-MMPR and 6-TGN levels were compared for patients with and without a *GSTM1-null* genotype.[14] In contrast to our findings, the authors found no differences in the 6-MMPR levels, possibly because dose adjustments were allowed, which resulted in a significantly lower AZA dose in mg/kg in the patients with one or two copies of the *GSTM1* gene. Furthermore, elevated 6-MMPR levels are associated with hepatotoxicity, whereas the 6-TGN levels are correlated with efficacy.[4,32] Two previous studies in inflammatory bowel disease patients on AZA showed a lower rate of side effects in patients with a *GSTM1-null* genotype.[13,31] Despite the differences in the 6-MMPR levels in patients on AZA with and without the *GSTM1-null* genotype in our study, we found no differences in the hepatotoxicity rates. One explanation for this

Table 2. Genotype frequencies of genetic variants in *GSTA1*, *GSTA2* and *GSTM1*

Genotype	*Frequencies*			
	Total (n = *199*)	*AZA* (n = *126*)	*MP* (n = *73*)	*Population reference*[a]
GSTM1 1/1 and 1/null	0.48	0.44	0.53	0.47
GSTM1 null/null	0.52	0.56	0.47	0.53
*GSTA1*A / GSTA1*A*	0.37	0.43	0.27	0.34
*GSTA1*A / GSTA1*B*	0.47	0.42	0.56	0.50
*GSTA1*B / GSTA1*B*	0.16	0.15	0.16	0.17
*GSTA2*B (c.629A > C)*				
AA	0.89	0.89	0.89	0.87
AC	0.09	0.09	0.08	0.13
CC	0.02	0.02	0.03	0.00
*GSTA2*C (c.335G > C)*				
GG	0.17	0.17	0.18	0.16
GC	0.47	0.42	0.55	0.54
CC	0.36	0.41	0.27	0.30
*GSTA2*E (c.328C > T)*				
CC	0.92	0.91	0.93	0.89
CT	0.08	0.09	0.07	0.11
TT	0.00	0.00	0.00	0.00

Abbreviations: AZA, azathioprine; GSTA, glutathione transferase alpha; GSTM, glutathione transferase mu; MP, mercaptopurine. [a]Based on Garte *et al.*[11] for GSTM1 and the HapMap-CEU for GSTA1 and GSTA2. *GSTM1 1/1* and *GSTM1 1/null* indicate homozygous and heterozygous carrier of the *GSTM1* gene. GSTM1 null/null indicates a homozygous gene deletion on the *GSTM1* gene. Detailed information about nomenclature of variants in *GSTA1* and *GSTA2* is provided in Supplementary File 1.

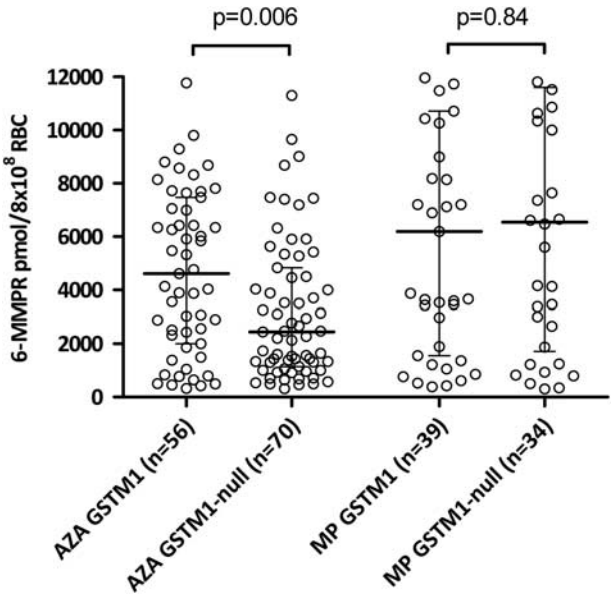

Figure 2. Boxplot showing the 6-MMPR levels for homo- and heterozygous carriers of the *GSTM1* gene (*GSTM1*) versus the homozygous *GSTM1* gene deletion (*GSTM1-null*) genotype for patients treated with AZA and MP. Median values with interquartile range are provided. AZA, azathioprine; GSTM1, glutathione transferase mu1; MP, mercaptopurine, 6-MMPR, 6-methylmercaptopurine ribonucleotides.

Table 3. Characteristics and metabolite levels of patients with and without the *GSTM1-null* genotype treated with AZA and MP

	AZA users			*MP users*		
	GSTM1	*GSTM1-null*	P-*value*	*GSTM1*	*GSTM1-null*	P-*value*
Total, *n* (%)	56 (44)	70 (56)		39 (53)	34 (47)	
Gender, male, *n* (%)	27 (48)	31 (44)	0.66	16 (41)	16 (47)	0.60
Age (years), median (IQR)	34 (25–52)	31 (23–43)	0.12	39 (26–46)	42 (26–57)	0.35
Weight, mean kg (SD)	74 (14)	71 (14)	0.20	71 (22)	72 (16)	0.70
Thiopurine dose in mg/kg, median (IQR)[a]	1.07 (1.02–1.13)	1.05 (1.00–1.11)	0.18	1.17 (1.07–1.31)	1.25 (1.14–1.30)	0.26
Disease type, CD, *n* (%)	36 (64)	40 (57)	0.35	21 (54)	20 (59)	0.46
TPMT activity in mg/mmol Hb.h, mean (SD)	95 (17)	97 (23)	0.46	94 (17)	95 (18)	0.91
Baseline disease activity						
CD (HBI), mean (SD)	3.2[b] (2.3)	3.0[c] (2.3)	0.79	3.2[d] (3.4)	3.8[e] (4.0)	0.68
UC (partial Mayo), mean (SD)	4.2[f] (2.0)	3.3[g] (1.8)	0.13	4.0[h] (2.1)	4.4[i] (1.6)	0.63
6-MMPR (IQR)	4371 (1897–7369)	2239 (1006–4587)	< 0.01	6195 (1551–10712)	6543 (1717–11600)	0.84
6-TGN (IQR)	217 (171–305)	232 (185–315)	0.57	267 (207–423)	282 (202–364)	0.68
6-MMPR/6-TGN ratio (IQR)	17.7 (6.8–33.1)	8.6 (4.0–20.4)	0.01	18.7 (5.8–44.3)	21.9 (9.0–41.3)	0.58

Abbreviations: AZA, azathioprine; CD, Crohn's disease; GSTM1, glutathione transferase mu1; HBI, Harvey-Bradshaw Index; MP, mercaptopurine; TPMT, thiopurine S-methyltransferase; UC, ulcerative colitis ; 6-MMPR, 6-methylmercaptopurine ribonucleotides; 6-TGN, 6-thioguanine nucleotides. [a]AZA dose was converted by 2.08. [b]Available for 32 patients. [c]Available for 31 patients. [d]Available for 17 patients. [e]Available for 14 patients. [f]Available for 18 patients. [g]Available for 29 patients. [h]Available for 15 patients. [i]Available for 11 patients.

Table 4. Patient characteristics and metabolite levels for the two most prevalent *GSTA1* and *GSTA2* haplotypes

	AZA users (n = *77*)			*MP users* (n = *42*)		
	Haplotype 1	*Haplotype 2*	P-*value*	*Haplotype 1*	*Haplotype 2*	P-*value*
Total, *n* (%)	42 (33)	35 (28)		12 (16)	30 (41)	
Weight, mean kg (SD)	73 (14)	71 (16)	0.57	69 (11)	70 (15)	0.92
Thiopurine dose in mg/kg, median (IQR)[a]	1.05 (1.00–1.11)	1.08 (1.00–1.12)	0.63	1.17 (1.09–1.37)	1.19 (1.10–1.29)	0.78
TPMT activity in mg/mmol Hb.h, mean (SD)	96 (19)	92 (15)	0.27	89 (12)	96 (18)	0.27
Gender, male, *n* (%)	22 (52)	16 (46)	0.56	5 (42)	12 (40)	0.92
Age (years), median (IQR)	32 (23–50)	36 (26–53)	0.30	36 (26–46)	42 (27–52)	0.47
Disease type, CD, *n* (%)	25 (60)	20 (57)	0.74	6 (50)	17 (57)	0.70
Baseline disease activity						
CD (HBI), mean (SD)	3.1[b] (2.2)	3.4[c] (1.8)	0.71	4.0[d] (1.6)	2.8[e] (4.2)	0.60
UC (partial Mayo), mean(SD)	3.5[f] (2.0)	3.6[g] (2.0)	0.92	4.2[h] (1.6)	4.4[i] (1.7)	0.79
6-MMPR (IQR)	2925 (1271–6505)	3258 (837–5630)	0.53	5386 (1103–11519)	7050 (3200–11453)	0.52
6-TGN (IQR)	218 (182–274)	219 (171–333)	0.88	216 (191–275)	297 (204–391)	0.07
6-MMPR/6-TGN ratio (IQR)	13.6 (4.6–29.7)	12.0 (3.1–27.1)	0.61	32.2 (4.2–49.6)	22.7 (10.5–34.5)	0.92

Abbreviations: AZA, azathioprine; CD, Crohn's disease; GSTA1, glutathione transferase alpha 1; GSTA2, glutathione transferase alpha 2, HBI, Harvey-Bradshaw Index; Haplotype 1, combination of homozygous *GSTA1*A* and homozygous *GSTA2*C*; Haplotype 2, heterozygous carrier of *GSTA1*B* and *GSTA2*C*; MP, mercaptopurine; TPMT, thiopurine S-methyltransferase; UC, ulcerative colitis. [a]AZA dose was converted by 2.08. [b]Available for 21 patients. [c]Available for 18 patients. [d]Available for 4 patients. [e]Available for 12 patients. [f]Available for 16 patients. [g]Available for 14 patients. [h]Available for 15 patients. [i]Available for 13 patients.

Table 5. Analysis of covariance in patients on AZA to estimate the effect of the *GSTM1-null* genotype on the log-transformed 6-methylmercaptopurine ribonucleotide (6-MMPR) levels controlling for factors with a known influence on 6-MMPR levels (age, gender, thiopurine dose and 5-aminosalicylic acid use)

Variable	*Effect*	*95% CI*	P-*value*
Female gender	0.54	0.29–0.79	< 0.01
GSTM1-null genotype	−0.21	−0.45–0.03	0.04
Age (years)	0.006	−0.002–0.014	0.13
Concomitant 5-ASA use	−0.21	−0.46–0.04	0.08
Thiopurine dose in mg kg^{-1}[a]	1.26	−0.13–2.64	0.08

Abbreviations: CI, confidence interval; GSTM1, glutathione transferase mu1. [a]Azathioprine dose was converted by 2.08 to compare with the mercaptopurine dose.

result might be that the median 6-MMPR levels were far below 5700 pmol × 10^8/RBCs in both groups, which is the threshold above which hepatotoxicity is more likely to occur.[4] We found no difference in the treatment response between patients with and without the *GSTM1-null* genotype, which seemed logical given that the 6-TGN levels were equal. The reduction in the 6-MMPR levels in combination with unaffected 6-TGN levels, results in a more beneficial 6-MMPR/6-TGN ratio. Theoretically, this might result in fewer side effects with an equal efficacy in half of the population (i.e., those with a *GSTM1-null* genotype) when treated with AZA.

This study is unique compared with preceding studies because we also included patients treated with MP.[5,33] The current conception is that glutathione transferases are involved in the conversion of AZA to MP but not in further downstream thiopurine metabolism, which makes patients on MP an ideal control group. Additionally, the prospective data collection and the exclusion of patients with a variant in *TPMT* or dose adjustments further increased the consistency of our work. Both the similar baseline characteristics between patients with and without the *GSTM1-null* genotype and the analysis of covariance support the notion that the observed differences can only be contributed to *GSTM1*.

A novel aspect of the present study is that we explored the effect of genetic variants in *GSTA1* and *GSTA2* on thiopurine metabolism.[6] Most of the variants occurred in two haplotypes, which was in agreement with the literature in Caucasians.[21] In our study, the metabolite levels did not differ between the two most prevalent haplotypes. A likely explanation is that the haplotypes probably neutralized the effects caused by individual genetic variants. This phenomenom is illustrated by Coles et al., who showed that *GSTA1* expression in the human liver was reduced in patients with one *GSTA1*B* variant and was further decreased in patients homozygous for *GSTA1*B*. However, the reduced *GSTA1* expression level was compensated by an increase in *GSTA2* expression: thus the total *GSTA1/GSTA2* expression was similar.[17] Moreover, large variability in *GSTA1* expression exists between humans with a similar *GSTA1* genotype. Together, this might explain why the metabolite levels cannot be predicted with the most prevalent *GSTA1/GSTA2* haplotypes.[17]

We show that the 6-MMPR levels are increased in AZA users that carry one or two copies of *GSTM1* compared with patients with a *GSTM1-null* genotype. Carriers of the *GSTM1* gene metabolize AZA more effectively, resulting in more MP being available for further metabolism.[33] Interestingly, although the 6-MMPR levels were two-fold lower in patients on AZA with a *GSTM1-null* genotype, no difference was observed in the 6-TGN levels. The most likely explanation for this finding is that inosine monophosphate dehydrogenase (IMPDH) is a rate-limiting enzyme in purine synthesis.[34–36] Since IMPDH is positioned more distally in the thiopurine pathway, hypothetically the 6-TGN levels are influenced to a lesser degree by a higher supply of MP.[37]

Genetic variants in the enzymes involved in thiopurine metabolism may have important clinical consequences.[38] The most relevant is the presence of homozygous variants in the *TPMT* gene, which lead to diminished or negligible TPMT activity and subsequently to an excessive 6-TGN level.[39,40] The homozygous presence of a variant in *TPMT* occurs in 0.3% of the population, whereas the *GSTM1-null* genotype is reported in approximately 50% of the population.[3,11] Therefore, this variant significantly contributes to the individual variation in AZA metabolism. Moreover, other factors, such as gender, age, thiopurine dose

and use of concomitant drugs, may influence thiopurine metabolism and subsequently have an effect on the thiopurine metabolite levels. In this study, only gender had a significant effect on the 6-MMPR levels: in contrast, the thiopurine dose and concomitant 5-ASA use had no significant effect but tended to result in higher and lower 6-MMPR levels, respectively.

A limitation of the present work is that the use of a melt-curve analysis might lead to patients being mistakenly assigned to the *GSTM1-null* genotype when the PCR technique fails. To minimize the influence of technical failure, all PCRs were performed in duplicate on different occasions. Another limitation is that the categorization of patients into subgroups may curtail the power of the analysis. This possibility is illustrated by some degree of variation in the genotype frequencies between the AZA and MP users compared with the reference population, although the overall genotype frequencies correspond to the population references.

In conclusion, we demonstrated that the common *GSTM1-null* genotype resulted in lower 6-MMPR levels in patients treated with AZA, whereas the 6-TGN levels remained unaffected, leading to a potentially more beneficial 6-MMPR/6-TGN ratio. In contrast, genetic variants in *GSTA1* and *GSTA2* do not influence the thiopurine metabolite levels. The reduction in the 6-MMPR levels in patients with a *GSTM1-null* genotype on AZA without a corresponding in efficacy may lead to a lower rate of side effects in these patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Study design: MB, GW, DW, MC, DJ. Performed experiments: MB, HR. Metabolite measurements: DW, PH. Data collection: MB, CM, MC, DW. Data analysis: MB, HR, MC. Writing of the manuscript: MB, DW, GW, DJ, MC. Interpretation of data and critical revision of the manuscript for important intellectual content: HG, AV, HS, LD, OK, HR. Study supervision: DJ, MC.

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