INFLAMMATORY BOWEL DISEASE

Pharmacogenetics during standardised initiation of thiopurine treatment in inflammatory bowel disease

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Background: Firm recommendations about the way thiopurine drugs are introduced and the use of thiopurine methyltransferase (TPMT) and metabolite measurements during treatment in inflammatory bowel disease (IBD) are lacking.

Aim: To evaluate pharmacokinetics and tolerance after initiation of thiopurine treatment with a fixed dosing schedule in patients with IBD.

Patients: 60 consecutive patients with Crohn's disease (n=33) or ulcerative colitis (n=27) were included in a 20 week open, prospective study.

Methods: Thiopurine treatment was introduced using a predefined dose escalation schedule, reaching a daily target dose at week 3 of 2.5 mg azathioprine or 1.25 mg 6-mercaptopurine per kg body weight. TPMT and ITPA genotypes, TPMT activity, TPMT gene expression, and thiopurine metabolites were determined. Clinical outcome and occurrence of adverse events were monitored.

Results: 27 patients completed the study per protocol, while 33 were withdrawn (early protocol violation (n=5), TPMT deficiency (n=1), thiopurine related adverse events (n=27)); 67% of patients with adverse events tolerated long term treatment on a lower dose (median 1.32 mg azathioprine/kg body weight). TPMT activity did not change during the 20 week course of the study but a significant decrease in TPMT gene expression was found (TPMT/huCYC ratio; p=0.02). Patients with meTIMP concentrations >11 450 pmol/8×10⁸ red blood cells during steady state at week 5 had an increased risk of developing myelotoxicity (odds ratio=45.0; p=0.015).

Conclusions: After initiation of thiopurine treatment using a fixed dosing schedule, no general induction of TPMT enzyme activity occurred, though TPMT gene expression decreased. The development of different types of toxicity was unpredictable, but we found that measurement of meTIMP early in the steady state phase helped to identify patients at risk of developing myelotoxicity.

The thiopurine drugs azathioprine and 6-mercaptopurine (6-MP) are well established in the treatment of inflammatory bowel disease (IBD) and have proven to be effective in both inducing and maintaining remission of Crohn's disease and ulcerative colitis.^{1–4} Adverse drug reactions occur in 9–28% of patients and often require dose reduction or discontinuation of the drug.^{4–8} Individual variations in drug metabolism are of importance for differences in tolerance to thiopurines (fig 1). Intracellular activation of 6-MP through an initial step catalysed by the enzyme hypoxanthine phosphoribosyl transferase (HPRT) results, after further metabolism, in the formation of active thioguanine nucleotides (TGNs).⁹ The TGN metabolites act as purine antagonists and induce cytotoxicity and immunosuppression by inhibition of RNA, DNA, and protein synthesis. These cytotoxic properties are, at least in part, due to the direct incorporation of TGN into DNA.¹⁰ Recently, it has been suggested that the immunosuppressive effects of thiopurines are mediated by binding of TGN triphosphate instead of guanine triphosphate to the Rac1 protein. This results in suppressed Rac1 activation and induction of apoptosis.¹¹ It has also been shown that thiopurines selectively inhibit inflammatory gene expression in activated T lymphocytes.¹²

Competing with TGN formation from the parent drugs are the oxidative and methylation pathways, both of which show interindividual variation.^{13 14} The methylation of thioinosine monophosphate (TIMP) is catalysed by thiopurine methyltransferase (TPMT), which leads to the production of methylthioinosine monophosphate (meTIMP). This

metabolite is found in concentrations that far exceed those of TGN^{15 16} and is a potent inhibitor of purine de novo synthesis in vitro.^{17 18} High meTIMP concentrations have, furthermore, been associated with an increased risk of hepatotoxicity.^{19 20}

Genetic polymorphisms in the TPMT gene (TPMT *2 to *18) are associated with decreased TPMT activity^{21 22} and with the development of myelotoxicity because of high TGN metabolite concentrations.²³

Recently, inosine triphosphate pyrophosphatase (ITPase) deficiency has been associated with the development of flue-like symptoms, rash, and pancreatitis.²⁴ A subsequent study could not support these findings.²⁵

Our aim in this study was to investigate the influence of thiopurine treatment on TPMT enzyme activity and TPMT gene expression, as well as pharmacokinetics and tolerance, after the initiation of thiopurine treatment in patients with IBD using a predetermined dose escalation protocol.

METHODS

Study design and patient selection

The trial design was an open, prospective multicentre study. Between January 2002 and October 2003, we recruited 60

Abbreviations: HBI, Harvey-Bradshaw index; HPRT, hypoxanthine phosphoribosyl transferase; IBD, inflammatory bowel disease; IQR, interquartile range; ITPA, inosine triphosphate pyrophosphohydrolase; ITPase, inosine triphosphate pyrophosphatase; meTIMP, methylthioinosine monophosphate; purine de novo synthesis; pRBC, packed red blood cells; TGN, thioguanine nucleotide; TIMP, thioinosine monophosphate; TPMT, thiopurine methyltransferase

Figure 1 The thiopurine metabolism. The phosphorylation and dephosphorylation reactions of TIMP involving the ITPA enzyme are not shown. The metabolites TGMP, TGDP, dTGDP, TGTP, and dTGTP are collectively named thioguanine nucleotides or TGN and are marked with a dark grey triangle. AZA, azathioprine; dTGTP, deoxy-TGTP; GMPS, guanosine monophosphate synthetase; HPRT, hypoxanthine guanine phosphoribosyl transferase; IMP, inosine monophosphate; IMPDH, inosine monophosphate dehydrogenase; meTGN, methyl-thioguanine; meTIMP, methyl-thioinosine monophosphate; MMP, methyl-mercaptopurine; PRA, phosphoribosylamine; PRPP, phosphoribosyl pyrophosphate; TGMP, thioguanine monophosphate; TGDP, thioguanine diphosphate; TGTP, thioguanine triphosphate; TIMP, thioinosine monophosphate; TPMT, thiopurine methyltransferase; TUA, thiouric acid; TX, thioxanthine; TXMP, thioxanthine monophosphate; XO, xanthine oxidase; 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine.

consecutive IBD patients aged over 18 years with Crohn's disease ($n = 33$) or ulcerative colitis ($n = 27$), in whom thiopurine treatment was indicated. Primary indications for thiopurine treatment were: steroid dependent and steroid resistant chronic active disease ($n = 31$), frequent relapses (≥ 2 per year) ($n = 16$), and fistulising disease ($n = 4$). Secondary indications were the maintenance of medically or surgically induced remission ($n = 9$).

Patients were not included if they had known TPMT deficiency, had received immunosuppression other than infliximab (azathioprine, 6-MP, methotrexate, mycophenolate mofetil) within four weeks before enrolment, had active infection or known malignancy (past or present), or were pregnant or breast-feeding. Diagnoses of Crohn's disease or ulcerative colitis were made by standard clinical, radiological, histological, and endoscopic criteria.²⁶ The demographics of the patients are given in table 1.

Baseline TPMT activity was determined. Patients with low TPMT activity (< 3 U per ml of packed red blood cells (pRBC)) were excluded. Patients with higher TPMT activity were included. Azathioprine (Imurel®, GlaxoSmithKline, Sweden) was given to all thiopurine-naïve patients. Patients who had previously experienced side effects other than pancreatitis when on azathioprine received 6-MP (Puri-Nethol®, GlaxoSmithKline, Sweden). Patients who had experienced pancreatitis were not included. The patients

followed a dose escalation schedule and the target dose for azathioprine (2.5 mg/kg body weight) and for 6-MP (1.25 mg/kg body weight) was reached by week 3 (table 2).

Patients visited the outpatient clinic at baseline (week 0) and at weeks 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, and 20 after the start of treatment. They were closely monitored for blood dyscrasias, adverse events, and clinical effects. Patients were advised to adhere strictly to the dose escalation schedule. They were excluded from further sampling the day after the last day of correct dosing, irrespective of the reason. All were, however, sampled at week 20 from the start of treatment. Blood was drawn at each visit for later analysis of TPMT gene expression and enzyme activity, TGN, meTIMP, and TPMT, and for ITPA 94C>A polymorphisms. In addition, 200 DNA samples (400 alleles) were obtained from a regional population based DNA biobank and used to assess allele frequencies for the ITPA 94C>A polymorphism.

Outcome definitions

Disease activity was assessed using the Harvey-Bradshaw index (HBI)²⁷ for Crohn's disease and the Walmsley index²⁸ for ulcerative colitis. Remission was defined as an HBI value of ≤ 5 for Crohn's disease or a Walmsley index of ≤ 5 for ulcerative colitis. Relapse was defined as an HBI value or a Walmsley index of > 5 in patients with a previous value below these limits. If patients developed leucopenia (defined

Table 1 Baseline characteristics of the patients at time of inclusion

Feature	Crohn's disease (n = 33)	Ulcerative colitis (n = 27)
Sex (male/female)	15/18	17/10
Age (y) (median (range))	42 (18 to 64)	39 (19 to 76)
Disease duration (y) (mean (range))	11.2 (0.5 to 33)	7.7 (0.5 to 31)
Disease activity at baseline		
Remission	21	19
Active disease	11	8
Not assessed	1	–
Disease location (n)		
Ileocolitis	18	–
Colitis	10	27
Small intestine	5	–
Thiopurine studied (AZA/6-MP)	27/6	21/6
Previous adverse effect of thiopurine treatment (n)	7	6
Primary indication for AZA/6-MP treatment (n)		
Steroid dependence	21	10
Frequent relapses	1	15
Maintenance therapy	7	2
Fistulising disease	4	–
Corticosteroids (n)	14	17
Sulfasalazine or 5-ASA (n)	14	13
Previous surgery (n)	12	1

AZA, azathioprine; y, years; 5-ASA, 5-aminosalicylic acid; 6-MP, 6-mercaptopurine.

as white blood cell count (WBC) $<3.0 \times 10^9/l$, neutropenia (absolute neutrophil count $<1.5 \times 10^9/l$), thrombocytopenia (platelet count $<100 \times 10^9/l$), or hepatotoxicity (aspartate transaminase or alanine transaminase more than five times the upper normal limit or alkaline phosphatase more than three times the upper normal limit), the dose was reduced. If any laboratory abnormality did not subside, the treatment was discontinued. Decisions about discontinuation or dose adjustment in patients who experienced other adverse events were taken by the responsible physician on a case-by-case basis.

Analytic procedures

Whole blood (4.5 ml) was collected in EDTA tubes. Genomic DNA was isolated with a QIAamp DNA Blood Minikit (Qiagen, VWR International, Stockholm, Sweden) according to the manufacturers' instructions. For RNA isolation, blood was collected in PAXgene^{TM} blood RNA tubes (PreAnalytix GmbH, VWR International, Stockholm, Sweden) which contain a cationic detergent and additive salts for stabilisation of RNA.²⁹ RNA was isolated with the PAXgene^{TM} RNA isolation kit according to the instructions from the manufacturer, with an additional on-column DNase treatment step. A volume corresponding to 250 ng RNA in a total volume of 50 µl was taken for cDNA synthesis using the cDNA high capacity archive kit (Applied Biosystems, Stockholm, Sweden).

Real time RT-PCR

Real time reverse transcriptase polymerase chain reaction (RT-PCR) for quantification of TPMT gene expression was carried out as described previously,³⁰ with the following modifications. The housekeeping gene cyclophilin (huCYC) was detected using a probe labelled with the fluorescent dye FAM (Applied Biosystems assay ID: Hs99999904_m1) instead of VIC, resulting in a higher fluorescence yield in the SDS 7700 instrument. The concentrations of TPMT primers were 800 nM and 200 nM for the TaqMan probe. For comparison between experiments, a cell line RNA sample was used as a calibrator and included from cDNA synthesis to

Table 2 Dose escalation schedule for azathioprine and 6-mercaptopurine

Body weight	Treatment week		
	1	2	3–20, Target dose
Dose escalation schedule for azathioprine (mg/day)			
45–55 kg	50	75	125
56–65 kg	50	100	150
66–75 kg	50	100	175
76–85 kg	50	125	200
>85 kg	75	150	225
Dose escalation schedule for 6-mercaptopurine (mg/day)			
45–50 kg	25	25	50
51–70 kg	25	50	75
>70 kg	25	75	100

The daily dose was given in the morning during week 1, divided into morning and evening doses during weeks 2 to 4, and in the evening from week 5 on.

real time RT-PCR amplification each time. The level of TPMT gene expression was given as a ratio in relation to the gene huCYC.

TPMT genotype

TPMT genotype was determined by a pyrosequencing method as described previously by our group.²¹ The patients were genotyped for the following nucleotide substitutions: 238G>C, 460G>A, 719A>G, 292G>T, intron IX/exon X splice site (G>A), 146T>C, 539A>T, 681T>G, 644G>A, and 430G>C (TPMT *2, *3A, *3B, *3C, *3D, *4, *5, *6, *7, *8, and *10). Genotyping for +1A>G (TPMT*14) and for IVS7-1G>A (TPMT*15) was undertaken as previously described.²¹ However, for TPMT*15, PCR primers for exon VIII were used.³¹

ITPA 94C>A polymorphism

The ITPA 94C>A polymorphism was investigated using pyrosequencing with PCR primers forward: 5'-TCG ATG AGA AAG GCG GAT GA-3' and reverse: 5'-biotin-ACG GTC AAT TTT CTG TGC CAC-3', and sequencing primer 5'-TTC AGA TTC TAG GAG ATA AG-3'.³² Primers were purchased from Invitrogen, Stockholm, Sweden.

Figure 2 Median TPMT enzyme activity (quartile 1; quartile 3) in the "per protocol" (PP) group (n=27) during the 20 week course of the study. TPMT, thiopurine methyltransferase.

TPMT activity assay

TPMT activity was determined as previously described.³³ Briefly, we measured the formation of 6-methylmercaptapurine from 6-mercaptopurine with radiolabelled *S*-adenosyl-L-methionine as the methyl donor. Product formation was measured by a liquid scintillation counter. One unit of enzyme activity represents the formation of 1 nmol of 6-methylmercaptapurine per ml pRBCs per hour of incubation. The interassay and intra-assay coefficients of variation were 4.7% and 3.3%, respectively.

Determination of thioguanine nucleotides and methylthioinosine monophosphate in red blood cells

The nucleotides were determined as previously described.³³ EDTA blood was centrifuged and red blood cells were washed and diluted in saline to a final concentration of 8×10^8 cells per 200 μ l before storage at -70°C . TGN and meTIMP were determined as purine bases by reverse phase high performance liquid chromatography at 330 nm after acid hydrolysis and an extraction procedure. The limit of quantification for TGN was 20 pmol/ 8×10^8 RBC and for meTIMP 300 pmol/ 8×10^8 RBC. At these levels, the interassay and intra-assay coefficients of variation were 12.2% and 5.7%, respectively, for TGN, and 17.4% and 16.7% for meTIMP. All samples from the same patient were analysed in the same run.

Ethical considerations

The protocol was approved by the local ethics committee. Informed consent was obtained from the patients before inclusion.

Statistics

Statistical analysis was carried out with the SPSS v.11.0 for Windows (SPSS Inc, Chicago, Illinois, USA). In calculations including drug doses, we converted the 6-MP dose into the equivalent azathioprine dose with a conversion factor of 2.08.³⁴ Differences between two independent groups were evaluated by the Mann-Whitney U test, and differences between multiple independent groups were evaluated by the Kruskal-Wallis test. Spearman rank order correlation coefficients were applied to test for correlations between variables. Results are expressed as medians with interquartile range (IQR; q1 to q3) throughout. Changes in enzyme activity and TPMT gene expression over time were analysed using two way analysis of variance (ANOVA), after testing that the residuals were close to normally distributed. In a subsequent

Figure 3 Median TPMT gene expression (quartile 1; quartile 3) expressed as a ratio between TPMT and the gene cyclophilin (huCYC) in the "per protocol" (PP) group (n=16) during the 20 week course of the study. TPMT, thiopurine methyltransferase.

post-hoc Dunnett's test, values at baseline were used as control, and other groups of measurements (values of different weeks) were compared against this control. A generalised Fisher's exact test was used when testing for associations between ITPA 94C>A alleles and the occurrence of adverse events. Fisher's exact test was used to test the association between TPMT genotype and the occurrence of adverse events. The increase in TGN and meTIMP concentrations from weeks 1 to 5 was calculated as a slope (regression coefficient) for each person, and the slopes were compared between groups using a two tailed *t* test. To evaluate the relation between myelotoxicity and metabolite concentrations a binary logistic regression was used and results expressed as odds ratios (OR) with 95% confidence intervals (CI). Receiver operating characteristics (ROC) curves were obtained to plot the sensitivity and specificity for various metabolite concentrations to predict the development of myelotoxicity. A Kaplan-Meier plot and a log-rank test were used to visualise and to test differences between TPMT genotypes in probability of remaining in the study. Two sided probability (*p*) values of <0.05 were considered significant.

RESULTS

One of 60 patients included was found to have TPMT activity below 3 U/ml pRBC and five did not adhere to the predetermined dose escalation schedule during the first three weeks (early protocol violation). These six patients were withdrawn from the study. Of the remaining 54 patients, 27 completed the 20 week period per protocol and 27 were

Figure 4 Median (quartile 1; quartile 3) levels of thioguanine nucleotide (TGN) (A) and methylthioinosine monophosphate (meTIMP) (B) during the 20 week course of the study in the "per protocol" (PP) group and "adverse events" (AE) group, respectively.

withdrawn because of thiopurine related side effects. These two groups were called the per protocol (PP) group and the adverse events (AE) group, respectively.

TPMT genotype and phenotype

Of the 60 patients, one was genotyped as TPMT*3A/*14 and seven (12%) were heterozygous for the nucleotide substitutions 460G>A and 719A>G and considered to be TPMT*1/*3A. Fifty two patients (87%) did not carry any of the 12 variant TPMT alleles tested for, and were considered to be wild type (TPMT*1/*1). There was incomplete concordance between TPMT activity (phenotype) and TPMT genotype. The TPMT deficient patient had a baseline TPMT activity of 2.6 U/ml pRBC, one the seven TPMT heterozygous patients had baseline TPMT activity above, and four of the 52 wild type patients had baseline TPMT activity below 8.9 U/ml pRBC, which is our cut off level for intermediate v high TPMT enzyme activity.³¹ The median TPMT activity at baseline for the PP group was 12.1 (IQR, 10.7 to 13.2) U/ml pRBC, and for the AE group, 11.8 (9.4 to 14.0) ($p = 0.45$). In the PP group, the TPMT enzyme activity did not differ in subsequent weeks compared with the baseline value ($p > 0.05$; fig 2). In all, 10 individuals had an initial TPMT enzyme activity level below 8.9 U/ml pRBC. Despite their low TPMT activity, four of these 10 individuals were genotyped as TPMT wild type and they all increased their activity during thiopurine treatment. Three of the four patients completed the study and increased their TPMT enzyme activity by between 22% and 61% from baseline until week 20. The fourth patient increased his TPMT activity by 12% to the time of exclusion at week 4.

TPMT gene expression

Samples with proven identical RNA stability ($n = 16$ from the PP group) were analysed. There was a decrease in TPMT gene expression during treatment (fig 3). Compared with baseline gene expression, significant differences ($p < 0.05$) were found for all subsequent weeks with the exception of week 6, and the TPMT/huCYC ratio decreased from 4.8 (IQR, 3.5 to 7.0) at baseline to 3.5 (2.7 to 5.1) at week 20 ($p < 0.02$).

ITPase polymorphism ITPA 94C>A

Of the 60 patients, 53 (88%) were wild type for ITPA 94C>A (C/C), six (10%) were heterozygous (C/A), and one (1.7%) was homozygous for the variant allele (A/A), giving the allelic frequencies for the C and A alleles of 0.93 and 0.06, respectively. In the DNA bank ($n = 200$), 174 individuals (87%) were wild type (C/C), 25 (12.5%) were heterozygous (C/A), and one (0.5%) was homozygous for the A allele (A/A). Allelic frequencies for the C and A alleles were 0.93 and 0.07, respectively. None of the seven patients genotyped as TPMT*1/*3A carried the variant ITPA 94A allele.

Thiopurine metabolite concentrations

Steady state levels of TGN and meTIMP were reached in the PP group after a period of two weeks on stable dose, corresponding to week 5 (fig 4). Median levels at week 5 for TGN and meTIMP were 180 (IQR, 145 to 259) and 4200 (2600 to 7100) pmol/ 8×10^8 RBC, respectively. Patients in the AE group formed higher concentrations of TGN and meTIMP (fig 4), but this difference was significant only for meTIMP at week 6 ($p = 0.04$). In the PP group, the meTIMP concentration decreased between week 5 and week 20 ($p = 0.009$). Patients with one variant TPMT allele (TPMT*1/*3A) formed significantly higher concentrations of TGN than wild type patients. In contrast, the meTIMP formation was lower and differed significantly only in week 3.

Clinical outcome

Of the 54 evaluable patients, 34 (17 with Crohn's disease, 17 with ulcerative colitis) were at baseline in remission and 19 (11 with Crohn's disease, eight with ulcerative colitis) had active disease. Information on disease activity was missing in one case. Of the 34 patients in remission, 22 were on steroid treatment, as were nine of 19 patients with active disease.

After the initial dose escalation, the 27 patients in the PP group were treated with a median azathioprine dose of 2.46 (IQR, 2.28 to 2.63) mg/kg body weight. Of 19 patients in remission at baseline (eight with Crohn's disease, 11 with ulcerative colitis), 17 remained in remission by week 20 and two had relapsed. Of eight patients with active disease at baseline (four with Crohn's disease, four with ulcerative colitis), six had gone into remission by week 20, while two still had active disease. Thus at the end of the study, 85% of the patients in the PP group (23/27) were in remission. There were no significant differences at week 20 in either TGN concentrations (176 (IQR, 119 to 226) v 163 (156 to 198) pmol/ 8×10^8 RBC ($p = 0.935$)) or meTIMP concentrations (3200 (1050 to 5150) v 2300 (600 to 3200) pmol/ 8×10^8 RBC ($p = 0.452$)) between patients in remission ($n = 23$) and those with active disease ($n = 4$).

There was no correlation between change in MCV from baseline to week 20 (Δ MCV) and either TGN ($r_s = -0.11$; $p = 0.62$) or meTIMP ($r_s = 0.18$; $p = 0.41$) concentrations after 20 weeks of thiopurine treatment.

At baseline 19 of the 27 patients (70%) were treated with steroids, compared with eight (30%) at the end of study. Of the eight patients on steroids at week 20, three had reduced the dose by more than 50% and five remained on an unchanged low dose.

Effects of co-administration of 5-aminosalicylates, corticosteroids, and antibiotics

The median levels of TGN and meTIMP at week 5 (steady state) for patients in the PP group co-treated with

Table 3 Details of different types of adverse events in 39 patients

	Total (n)	Study PP (n)	Withdrawn (n)	Maximum meTIMP (pmol/ 8×10^8 RBC)	Maximum TGN (pmol/ 8×10^8 RBC)	Time to AE (weeks)
Myelotoxicity	10	1	9	10 450 (4025 to 23 625)	312 (185 to 477)	9 (5.75 to 12.5)
Gastrointestinal intolerance	10	3	7	4550 (2725 to 10 700)	192 (125 to 251)	3 (1.75 to 4.25)
Hepatotoxicity	5	3	2	12 400 (2650 to 13 750)	266 (177 to 441)	5 (4 to 8)
Myalgia/arthritis	4	1	3	1850 (425 to 6125)	124 (23 to 188)	2.5 (1.25 to 3.75)
Pancreatitis	4	0	4	1650 (800 to 3400)	188 (61 to 248)	3 (2.25 to 3.75)
Other	6	4	2	5350 (4300 to 6925)	255 (104 to 369)	4.5 (2.5 to 20)

Values are median (interquartile range).

Other adverse events includes rash ($n = 1$), palpitations and sleeping disturbances ($n = 1$), sleeping disturbances and depression ($n = 1$), fever and diarrhoea ($n = 1$), headache ($n = 1$), and dizziness ($n = 1$).

AE, adverse event; meTIMP, methylthioinosine monophosphate; PP, per protocol; RBC, red blood cells; TGN, thioguanine nucleotide.

Table 4 Details for patients with myelotoxicity (n = 10)

Patient No	WBC ($\times 10^9/l$)	Neutrophil count ($\times 10^9/l$)	Platelet count ($\times 10^9/l$)	TPMT genotype	TPMT activity (U/ml RBC)	Max TGN (pmol/ 8×10^8 RBC)	Max meTIMP (pmol/ 8×10^8 RBC)	Time point AE (week)	Medical decision
4	2.3	1.6	186	*1/*1	13.9	150	3800	8	DIS+LR
6	2.1	1.2	204	*1/*1	15.0	420	19 800	5	RED
8	2.4	1.3	223	*1/*1	11.9	315	43 900	12	DIS+LR
15	1.8	1.1	249	*1/*1	15.5	389	5600	12	DIS
16	2.8	1.6	306	*1/*1	10.5	174	13 000	20	COM
27	3.1	2.3	181	*1/*1	17.1	647	12 800	10	DIS+LR
34	4.2	2.9	64	*1/*3A	4.8	859	1500	3	DIS
44	3.3	1.2	169	*1/*1	10.9	286	35 100	7	RED
46	3.3	2.2	75	*1/*1	11.8	189	4100	14	DIS+LR
51	2.2	1.3	128	*1/*3A	7.3	310	8100	6	DIS+LR

Values for WBC, neutrophil count, and platelet count are the lowest recorded during the 20 week study period, while TGN and meTIMP concentrations are maximum values.

AE, adverse event; max, maximum; COM, completed study per protocol with dose reduction after week 20; DIS, discontinued treatment; LR, reintroduction of a thiopurine drug at a lower dose; meTIMP, methylthiinosine monophosphate; RBC, red blood cells; RED, dose reduction; TGN, thioguanine nucleotide; TPMT, thiopurine methyltransferase; WBC, white blood cell count.

5-aminosalicylates (n = 11) were 167 (IQR, 147 to 247) and 4200 (2600 to 5200) pmol/ 8×10^8 RBC, respectively, compared with 189 (140 to 331) pmol/ 8×10^8 RBC ($p = 0.51$) and 4250 (2800 to 7850) ($p = 0.61$) in patients not on 5-aminosalicylates (n = 16). No significant differences between either TGN or meTIMP levels were found in patients with and without concomitant steroid use or concomitant antibiotic use.

Adverse events

Thirty nine patients (72%) experienced an adverse event. In 12, the adverse events subsided without any changes in the thiopurine dose. In the other 27, adverse events necessitated dose reduction in five and discontinuation in 22. By week 20, however, thiopurine treatment had been successfully reintroduced at a lower dose in 13 of the latter 22 patients. Thus, 18 of 27 patients (67%) who had their treatment decreased or stopped because of adverse events were treated with a median of 1.32 (IQR, 1.15 to 1.71) mg azathioprine/kg body weight at week 20. Of the adverse events observed, myelotoxicity (n = 10) and gastrointestinal intolerance (n = 10) were most common, followed by hepatotoxicity (n = 5), myalgia/arthritis (n = 4), and pancreatitis (n = 4) (table 3). The time points for development of adverse events differed according to type of toxicity ($p = 0.045$).

Myelotoxicity

Ten patients developed myelotoxicity (leucopenia or neutropenia or both in eight, thrombocytopenia in two) after a median of 9 (IQR, 5.8 to 12.5) weeks. Anaemia, defined as a haemoglobin level < 120 g/l, was evident during the study in six of 10 patients with myelotoxicity, compared with 19 of 44 without myelotoxicity ($p = 0.34$). One patient with previous myelotoxicity the year before had her thiopurine dose reduced when her leucocyte count was $3.1 \times 10^9/l$, which was considered to represent leucopenia by her treating physician. She was treated in the analysis as having myelotoxicity, although formally she was above the predetermined level for leucopenia. Details for the 10 patients are summarised in table 4.

Six of these patients were treated with azathioprine and four with 6-MP (6/43 v 4/11; $p = 0.011$). Both maximum meTIMP levels (10 450 (IQR, 4025 to 23 625) v 4550 (2325 to 7800) pmol/ 8×10^8 RBC ($p = 0.046$)) and maximum TGN levels (312 (185 to 477) v 214 (155 to 290) pmol/ 8×10^8 RBC ($p = 0.040$)) were higher in patients with myelotoxicity than in patients without myelotoxicity (fig 5). Two of the 10 patients who developed myelotoxicity (at weeks 3 and 6) were heterozygous for a defective TPMT allele (TPMT*1/*3A).

Figure 5 Scattergram of maximum metabolite concentrations in patients with (n = 10) and without (n = 44) myelotoxicity. meTIMP, methylthiinosine monophosphate; TGN, thioguanine nucleotide.

We undertook a logistic regression analysis to elucidate factors associated with myelotoxicity. The independent variables were disease type (Crohn's disease/ulcerative colitis), sex, TPMT genotype, TPMT activity at baseline, 5-ASA treatment, steroid treatment at baseline, and maximum TGN (log max TGN) and meTIMP (log max meTIMP); the dependent variable was myelotoxicity. In this regression model only maximum meTIMP concentration (OR = 25.1 (95% CI, 1.4 to 467.1), $p = 0.031$) was significantly associated with myelotoxicity. The area under the receiver operating characteristic (ROC) curve for maximum meTIMP was 0.70 (95% CI, 0.51 to 0.90), $p = 0.046$. A specificity of 100% was seen at the cut off level of 18 550 pmol/ 8×10^8 RBC, but at the expense of a sensitivity of 30%.

To test the possibility of predicting myelotoxicity we used metabolite concentrations when steady state was reached at week 5. As cut off values we used the lower limit of the upper quartile (75th centile) for maximum meTIMP and TGN concentrations. Patients with a meTIMP concentration exceeding 11 450 pmol/ 8×10^8 RBC at week 5 had an

increased risk of developing myelotoxicity (OR = 45.0 (95% CI, 2.1 to 957.6), $p = 0.015$), while TGN concentrations in the upper quartile (>316 pmol/ 8×10^8 RBC) were not predictive of the development of myelotoxicity. The area under the ROC curve for meTIMP $>11\,450$ at week 5 was 0.74 (95% CI, 0.51 to 0.97), $p = 0.030$). Thus a meTIMP value above 11 450 at this time point had a specificity of 97% and a sensitivity of 44% for predicting myelotoxicity.

We also calculated the meTIMP/TGN ratios at week 5 and found no differences between patients with and without myelotoxicity ($p = 0.45$).

Hepatotoxicity

Five patients (four on azathioprine, one on 6-MP) had abnormal liver function tests after a median of 5 (IQR, 4 to 8) weeks. Maximum metabolite concentrations were not significantly different in patients with and without hepatotoxicity. Among the three patients in whom signs of hepatotoxicity subsided without change in the drug treatment, there was no clear relation to the meTIMP levels. One patient had a maximum concentration of 12 400 pmol/ 8×10^8 RBC, but 4600 pmol/ 8×10^8 RBC when signs of hepatotoxicity became evident. In one patient abnormal liver function tests were noted at meTIMP concentrations of 3300 pmol/ 8×10^8 RBC but subsided despite meTIMP continuing to rise to a maximum of 13 900 pmol/ 8×10^8 RBC. In one patient who had her 6-MP dose reduced from 100 to 37.5 mg, signs of hepatotoxicity subsided and meTIMP concentration fell from 13 600 to 2700 pmol/ 8×10^8 RBC, while the patient who discontinued treatment had a maximum meTIMP concentration of 1200 pmol/ 8×10^8 RBC.

Pancreatitis

Four patients with Crohn's disease developed pancreatitis after a median time of 3.0 (IQR, 2.25 to 3.75) weeks. All four patients were treated with azathioprine, which was discontinued when the diagnosis became evident. Maximum meTIMP concentrations were significantly lower in patients with pancreatitis than in those without pancreatitis (1650 (IQR, 800 to 3400) v 5600 (3575 to 11 950) pmol/ 8×10^8 RBC; $p = 0.011$), while no significant differences in maximum TGN concentrations were noted.

Patients with previous adverse events

Twelve patients had previously experienced side effects while being treated with azathioprine. Under the present protocol all except one of these patients were treated with 6-MP. Previous adverse events included myelotoxicity ($n = 5$), hepatotoxicity ($n = 1$), gastrointestinal intolerance ($n = 3$), fever ($n = 1$), and other ($n = 2$). Nine patients developed adverse events again. Among the five patients with previous myelotoxicity four developed myelotoxicity. Of the three with previous gastrointestinal intolerance (either abdominal pain or nausea), one had recurrent symptoms of abdominal pain while the other two developed hepatotoxicity and myelotoxicity, respectively; the patient with previous hepatotoxicity developed myalgia.

TPMT genotype, ITPA 94C>A polymorphism, and adverse events

Five of six TPMT heterozygous patients failed to complete the study because of adverse events. Compared with TPMT wild type patients, they had a lower probability of remaining in the study ($p = 0.039$). TPMT genotype did not predict the occurrence of subgroups of adverse events (myelotoxicity ($p = 0.31$), gastrointestinal intolerance ($p = 1.0$), hepatotoxicity ($p = 0.46$), myalgia/arthritis ($p = 1.0$), pancreatitis ($p = 0.39$)). Neither was the ITPA polymorphism 94C>A associated with the development of adverse events overall

($p = 0.35$) or subgroups of adverse events (myelotoxicity ($p = 1.0$), gastrointestinal intolerance ($p = 1.0$), hepatotoxicity ($p = 1.0$), myalgia/arthritis ($p = 0.39$), pancreatitis ($p = 0.39$)).

Long term follow up

At the one year follow up, 42 of 54 patients (78%) were still on treatment with azathioprine ($n = 30$) or 6-MP ($n = 12$), with a median azathioprine dose of 2.2 (IQR, 1.7 to 2.5) mg/kg body weight.

DISCUSSION

This is the first study in which thiopurines have been introduced according to a predetermined dose escalation schedule with regular monitoring of TPMT enzyme status and thiopurine metabolites.

We aimed at a fixed dose of 2.5 mg/kg body weight for azathioprine or 1.25 mg/kg body weight for 6-MP, and started during the first week with one third of the full dose, during the second week with two thirds of the full dose, and reached the full dose by the third week. Treatment was discontinued in five of 54 patients (9%) because of adverse events before full doses were reached by week 3, and in 16 of 54 patients (30%) before steady state concentrations were reached at week 5. Overall, the frequency of adverse events was higher in this study than in most other published reports.⁴⁻⁸ One exception is the study by Sandborn *et al*, in which patients were started on a full dose of azathioprine (2 mg/kg body weight) after an intravenous azathioprine or placebo infusion, with withdrawal rates of 37% and 42% in the respective groups.³⁵ A recent study by Derijks *et al*, in which all patients were started on 50 mg of 6-MP, found 33% of drug related study failures during the eight week follow up.³⁶ The majority of adverse events, both in our study and in that by Sandborn *et al*, were gastrointestinal or idiosyncratic side effects, or both. One possible explanation for the high rate of toxicity might be relatively rapid dose increment to a rather high thiopurine dose, though still in the range of normally recommended dosage. In most other studies the thiopurine dosage has tended to be lower and, traditionally, the time to reach the target dose has been longer. Thus it is possible that a less intense treatment schedule might prevent some of the side effects encountered. The fact that in the long run 67% of the patients withdrawn because of thiopurine related adverse events were able to tolerate thiopurine treatment at a lower dose supports this assumption.

We found a significant decrease in TPMT gene expression during thiopurine treatment. One explanation for this decrease is the inhibition of purine de novo synthesis by the meTIMP formed, leading to a decreased amount of purines available for DNA and RNA synthesis.^{37,38} Another reason could be downregulation of TPMT transcription, in keeping with the fact that thiopurines have been shown to downregulate transcription of several genes involved in the response to inflammation.¹² We have not investigated whether there are differences in TPMT gene expression between different populations of white blood cells; the change in expression during treatment could be ascribed to changes in white blood cell populations rather than to inhibition of purine de novo synthesis.

In general, TPMT activity did not change during the treatment, but we observed marked inter-individual differences. In four patients there was a discrepancy between genotype and phenotype. In these patients, even if the increase in TPMT enzyme activity was profound (12–66%), the TPMT gene expression decreased. The natural circadian variability in TPMT activity in healthy individuals has been reported to be 6.4%, and the variability over weeks to be 6.5%.³⁹ The intra-assay variation of our TPMT assay is 3.3%.

In the light of such small variations, we feel confident that the increases in enzyme activity seen in these four patients were caused by the treatment.

In parallel with our findings, measurement of TPMT activity in IBD patients during a longer follow up has led to a variable decrease or increase in TPMT activity, but to no general induction.⁴⁰

Drug treatment other than thiopurines, as well as red blood cell age and transfusions, are important factors influencing TPMT enzyme activity.^{41–44} One study observed that TPMT activity was already significantly increased after the induction treatment for childhood acute lymphatic leukaemia, even before the patients received 6-MP.⁴⁵ The promoter of the TPMT gene contains regions with variable numbers of tandem repeats which originally were proposed to modulate TPMT enzyme activity.^{46–47} It has now been shown that these regions explain neither the variations in TPMT enzyme activity in untreated individuals⁴⁸ nor the increase in TPMT enzyme activity during thiopurine treatment.⁴⁰

Among our patients with myelotoxicity, both meTIMP and TGN levels were higher than in patients without myelotoxicity, but in the regression analysis only meTIMP levels were associated with myelotoxicity. This is a new observation in humans. meTIMP has been shown to be cytotoxic in cell cultures and to inhibit purine de novo synthesis^{17–18} and in an in vitro study by Dervieux *et al*, MeMP concentrations of 224 pmol/5 × 10⁶ RBC (corresponding to 35 840 pmol/8 × 10⁸ RBC) produced a 50% inhibition of purine de novo synthesis.¹⁷ In our study concentrations of this magnitude were seen only in patients who developed myelotoxicity. Clinical studies in patients with ALL have shown that 6-MP is capable of fully inhibiting purine de novo synthesis in 20% of the patients and that this inhibition is associated with a significantly greater proportional decrease in the number of circulating leucocytes.⁴⁹ Although markedly lower doses of thiopurine drugs must be used in treatment of TPMT deficient patients to avoid bone marrow toxicity, such patients do not form meTIMP and can tolerate higher TGN concentrations than patients with normal TPMT activity.⁵⁰ Furthermore, patients treated with 6-thioguanine can tolerate higher TGN concentrations without developing signs of myelotoxicity.¹⁵ This might be because meTGN, the methylated metabolite produced in 6-thioguanine therapy, is a less potent inhibitor of purine de novo synthesis than meTIMP,⁵¹ and that the meTGN/TGN ratio in 6-thioguanine treatment is much lower than the meTIMP/TGN ratio during 6-MP treatment.¹⁷ Thus, by inhibiting PRPP amidotransferase (the first enzyme in purine de novo synthesis), theoretically high meTIMP concentrations could facilitate the incorporation of TGN into DNA, as less endogenous purines are available, making seemingly low to normal TGN concentrations cytotoxic. It has also been suggested that inhibition of purine de novo synthesis is the main mechanism of cytotoxicity during treatment with azathioprine and 6-MP, while 6-TG treatment is dependent on DNA-TGN incorporation.¹⁸

Myelotoxicity occurred significantly later in time than both gastrointestinal and allergic adverse events, at a median of nine weeks after the start of treatment. As myelotoxicity was independently associated with high meTIMP concentrations we investigated whether it was possible to predict the development of myelotoxicity using meTIMP determinations early in the course of treatment. By using a stipulated cut-off value of >11450 pmol/8 × 10⁸ RBC (corresponding to maximum meTIMP values in the upper quartile) at steady-state (i.e. week 5), we found that patients with concentrations above this cut-off level had a dramatically increased risk of developing bone marrow toxicity OR = 45.0 (95% CI, 2.1 to 957.6). This observation may influence the clinical application of metabolite measurements. Furthermore, the ROC

analysis showed that meTIMP concentrations above this cut-off level has high sensitivity (97%) but low specificity (44%) for predicting myelotoxicity.

Patients treated with 6-MP had an increased risk of developing myelotoxicity compared to those treated with azathioprine. This finding is probably explained by a selection bias as four of five patients with previous myelotoxicity during azathioprine treatment were given 6-MP when included in this study. When an equivalent or higher dose of 6-MP was given, the reoccurrence of this dose dependent toxicity would be expected. However, all five patients tolerated long term treatment on a lower thiopurine dose.

The previously described association between hepatotoxicity and high MeMP levels (that is, meTIMP levels)^{19–20} were not obvious. In one patient with relatively high meTIMP concentrations the signs of hepatotoxicity subsided while the meTIMP levels decreased after reduction in the thiopurine dose. In the other patients there were no clear cut association between meTIMP concentration and the development of hepatotoxicity. Thus these findings are not in accordance with those studies that have suggested high meTIMP (MeMP) as a risk factor for the development of hepatotoxicity.^{19–20} However, the number of patients in our study is small and the results have to be interpreted with caution.

All four individuals who developed pancreatitis had Crohn's disease. The meTIMP concentrations were significantly lower in this group than in patients without pancreatitis. One of four patients with pancreatitis was heterozygous for the ITPase polymorphism 94C>A (C/A) and another was heterozygous for TPMT (*1/*3A). These findings argue against the mechanism suggested by Marinaki *et al*, that a raised concentration of me-6-thio-ITP is a cause of pancreatitis.²⁴

We found no difference in either TGN or meTIMP levels between patients in clinical remission and those with active disease after 20 weeks of treatment. This finding has to be interpreted with caution as only 27 patients were evaluable at this time point, and of these only four had active disease. If this finding is true, it militates against the clinical value of metabolite measurements for assessing treatment efficacy.

In summary, the most important finding in our study was that high meTIMP concentrations are associated with the risk of developing myelotoxicity, and that patients who have meTIMP concentrations exceeding a stipulated cut off level of 11 450 pmol/8 × 10⁸ RBC early in steady state have a dramatically increased risk of developing bone marrow toxicity. As myelotoxicity tended to develop several weeks after maximum meTIMP concentrations were reached it might be possible to identify patients at risk early in the course of treatment, thereby preventing the development of myelotoxicity and the associated risk of infections.

We conclude that metabolite measurements at the time point when steady state concentrations are reached are of value for identifying patients at risk of developing myelotoxicity. A larger prospective study is now needed to verify this observation.

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