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Abstract

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mean reduction 109 pmol/8 × 10^6 erythrocytes) was observed (median 221 pmol/8 × 10^6 erythrocytes, range: 96-427, $P$ value linear mixed effects model 0.0011). Demographic and clinical covariates were not related to thiopurine metabolites concentrations. All patients were wild-type for the most relevant ITPA and TPMT variants. For NAT1 genotyping, 7 subjects presented an allele combination corresponding to fast enzymatic activity and 5 to slow activity. NAT1 genotypes corresponding to fast enzymatic activity were associated with reduced TGN concentration ($P$ value linear mixed effects model 0.033), putatively because of increased 5-ASA inactivation and consequent reduced inhibition of thiopurine metabolism. The effect of NAT1 status on TGN seems to be persistent even after one month since the interruption of the aminosalicylate.

No effect of NAT1 genotypes was shown on MMPN concentrations. NAT2 genotyping revealed that 6 patients presented a genotype corresponding to fast enzymatic activity and 6 to slow activity; NAT2 genotypes were not related to thiopurine metabolites concentration in this study.

**CONCLUSION:** NAT1 genotype affects TGN levels in patients treated with thiopurines and aminosalicylates and could therefore influence the toxicity and efficacy of these drugs; however the number of patients evaluated is limited and this has to be considered a pilot study.

**Key words:** Thiopurines; Aminosalicylates; Inflammatory bowel diseases; N-acetyl transferase; Pharmacogenomics

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Core tip: During treatment of inflammatory bowel disease with thiopurines and aminosalicylates, interruption of the aminosalicylate results in a significant decrease in thiopurines’ thioguanine nucleotides (TGN) active metabolites. Genetic polymorphisms in genes involved in aminosalicylates biotransformation (NAT1 genotype) affects TGN levels in patients treated with thiopurines and aminosalicylates and could therefore influence the toxicity and efficacy of these drugs.


**INTRODUCTION**

Thiopurines and aminosalicylates are the two most widely used drugs in inflammatory bowel disease (IBD) and are often used in combination. The thiopurines 6-mercaptopurine (6MP) and its prodrug azathioprine (AZA) are effective in inducing and maintaining remission and are considered steroid sparing agents. 6MP is metabolized by a multistep enzymatic pathway, initiated by hypoxanthine phosphoribosyl transferase that leads to formation of thioguanine nucleotides (TGNs). These active metabolites act as purine antagonist and inhibit DNA, RNA and protein synthesis, inducing cytotoxicity and immunosuppression. Blood levels of thiopurine metabolites have been correlated with the efficacy and toxicity of these drugs in patients with IBD: TGN levels higher than 235 pmol/8 × 10^6 red blood cells are considered therapeutic, and methyl mercaptopurine nucleotides (MMPNs) levels above 5700 pmol/8 × 10^6 red blood cells have been associated with hepatotoxicity.[1-3].

The aminosalicylate 5-aminosalicylic acid (mesalazine, 5-ASA) is used in the induction and maintenance of remission in ulcerative colitis.[4-6]. In Crohn’s disease, the use of aminosalicylates is controversial, however studies suggest that they could have a role in the postoperative maintenance of remission also in this IBD[6]. In addition, a chemopreventive role of 5-ASA in IBD against colon cancer has been suggested[7].

An increase in mean TGN blood levels has been reported in patients on 6MP or AZA co-treated with 5-ASA.[2-8-12]. Even more important, a higher rate of myelotoxicity was observed in patients treated with this combination in comparison with those treated with the thiopurine alone.[2,8-12].

6MP is inactivated by the enzyme thiopurine methyltransferase (TPMT, EC 2.1.1.67) that catalyzes its S-methylation to 6-methylmercaptopurine and, at least in part by inosine triphosphate pyrophosphatase (ITPA, EC 3.6.1.19). *In vitro* studies have shown that aminosalicylates and their metabolites can inhibit the activity of TPMT[14,15], however, this observation has not been confirmed *in vivo*.[2,8,9].

The enzymes N-acetyltransferases (NAT1 and NAT2, EC 2.3.1.5) are responsible for the N-acetylation of a number of xenobiotics and drugs including the aminosalicylates. Even the activity of NAT1 and NAT2 is genetically determined and subjects are classified as rapid, intermediate or slow acetylators. Although NAT1 and NAT2 polymorphisms have been associated with the incidence of some diseases, no significant effect has been reported for IBD[16]. 5-ASA is inactivated primarily by the NAT1 isoform in the colonic mucosa, and the drug and its metabolites are excreted in the urine[17-19]. The inheritance of a slow acetylator genotype for NAT1 could therefore lead to a reduced inactivation of 5-ASA, and hence to higher blood levels of the drug.

The aim of this study was to measure variation of the concentration of thiopurine metabolites after 5-ASA interruption and to evaluate the role of genetic polymorphisms of NAT1 and NAT2 on this phenomenon.
Table 1  Genotypes and methods of analysis considered in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT1</td>
<td>T1088A</td>
<td>Sequencing</td>
<td>With primer forward: 5'-TGCCC AAACATGGTGATAGATT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>With primer reverse: 5'-CCATAA AACTTTCAGGAATTCACA</td>
</tr>
<tr>
<td>NAT1</td>
<td>C1095A</td>
<td>Sequencing</td>
<td>As above</td>
</tr>
<tr>
<td>NAT2</td>
<td>C282T</td>
<td>PCR-RFLP</td>
<td>[25,24]</td>
</tr>
<tr>
<td>NAT2</td>
<td>T341C</td>
<td>PCR-RFLP</td>
<td>[25,24]</td>
</tr>
<tr>
<td>TPMT</td>
<td>G238C</td>
<td>PCR-ASO</td>
<td>[27]</td>
</tr>
<tr>
<td>TPMT</td>
<td>G460A</td>
<td>PCR-RFLP</td>
<td>[27]</td>
</tr>
<tr>
<td>TPMT</td>
<td>A719G</td>
<td>PCR-RFLP</td>
<td>[27]</td>
</tr>
<tr>
<td>ITPA</td>
<td>C94A</td>
<td>TaqMan</td>
<td>TaqMan SNP genotyping assay from Applied biosystems (C_27465000_10)</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

**Patients and inclusion criteria**

Twelve patients with IBD were enrolled by the Gastroenterology Unit of the Pediatric Hospital “Burlo Garofolo” in Trieste, and by the Research Children’s Hospital “Meyer”, Florence, Italy. These patients have been retrospectively selected considering the following criteria: previous diagnosis of IBD and treatment with AZA or 6MP plus 5-ASA for at least three months. 5-ASA therapy was interrupted, and a minimum of two blood samples for thiopurine metabolites measurement were taken one month before and one month after 5-ASA interruption. The study was approved by the local ethical committees and appropriate informed consent was obtained from all patients or their parents or guardians.

**Measurement of azathioprine metabolites**

Azathioprine metabolites (TGN and MMPN) were measured in patients’ erythrocytes using an HPLC assay by Dervieux and Boullieu[20] within few weeks from the sample collection. The ratio between TGN and the dose of azathioprine was calculated to account for the respective dose each patient was taking on the day that the metabolite testing was performed.

**Genotypes**

Genomic DNA was extracted from peripheral blood samples using a commercial kit (SIGMA, Milan, Italy), in order to characterize genetic polymorphisms in the candidate genes NAT1, NAT2, TPMT and ITPA. The considered genotypes and method of analysis are described in Table 1.

**NAT acetylator status determination**

NAT acetylator status (i.e., rapid or slow) was assessed from the genotyping results. In particular, for NAT1, patients with an A nucleotide at both 1088 and 1095 nucleotides, corresponding to NAT1*10 allele, were considered as fast NAT1 acetylators while all other genotype combinations were considered as slow NAT1 acetylators[21,22]. For NAT2, patients homzygous for the wild-type allele at either the 282 or 341 position or patients heterozygous for the variant allele at just one of these two positions were considered as fast NAT2 acetylators, all other genotypes combinations were considered as slow NAT2 acetylators[23,24].

**Statistical analysis**

Statistical analysis was performed using the software R (version 3.0.1). The primary intended outcome of this study was to evaluate variations of the concentration of thiopurine metabolites after 5-ASA interruption and the role of genetic polymorphisms of NAT 1 and 2.

Power analyses on preliminary data available indicate that given the difference in means and the distribution’s standard deviation, the minimum sample size to identify a statistically significant (P = 0.05, power 80%) result is 9 for the paired test comparing azathioprine metabolites during aminosalicylate treatment and after the suspension. For the analysis comparing thiopurine metabolites concentration in NAT1 fast acetylators compared to slow acetylators, the minimum number of patients to detect a statistically significant (P = 0.05, power 80%) result is 5 for each NAT1 activity status.

The association between pharmacological phenotypes of interest (i.e., TGN metabolites concentrations, MMPN metabolites concentrations) and the considered demographic variables, IBD type, co-treatment with aminosalicylate or genotypes in a univariate analysis, was evaluated by considering for each phenotype and patient the individual observations and evaluating the effect of each covariate by calculating the P value from a linear mixed effects model built using the phenotype as the dependent variable, each covariate as the fixed effect and the patients as the random effect in the model. Multivariate analysis was done to test the independence of the effects of the covariates significant in the univariate analysis on the phenotypes considered by using linear mixed effects models with the phenotype of interest as the dependent variable and the covariates selected in the univariate analysis as the independent variables. For all parametric analyses (i.e., linear mixed effects models used in the univariate analysis and the multivariate analysis), normality of the phenotype was tested by the Shapiro test and log10 transformation was applied if needed, in order to achieve normality of the distribution.

**RESULTS**

**Patients enrolled and samples collected**

The present study recruited 12 young patients (3 females and 9 males, median age 16 years) with IBD (6 Crohn’s disease and 6 ulcerative colitis). Seven
patients were treated with 6MP (median dosage 1.0 mg/kg, range: 0.5-1.0, equivalent to a median AZA dose of 2.08 mg/kg, range: 1.04-2.08) and 5 with AZA (median dosage 2.2 mg/kg, range: 1.6-2.6). All patients were co-treated with 5-ASA for at least three months at standard doses (50 mg/kg). A total of 36 samples of peripheral blood were collected to measure azathioprine metabolites; on average, 3 samples for patient were collected (range: 2-4). For 8 patients it was not possible to collect two samples before 5-ASA interruption and 2 samples after 5-ASA interruption, because of clinical reasons: therefore 12 samples (5 before and 7 after) were missing; however each patient had at least one sample before and one after 5-ASA interruption. Among these, 19 were obtained during treatment with the thiopurines and 5-ASA, before 5-ASA interruption, and 17 after interruption of 5-ASA and therefore during treatment with the thiopurine alone. The reason for 5-ASA interruption was clinical, mostly simplification of therapy to increase compliance, which is particularly useful in pediatric patients. Samples were taken from the same patient with an interval of at least one month.

### Measurement of azathioprine metabolites

Median TGN concentrations before 5-ASA interruption was 270 pmol/8 × 10⁸ erythrocytes (range: 145-750); after the interruption of the aminosalicylate, a 35% reduction (mean absolute value 109 pmol/8 × 10⁸ erythrocytes) in TGN mean concentrations was observed (median 221 pmol/8 × 10⁸ erythrocytes, range: 96-427, coefficient = -0.18, 95%CI: -0.27-(-0.09), P value linear mixed effects model 0.14). There was a significant correlation between TGN and MMPN concentrations (coefficient 0.3, 95%CI: 0.15-0.45, linear mixed effect P value 0.0007) while the dose of thiopurine did not correlate with TGN and MMPN concentrations in these patients. The dose of thiopurine did not change before and after the interruption of the aminosalicylate (median value of azathioprine dose and range 2.08 mg/kg, range: 1.04-2.6).

### Demographic and clinical covariates and azathioprine dose and metabolites

For the demographic (gender and age) and clinical (type of IBD and treatment duration) covariates considered, none showed a fully significant effect on the median TGN or MMPN concentrations in a univariate analysis (Table 2).

### Genotyping

All polymorphisms considered were respecting Hardy-Weinberg equilibrium and their distribution is comparable to what has been reported in the literature for patients of Caucasian ethnicity. All patients were wild-type for the most relevant ITPA and TPMT variants. For NAT1 genotyping, 7 presented an allele combination corresponding to fast enzymatic activity and 5 to slow activity. NAT2 genotyping revealed that 6 patients presented a genotype corresponding to fast enzymatic activity and 6 to slow activity.

### Genotypes and thiopurine metabolites

NAT1 genotypes corresponding to fast enzymatic activity was associated with reduced TGN concentration [coefficient = -0.159, 95%CI: -0.28-(-0.04), P value linear mixed effects model 0.033]: mean values were 269.3 and 400.8 respectively in patients with fast and slow NAT1 status before 5-ASA interruption, and 181.9 and 261.6 after 5-ASA interruption. Unexpectedly, the effect of NAT1 status on TGN seems to be persistent even one month since the interruption.

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**Table 2** Demographic, clinical and pharmacological data for the 12 patients enrolled

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at enrollment (yr)</th>
<th>Disease</th>
<th>Thiopurine dose (mg/kg per day)</th>
<th>5-ASA dose (mg/d)</th>
<th>TGN concentration¹ Before</th>
<th>% TGN change</th>
<th>NAT1 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.7</td>
<td>CD</td>
<td>AZA 2.6</td>
<td>50</td>
<td>244</td>
<td>11%</td>
<td>Rapid</td>
</tr>
<tr>
<td>2</td>
<td>17.3</td>
<td>CD</td>
<td>AZA 2.2</td>
<td>50</td>
<td>210²</td>
<td>-16%</td>
<td>Rapid</td>
</tr>
<tr>
<td>3</td>
<td>17.8</td>
<td>CD</td>
<td>AZA 1.6</td>
<td>50</td>
<td>276²</td>
<td>-64%</td>
<td>Rapid</td>
</tr>
<tr>
<td>4</td>
<td>14.7</td>
<td>CD</td>
<td>6MP 1.0</td>
<td>50</td>
<td>288²</td>
<td>-51%</td>
<td>Rapid</td>
</tr>
<tr>
<td>5</td>
<td>10.4</td>
<td>UC</td>
<td>6MP 0.6</td>
<td>50</td>
<td>310²</td>
<td>-34%</td>
<td>Rapid</td>
</tr>
<tr>
<td>6</td>
<td>14.5</td>
<td>UC</td>
<td>6MP 0.5</td>
<td>50</td>
<td>330²</td>
<td>-43%</td>
<td>Rapid</td>
</tr>
<tr>
<td>7</td>
<td>11.9</td>
<td>UC</td>
<td>6MP 1.0</td>
<td>50</td>
<td>228</td>
<td>+7%</td>
<td>Rapid</td>
</tr>
<tr>
<td>8</td>
<td>17.4</td>
<td>CD</td>
<td>AZA 2.2</td>
<td>50</td>
<td>217²</td>
<td>+2%</td>
<td>Slow</td>
</tr>
<tr>
<td>9</td>
<td>6.3</td>
<td>UC</td>
<td>AZA 2.3</td>
<td>50</td>
<td>375</td>
<td>-63%</td>
<td>Slow</td>
</tr>
<tr>
<td>10</td>
<td>17.5</td>
<td>UC</td>
<td>6MP 1.0</td>
<td>50</td>
<td>647²</td>
<td>-38%</td>
<td>Slow</td>
</tr>
<tr>
<td>11</td>
<td>17.3</td>
<td>UC</td>
<td>6MP 1.0</td>
<td>50</td>
<td>264</td>
<td>+5%</td>
<td>Slow</td>
</tr>
<tr>
<td>12</td>
<td>16.6</td>
<td>CD</td>
<td>6MP 0.5</td>
<td>50</td>
<td>501</td>
<td>-47%</td>
<td>Slow</td>
</tr>
</tbody>
</table>

¹ Pmol/8 × 10⁸ erythrocytes; ² This value is the average of two measurements. 5-ASA: 5-aminosalicylate; TGN: 6-thioguanine nucleotides; NAT1: N-acetylttransferase 1; CD: Crohn's disease; UC: Ulcerative colitis; 6MP: 6-mercaptopurine; AZA: Azathioprine.
of the aminosalicylate (Figure 1). No effect of NAT1 genotypes was shown on MMPN concentrations. NAT2 genotypes were not related to thiopurine metabolites’ concentration in this study.

**DISCUSSION**

The clinical use of thiopurines in IBDs has increased substantially in recent years; these drugs have indeed a steroid sparing effect and their use in combination with infliximab has been also advocated.

Coprescription of 5-ASA is also common, and up to 60% of patients on thiopurines are also treated with an aminosalicylate.

Thiopurines are generally well tolerated, however, 15%-20% of patients side effects such as leukopenia, hepatitis and pancreatitis. Two key enzymes, TPMT and ITPA, are important for 6MP metabolism: TPMT catalyzes the S-methylation to 6MP and genetic polymorphisms in the TPMT gene are associated with a reduced enzymatic activity and an increased production of the active TGNs; indeed, patients with the homozygous mutation are at high risk of severe and sometimes fatal immuno-suppression. For this reason TPMT genotyping or phenotyping is recommended prior to the initiation of therapy. Another important enzyme in thiopurines’ metabolism is ITPA; a polymorphism in this gene leads to accumulation of the metabolite 6-thiino-sine triphosphate and has been associated with an increased risk of toxicity, in particular pancreatitis, flu like symptoms, rash and gastrointestinal toxicity; this observation was however not confirmed by other studies.

All patients included in our study had a normal TPMT genotype and were wild-type for the most common mutation of ITPA, hence excluding bias due to the influence of these genotypes.

In IBD patients treated with thiopurines an additional risk results from the co-administration of other drugs, such as the aminosalicylates. In the present study we confirm previous observations of a significant decrease in TGN levels after discontinuation of 5-ASA. Furthermore, a dose dependent effect was previously reported for two different 5-ASA doses on thiopurine metabolites levels.

In our study all patients were treated with a dose of 5-ASA of 50 mg/kg, equivalent to the higher dose reported by de Graaf et al. Consistently with this study, after interruption of 5-ASA we observed an effect on TGN and not on MMPN concentration. This may be due to the different populations considered: TPMT activity indeed is significantly higher in wild-type children (0.08-17 years) than in wild-type adults (aged 18-68 years).

The mechanism of this interaction is however still unclear. It has been demonstrated that the aminosalicylates inhibit the activity of recombinant TPMT in vitro, with IC50 values of 78 and 1240 µmol/L for sulfasalazine and 5-ASA respectively. In our study all patients included in our study had a normal TPMT genotype and were wild-type for the most common mutation of ITPA, hence excluding bias due to the influence of these genotypes.

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of xenobiotics and drugs including the aminosalicylates. The activity of NAT1 and NAT2 is genetically determined; both genes are located on chromosome 8p22 and a number of polymorphisms have been reported, allowing subjects to be classified as rapid or slow acetylators. The isozymes NAT1 and NAT2 have distinct substrate specificity and the NAT1 isozyme is more important (19000-fold more active) than NAT2 in 5-ASA acetylation in vitro[42]. Interestingly, in our study, patients with the NAT1 slow metabolizer phenotype had significantly higher TGN levels in comparison with rapid metabolizers. The inheritance of a slow acetylator genotype for NAT1 could therefore lead to a reduced inactivation of 5-ASA, and hence to higher blood levels of the aminosalicylate. This could result in a reduction of 6MP inactivation, via a still unclear mechanism, with consequent increase in TGN levels. Quite unexpectedly however, this difference was maintained when measurements were performed one month after 5-ASA discontinuation. This may be due to the long half-life of TGN and the fact that a longer period is needed to overcome the reduction in TGN concentrations determined by the increased metabolism of 5-ASA. It is however possible that NAT1 influences TGN concentrations by a different mechanism, not involving 5-ASA metabolism.

As expected, no effect of the NAT2 polymorphism was observed in these patients.

In conclusion, co-administration of 5-ASA and thiopurines is common and probably this association will continue to be prescribed in light of the demonstrated chemopreventive activity for IBD associated colorectal cancer[37]. Since the number of patients enrolled in this study is limited, this has to be considered a pilot study and more research should be performed to evaluate if the difference in TGN levels observed in patients with the NAT1 slow acetylator phenotype are also related to an increased incidence of thiopurine induced side effects. If this were true, it might be useful to assess the NAT1 genotype before starting therapy and, in those patients with a slow acetylator genotype, it might be prudent to start therapy with a reduced dose of AZA. Moreover, further studies should be performed to evaluate a dose dependent effect of 5-ASA or thiopurine dose on the association between NAT1 status and the pharmacokinetic interaction between 5-ASA and thiopurines: indeed patients with adverse NAT1 status may be treated with low doses of aminosalicylates, maintaining their chemopreventive effect. The effect of NAT1 acetylator status on TGN concentration in patients treated only with thiopurine could also be investigated.

NAT1 genotyping, in addition to careful clinical monitoring and evaluation of thiopurine metabolites, might be a useful guide in those patients receiving azathioprine and aminosalicylates.

**COMMENTS**

**Background**

Thiopurines and aminosalicylates are the two most widely used drugs in inflammatory bowel disease (IBD) and are often used in combination. A significant pharmacokinetic interaction has been described for these medications, since this association increases the concentration of thiopurines active metabolites (TGN).

**Research frontiers**

Treatment of IBD with thiopurines and aminosalicylates displays significant inter-patient variability in terms of efficacy and incidence of adverse events. Identification of determinants to predict effects of these treatments, such as genetic polymorphisms of enzymes involved in thiopurines and aminosalicylates biotransformation, is of clinical interest.

**Innovations and breakthroughs**

This article confirms that after interruption of aminosalicylate, concentration of TGNs decrease significantly. Moreover, N-acetyl-transferase 1 (NAT1) acetylator status, relevant for aminosalicylates biotransformation, influences TGN concentration during co-treatment and after the interruption of the aminosalicylate.

**Applications**

If supported by further clinical studies, NAT1 may be incorporated in multilocus signatures of genotypes useful to predict the efficacy and safety of thiopurine and aminosalicylate co-treatment in young patients with IBD.

**Terminology**

TGN, the active metabolites of thiopurines, formed after biotransformation of mercaptopurine by enzymes of nucleotides salvage pathway. NAT1, an enzyme that catalyzes the acetylation of amino groups of aminosalicylates such as 5-ASA.

**Peer-review**

This is an interesting and clinically relevant paper. Obviously the study has a small sample size and thus it is best described as a pilot study. The results of this small study are of interest in that there seems to be a pharmacokinetic interaction between 5-aminosalicylate and thiopurines, there also seems to be a potentially important and previously unexplored effect of NAT polymorphisms.

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