Therapeutic drug monitoring of thiopurine metabolites in adult thiopurine tolerant IBD patients on maintenance therapy

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Received 14 June 2011; received in revised form 28 November 2011; accepted 5 December 2011

KEYWORDS
Inflammatory bowel disease; Thiopurines; Azathioprine;

Abstract

Background and aims: Therapeutic drug monitoring of active metabolites of thiopurines, azathioprine and 6-mercaptopurine, is relatively new. The proposed therapeutic threshold level of the thiopurine 5-methyltransferase (TPMT) is currently not available. The aim of this study was to investigate whether TDM of active metabolites of thiopurines can be an advantage in patients on maintenance therapy.

Abbreviations AZA, azathioprine; CAI, Colitis Activity Index; CDAI, Crohn’s Disease Activity Index; CI 95%, 95% confidence interval; HPLC, high performance liquid chromatography; IBD, Inflammatory bowel disease; 6-MMPR, 6-methylmercaptopurine ribonucleotides; 6-MP, 6-mercaptopurine; N, patient number; OR, odds ratio; RBC, red blood cells; TDM, therapeutic drug monitoring; 6-TGN, 6-thioguanine nucleotides; TPMT, thiopurine S-methyltransferase.

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1. Introduction

Immunosuppression with thiopurines such as, azathioprine (AZA) and 6-mercaptopurine (6-MP) has become standard in the maintenance therapy of inflammatory bowel disease (IBD) patients. Thiopurine metabolism in humans is complex due to involvement of various enzymes. Neither AZA nor 6-MP has intrinsic activity, hence both drugs have to undergo extensive metabolic transformations, yielding to a variety of pharmacological active metabolites, of which the 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine ribonucleotides (6-MMPR) are considered to be the most important (Fig. 1).1

Thiopurine S-methyltransferase (TPMT) geno- or phenotyping prior to thiopurine therapy may help to recognize TPMT polymorphism and prevent severe myelotoxicity.2,3,4

Therapeutic drug monitoring (TDM) of the active thiopurine metabolites 6-TGN and 6-MMPR is a neglected, but attractive option that may help to optimize drug therapy.5–7 In IBD patients with an exacerbation while on thiopurine maintenance therapy, a switch to biologicals is usually made without further attempt to optimize thiopurine dosage based on thiopurine metabolite levels.

In recent years, therapeutic ranges of thiopurine metabolite concentrations have been described by several groups: 6-TGN metabolites between 235 and 490 pmol/8×10^8 red blood cells (RBC) are associated with clinical response.5–8 The risk for leucocytopenia increases with 6-TGN levels above 490 pmol/8×10^8 RBC, whereas 6-MMPR levels higher than 5700 pmol/8×10^8 RBC are associated with hepatotoxicity.5,6 A 6-MMPR/6-TGN ratio higher than 11 is correlated with a lower frequency of clinical response.6,9

Although these studies found no correlation between metabolite levels and drug dose resulting from inter-individual differences in metabolism, in daily clinical practice thiopurine drugs have still been dosed based on the patient’s body-weight (AZA 2–3 mg/kg and 6-MP 1–1.5 mg/kg).10

Osterman and colleagues performed a meta-analysis of six studies and showed that higher 6-TGN levels are correlated with clinical remission of IBD: 62% of the patients with 6-TGN levels above 230–260 pmol/8×10^8 RBC were in remission versus 36% with 6-TGN levels lower than 230 pmol/8×10^8 RBC, with a pooled odds ratio of 3.3 (CI 95%, 1.71–6.27; p<0.001).18 Most of the data on 6-TGN metabolite therapeutic thresholds have been obtained in smaller subsets of IBD patients, in the early phase after initiating thiopurine therapy or in pediatric IBD cohorts.5,6,9,11–19

The present study was undertaken to compare thiopurine metabolite levels in a larger group of adult thiopurine tolerant IBD patients on maintenance therapy with an exacerbation and those in clinical remission, in order to be able to find the optimal threshold 6-TGN level for therapeutic efficacy. Such parameters may help to determine the role of TDM in clinical decision making: either to optimize thiopurine dosage, to avoid toxicity or to switch to an alternative treatment.

2. Materials and methods

2.1. Patient selection

A prospective cross-sectional study was performed in a group of IBD patients in four hospitals in the southern part of the Netherlands: one university hospital (Maastricht University Medical Centre) and three general district hospitals (Maasland Hospital Sittard, Laurentius Hospital Roermond and Catharina Hospital Eindhoven). Adult IBD patients presenting an exacerbation while on tolerated maintenance dose of AZA or 6-MP for at least 3 months, were asked to participate. Meanwhile, consecutive IBD patients in clinical remission while on maintenance AZA or 6-MP for at least 3 months, were also asked to participate. Concomitant IBD medication had to be stable for at least 3 months and was registered, including medication known or suspected to interfere with thiopurine metabolism in vivo (i.e. mesalazine and infliximab).20,21

At inclusion, the following parameters were assessed: hemoglobin count, hematocrit, leucocytes, thrombocytes, bilirubin,
aspartate transaminase, amylase, C-reactive protein and serum albumin. Also, blood samples were collected for 6-TGN and 6-MMPR metabolite levels and TPMT activity measurement. Disease activity was scored by the Crohn’s Disease Activity Index (CDAI) or Colitis Activity Index (CAI).22 Written informed consent was obtained from all participating patients.

2.2. Outcome parameters

Primary outcome parameters were 6-TGN and 6-MMPR metabolite levels, to be used for correlations with CDAI or CAI scores and the calculation of a therapeutic 6-TGN threshold level. Zero metabolite levels were defined as non-detectable 6-TGN and 6-MMPR levels, and were considered to indicate non-compliance. Very low levels, defined as combined 6-TGN levels \(< 150\) pmol/8×10⁸ RBC and 6-MMPR levels \(< 900\) pmol/8×10⁸ RBC, without diminished TPMT activity, were considered indicative for non-compliance or low compliance.

An exacerbation was defined by a CDAI score \(\geq 200\) for Crohn’s disease patients or a CAI score \(\geq 8\) for patients with ulcerative colitis (UC) or indeterminate colitis.

Remission was defined by a CDAI score \(< 150\) or CAI \(< 8\). Patients with CDAI scores between 150 and 200 were not included to more clearly separate exacerbations or active disease from clinical remissions.

Secondary outcome parameters were CDAI and CAI scores, TPMT activity, 6-MMPR/6-TGN ratio and correlations between 6-TGN and 6-MMPR metabolite levels.

2.3. Analytical procedures

6-TGN and 6-MMPR levels were measured with the modified high performance liquid chromatography (HPLC) method of Lennard et al. as published previously.5,6,8,23 Blood samples were immediately stored in the refrigerator (2–8 °C) and subsequently sent to the laboratory of the Department of Clinical Pharmacy & Toxicology of the Maasland Hospital (Sittard, The Netherlands) where the samples were stored at \(-20\) °C, so that stability of the metabolites was assured until measurement occurred.

The lower limit of quantification of the assay was determined at 30 pmol/8×10⁸ RBC for 6-TGN and 300 pmol/8×10⁸ RBC for 6-MMPR.6

TPMT activity was measured in erythrocyte lysates essentially as described by Jacques-Aigrain,24 using reversed phase HPLC for quantification. The inter-assay co-variation

Figure 1  Proposed thiopurine metabolism. AZA, azathioprine; 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptopurine; 8-OHMP, 8-hydroxy-6-mercaptopurine; 6-TUA, 6-thiouric acid; 6-MTIMP, 6-methylthioinosine monophosphate; 6-MTIDP, 6-methylthioinosine diphosphate; 6-MTITP, 6-methylthioinosine triphosphate; 6-TIMP, 6-thioinosine monophosphate; 6-TIDP, 6-thioinosine diphosphate; 6-TTP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-MTITP, 6-methylthioinosine triphosphate; 6-TIMP, 6-thioinosine monophosphate; 6-TIDP, 6-thioinosine diphosphate; 6-TTP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioguanine monophosphate; 6-MTGDP, 6-methylthioguanine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TXMP, 6-thioxanthosine monophosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGTP, 6-thioguanine triphosphate; 6-MTGMP, 6-methylthioinosine ribonucleotides (6-MMPR). 6-TGMP, 6-TGDP and 6-TGTP together form the 6-thioguaninenucleotides (6-TGN). Enzymes encoded by genes that are subject to known genetic polymorphisms are circled in gray. Adapted from Derijks et al.1
of the procedure was less than 10%, the typical recovery was 99%.

**TMPT genotyping** was performed using DNA extracted from whole blood using the Qiagen FlexiGene DNA kit (Qiagen, Venlo, The Netherlands) and the automated DNA isolation robot Autogenflex 3000 (Westburg, Leusden, The Netherlands). Exons 5, 7 and 10 of the TMPT gene and flanking intronic regions were screened by direct sequencing in order to detect the most prevalent Caucasian functional TPMT polymorphisms A80P (*2), A154T (*3A/B) and Y240C (*3B/C), although any other known or unknown variant in these exons would also be detected. Sequences were aligned with TPMT mRNA NCBI reference sequence NM_000367.

### 2.4. Statistical analysis

Normality was tested by the Kolmogorov–Smirnov test. Data are expressed as means with 95% confidence interval (CI 95%) or ranges. Pearson’s correlation test was used for correlations. The frequency distributions of the different variables between the patients with an exacerbation and a remission were compared by means of the likelihood ratio Chi-square test or, when expected counts were less than five, Fisher’s Exact test. The t-test for independent samples was used to compare continuous variables; the Mann–Whitney test was used when the assumption of a normal distribution did not hold.

P-values < 0.05 were considered statistically significant.

Power calculation was based on an analytical relevant 6-TGN level difference of 50 pmol/8×10^8 RBC and a 2 x standard deviation of 75 pmol/8×10^8 RBC, to exclude variation introduced by the HPLC method based on data of previous reports from our group. Based on these data 74 patients (at least 37 in each group) should be included to be able to demonstrate a significant difference in 6-TGN levels between both groups with a probability of 80%. A total of 100 patients (preferably 50 patients per study group) was aimed at. To find a therapeutic threshold 6-TGN level, separate analyses were performed. Receiver Operating Characteristic curves of the 6-TGN levels have been plotted to find a 6-TGN cut-off level with an optimal accuracy. At last, a quartile analysis was performed according to the original report of Dubinsky, to compare 6-TGN threshold levels.

### 2.5. Medical ethics

The study was approved by the local Medical Ethical Committee of the Maasland Hospital Sittard.

### 3. Results

#### 3.1. Patients

During 12 months, 123 IBD patients were asked to participate, see Fig. 2. A total of 100 patients were included for primary analysis, 41 patients with IBD exacerbation and 59 patients with IBD in remission. Patient characteristics are given in Table 1.

#### 3.2. Thiopurine metabolite levels

No significant differences were found in drug doses or thiopurine metabolite levels between patients with Crohn’s disease and ulcerative colitis. Therefore, in all further analyses Crohn’s disease and UC patients were analyzed as one group (IBD). Neither leukopenia, thrombocytopenia, disturbed liver enzymes nor elevated levels of serum amylase were observed in the IBD patients.

No significant differences were observed between IBD patients with exacerbation and IBD in remission concerning thiopurine dose, duration of thiopurine exposure, thiopurine metabolite levels, 6-MMPR/6-TGN ratio or TPMT activity. Data on thiopurine metabolites are given in Table 2.

The 6-TGN levels and TPMT activity were negatively correlated (r = -0.318, p = 0.01), although mean TPMT activity did not differ between the group with exacerbations and remissions.

Non-detectable levels were found in 9 of all included patients (9%). In 5 of 41 patients (12%) with an exacerbation and in 4 of 59 patients (7%) in remission no 6-TGN and 6-MMPR metabolite levels could be detected (Fig. 2). Very low levels were found in 1 of 41 patients (2%) with an exacerbation and 7 of 59 patients (12%) in remission.

Based on non-detectable and very low metabolite levels, the (suspected) non-compliance rate for all 100 patients was 17%. No significant difference was found in non-compliance between patients with an exacerbation and remission (15% versus 19%, Table 2). No correlations were found between 6-TGN levels and disease activity scores.

Based on the Receiver Operating Characteristic curve analysis the optimal 6-TGN cut-off level was 235 pmol/8×10^8 RBC with a positive predictive value of 52% for exacerbations with 6-TGN levels below 235 pmol/8×10^8 RBC and a negative predictive value of 70% with 6-TGN levels above 235 pmol/8×10^8 RBC in case of remissions (Table 3).

Twenty-six of the 41 patients with active disease (63%) had 6-TGN levels below the therapeutic threshold of 235 pmol/8×10^8 RBC, and 24 of the 59 patients in clinical remission (41%) had levels below this threshold (p = 0.04).

Regarding 6-TGN levels, 30% of the patients with 6-TGN levels above 235 pmol/8×10^8 RBC had an exacerbation versus 52% of the IBD patients with 6-TGN levels below 235 pmol/8×10^8 RBC (p = 0.04, Fig. 3). The odds ratio (OR) for having active disease in case of a 6-TGN level below 235 pmol/8×10^8 RBC was 2.5 (CI 95% 1.1–5.8) for all included patients.

Excluding all non-compliant patients in both groups, also showed no significant differences in median 6-TGN levels (230 pmol/8×10^8 RBC (0–934) versus 294 pmol/8×10^8 RBC (0–896) for the exacerbation and remission group, respectively; p = 0.15). The optimal threshold 6-TGN level appeared to be 235 pmol/8×10^8 RBC (Table 3). The OR for having active disease in case of a 6-TGN level below 235 pmol/8×10^8 RBC in the compliant patients was 3.8 (CI 95% 1.5–9.4).

#### 3.3. TPMT activity and genotype

High TPMT activity was found in 5 patients (5%, including one patient with an exacerbation): two of these patients (one
exacerbation) demonstrated zero levels and one patient very low thiopurine metabolite levels, indicating non-compliance. In the other two patients therapeutic metabolite levels were found.

Low TPMT activity was found in 2 patients (2%, 1 exacerbation). Both patients with low TPMT activity had high 6-TGN levels (712 and 934 pmol/8×10^8 RBC) and low 6-MMPR levels (both < 300 pmol/8×10^8 RBC), as expected. Low TPMT activity was not associated with myelotoxicity in this study.

TPMT genotyping revealed seven patients (7%) with heterozygote TPMT *1/*3A genotype. No homozygote mutant TPMT genotype was found in this cohort.

3.4. Concomitant IBD medication

No significant differences were found between both patient groups concerning the use of oral mesalazine, rectal mesalazine, infliximab, the combined use of mesalazine and infliximab or topical steroid treatment (Table 1). A significant difference for oral steroid treatment was found between both IBD patient groups: 24% of the patients with an exacerbation and 7% of the patients with quiescent disease (p=0.01) were using low-dose prednisolone.

4. Discussion

In this cross-sectional prospective study with IBD patients on thiopurine maintenance therapy we have shown that patients with 6-TGN levels above 235 pmol/8×10^8 RBC have a significantly higher chance of being in remission compared to IBD patients with 6-TGN levels below 235 pmol/8×10^8 RBC.

No significant differences were found in median 6-TGN or 6-MMPR metabolite levels between IBD patients with exacerbation and remission. Neither a correlation was found between 6-TGN levels, 6-MMPR/6-TGN ratio and disease activity. In this respect, our data are consistent with conclusions of other reports on TDM of thiopurines.\(^{12,16-18}\)

The defined therapeutic threshold 6-TGN level of 235 pmol/8×10^8 RBC had poor sensitivity, specificity and positive predictive value of maximum 72, 60 and 52% respectively, resulting in an accuracy of 67%. The therapeutic threshold level we defined is similar to the proposed 6-TGN threshold of 235 pmol/8×10^8 RBC in a study on pediatric IBD patients.\(^5\)

Employing an analysis with quartiles, according to Dubinsky et al.,\(^5\) revealed a corresponding therapeutic threshold 6-TGN level (Fig. 3). In that study median metabolite levels in IBD patients with exacerbations and remissions were significantly different (199 versus 312 pmol/8×10^8 RBC).\(^5\)

We found a significant difference in the percentage of IBD patients with exacerbations and remissions with 6-TGN levels above the threshold of 235 pmol/8×10^8 RBC: 37% of IBD patients with an exacerbation and 59% in remission. This finding is in accordance with the results of a recent meta-analysis performed by Osterman et al., describing 38% and 62% respectively.\(^{19}\)

The OR for being in clinical remission in case of 6-TGN levels above the threshold was 2.5 for all included IBD patients.
and is in agreement with the results of the meta-analysis, in which an OR of 3.3 was found.19

As expected, non-compliance will negatively influence clinical efficacy of pharmacotherapy and is associated with long-term maintenance therapy of chronic diseases, such as diabetes mellitus and hypertension. In the population of IBD patients we studied zero or very low thiopurine metabolite levels suggested non-compliance in 17% of all patients,

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of the patient population.</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>Exacerbation</td>
</tr>
<tr>
<td>Patient</td>
<td></td>
</tr>
<tr>
<td>Patient number: N</td>
<td>100</td>
</tr>
<tr>
<td>Mean age in years (range)</td>
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<tr>
<td>Male/female</td>
<td>60/40</td>
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<tr>
<td>Mean bodyweight (kg)</td>
<td>75.4</td>
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<tr>
<td>CDAl (mean)</td>
<td>157</td>
</tr>
<tr>
<td>CAI (mean)</td>
<td>6.8</td>
</tr>
</tbody>
</table>

| IBD | | | |
| Type | | | 1.0 |
| Crohn | 57 | 23 | 34 |
| Ulcerative colitis | 40 | 16 | 24 |
| Indeterminate colitis | 3 | 2 | 1 |

| Location | | | 0.99 |
| Proctosigmoid | 35 (35%) | 15 (37%) | 20 (34%) |
| Colon | 24 (24%) | 9 (22%) | 16 (27%) |
| Ileocolon | 23 (23%) | 11 (27%) | 12 (20%) |
| Terminal ileum | 12 (12%) | 5 (12%) | 7 (12%) |
| Perianal disease | 7 (7%) | 3 (7%) | 4 (7%) |
| Proximal Gl tract | 2 (2%) | 1 (2%) | 1 (2%) |

| Mean disease duration in months (range) | 98.7 (3–624) | 99.9 (3–432) | 97.9 (5–624) | 0.93 |

| Thiopurine therapy | | | |
| Azathioprine (N) | 67 | 33 (80%) | 34 (58%) | 0.02 |
| Mean AZA dose in mg (range) | 140 (50–225) | 136 (50–200) | 145 (50–225) | 0.33 |
| Mean AZA dose in mg/kg (range) | 1.8 (0.6–3.0) | 1.9 (0.8–2.5) | 1.8 (0.6–3.0) | 0.75 |
| 6-Mercaptopurine (N) | 33 | 8 (20%) | 25 (42%) | 0.02 |
| Mean 6-MP dose in mg (range) | 56 (50–100) | 56 (50–100) | 56 (50–100) | 0.96 |
| Mean 6-MP dose in mg/kg (range) | 0.8 (0.5–1.2) | 0.8 (0.6–1.2) | 0.8 (0.4–1.2) | 0.99 |
| Median thiopurine exposure in months (range) | 21.2 (3.0–314) | 19.4 (3.2–314) | 23.6 (3.0–120) | 0.425 |

| Concomitant IBD medication | | | |
| Oral mesalazine | 77 | 33 (80%) | 44 (75%) | 0.49 |
| Rectal mesalazine | 16 | 6 (15%) | 10 (17%) | 0.76 |
| Anti - TNF-α (i.e. infliximab) | 1 | 0 (0%) | 1 (2%) | 1.00 |
| Oral mesalazine + Anti-TNF-α (i.e. infliximab) | 7 | 3 (7%) | 4 (7%) | 1.00 |
| Oral steroids (i.e. prednisolone) | 14 | 10 (24%) | 4 (7%) | 0.01 |
| Topical steroids (oral/rectal) | 32 | 14 (34%) | 18 (31%) | 0.70 |

* P-value <0.05 is considered as statistically significant.

and is in agreement with the results of the meta-analysis, in which an OR of 3.3 was found.19

As expected, non-compliance will negatively influence clinical efficacy of pharmacotherapy and is associated with long-term maintenance therapy of chronic diseases, such as diabetes mellitus and hypertension. In the population of IBD patients we studied zero or very low thiopurine metabolite levels suggested non-compliance in 17% of all patients,

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Outcome measures of all included patients.</th>
</tr>
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<tbody>
<tr>
<td>Outcome measures</td>
<td>All patients</td>
</tr>
<tr>
<td>Median 6-TGN (pmol/8 × 10⁸ RBC); ranges*</td>
<td>235 (0–934)</td>
</tr>
<tr>
<td>Median 6-MMPR (pmol/8 × 10⁸ RBC); ranges*</td>
<td>645 (0–11,418)</td>
</tr>
<tr>
<td>Median 6-MMPR/6-TGN ratio</td>
<td>2.35 (0–63)</td>
</tr>
<tr>
<td>Number of non-compliant patients (%)</td>
<td>17 (17)</td>
</tr>
<tr>
<td>Mean TPMT activity (pmol/mg Hb/h)</td>
<td>7.0</td>
</tr>
</tbody>
</table>

N = number of patients; kg = kilograms bodyweight; mg = milligrams; 6-TGN = 6-thioguanine nucleotides level; 6-MMPR = 6-methylmercaptopurine ribonucleotides level; pmol = picomoles; RBC = red blood cells; TPMT = thiopurine S-methyltransferase; Hb = hemoglobin; h = hour. * Both 6-TGN and 6-MMPR levels were normally distributed, as given in the attached file next to table 1 of this proof. p-Value <0.05 is considered as statistically significant. n.s. = not significant.
emphasizing that clinicians should be always well aware that non-adherence also frequently occurs in IBD.\textsuperscript{26–28} Interestingly, exclusion of all non-compliant patients in the present study increased the OR to 3.8 for being in clinical remission for patients with 6-TGN levels above the therapeutic threshold level of 235 pmol/8×10\textsuperscript{8} RBC.

TDM is the only method to reveal non-compliance of thiopurine therapy, pointing to an important role for TDM in case of refractory IBD. Theoretically, zero or very low 6-TGN levels may result from other factors, such as thiopurine malabsorption or yet unknown enzyme defects or extremely high enzyme activity in the thiopurine metabolism pathway. Recently, a very high xanthine oxidase enzyme activity has been suggested to be the cause of zero 6-TGN and 6-MMPR levels in an individual who was treated with high-dose 6-MP.\textsuperscript{29} It should be noticed here that the thiopurine metabolic pathway is very complex and not yet fully discovered.\textsuperscript{1,29,30}

Low TPMT activity was observed in 7% of the IBD patients, which is relatively low, compared with results from previous reports.\textsuperscript{4,5,9,31–34} This discrepancy may be a result of the fact that all patients in our study were on thiopurine maintenance therapy for at least 3 months, so that patients with early toxicity due to TPMT polymorphism already discontinued therapy. An expected negative correlation between 6-TGN levels and TPMT activity was found ($r=−0.318$ and $p=0.01$) and the two patients with low TPMT activity both had zero 6-MMPR and high 6-TGN levels. This is in accordance with a recent publication by Kwan et al.\textsuperscript{34} High TPMT activity did not demonstrate higher 6-MMPR levels in our population ($n=5$), although two patients demonstrated zero levels.

Some remarks should be made about our study design and patient selection. First, we included patients visiting the outpatient department during regularly planned visits. All patients were on thiopurine maintenance therapy for at least 3 months, thereby excluding intolerant patients with early adverse events or toxicity. The delayed onset of therapeutic response on thiopurines was taken into account, as clinical efficacy occurs in general after 3–4 months after initiation of thiopurine therapy.\textsuperscript{35}

The obtained results are therefore only applicable to thiopurine tolerant IBD patients on maintenance therapy after at least 3 months. Second, patients with mild active Crohn’s disease were not included, in order to be able to clearly differentiate exacerbations from remissions. The power analysis requested the inclusion of a substantial number of patients with active disease in order to draw conclusions on thiopurine metabolite levels in relation to disease activity. Studies that have included many patients with intermediate disease activity were not able to draw any conclusions with respect to TDM.\textsuperscript{36,37} Third, the patient group was heterogenic with a broad spectrum of IBD phenotypes and two different disease activity scores have been used. Fourth, both AZA and 6-MP treated patients were included. AZA is a prodrug of 6-MP (Fig. 1). When using a conversion factor of 0.5, AZA dose is

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#### Table 3

<table>
<thead>
<tr>
<th>Cut-off 6-TGN level</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>p-value</th>
<th>Likelihood ratio</th>
<th>Odds ratio (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IBD patients (N=100)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>12</td>
<td>88</td>
<td>42</td>
<td>59</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0 (0.3–1.5)</td>
</tr>
<tr>
<td>150</td>
<td>24</td>
<td>78</td>
<td>43</td>
<td>59</td>
<td>0.8</td>
<td>1.1</td>
<td>1.1 (0.4–2.9)</td>
</tr>
<tr>
<td>200</td>
<td>39</td>
<td>69</td>
<td>47</td>
<td>62</td>
<td>0.4</td>
<td>1.3</td>
<td>1.5 (0.6–3.4)</td>
</tr>
<tr>
<td>235</td>
<td>63</td>
<td>59</td>
<td>52</td>
<td>70</td>
<td>0.04</td>
<td>1.6</td>
<td>2.3 (1.1–5.7)</td>
</tr>
<tr>
<td>250</td>
<td>71</td>
<td>54</td>
<td>52</td>
<td>73</td>
<td>0.02</td>
<td>1.5</td>
<td>2.9 (1.6–6.7)</td>
</tr>
<tr>
<td>300</td>
<td>83</td>
<td>37</td>
<td>48</td>
<td>76</td>
<td>0.04</td>
<td>1.3</td>
<td>2.9 (1.1–7.6)</td>
</tr>
<tr>
<td><strong>All compliant patients (N=83)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>98</td>
<td>0</td>
<td>57</td>
<td>1.0</td>
<td>0</td>
<td>0.02 (0.04–11.0)</td>
</tr>
<tr>
<td>150</td>
<td>14</td>
<td>94</td>
<td>63</td>
<td>59</td>
<td>0.3</td>
<td>2.2</td>
<td>2.4 (0.5–10.9)</td>
</tr>
<tr>
<td>200</td>
<td>31</td>
<td>85</td>
<td>61</td>
<td>62</td>
<td>0.1</td>
<td>2.1</td>
<td>2.6 (0.9–7.5)</td>
</tr>
<tr>
<td>235</td>
<td>58</td>
<td>73</td>
<td>62</td>
<td>70</td>
<td>0.007</td>
<td>2.2</td>
<td>3.8 (1.5–9.4)</td>
</tr>
<tr>
<td>250</td>
<td>67</td>
<td>67</td>
<td>60</td>
<td>73</td>
<td>0.004</td>
<td>2.0</td>
<td>4.0 (1.6–10.0)</td>
</tr>
<tr>
<td>300</td>
<td>81</td>
<td>46</td>
<td>53</td>
<td>76</td>
<td>0.02</td>
<td>1.5</td>
<td>3.5 (1.3–9.5)</td>
</tr>
<tr>
<td>350</td>
<td>83</td>
<td>27</td>
<td>46</td>
<td>68</td>
<td>0.3</td>
<td>1.1</td>
<td>1.9 (0.6–5.5)</td>
</tr>
</tbody>
</table>

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**Figure 3** Quartile analysis of 6-TGN levels. Exacerbation frequency of all patients is given for each 6-TGN level-quartile as performed by Dubinsky et al.\textsuperscript{5} Frequency (y-axis) was calculated by dividing the number of exacerbations by all patients in the specific 6-TGN level quartile. Thirty percent of all patients with 6-TGN levels $>235$ pmol/8×10\textsuperscript{8} RBC had an exacerbation versus 52% of the IBD patients with 6-TGN levels $<235$ pmol/8×10\textsuperscript{8} RBC ($p=0.04$).
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IBD treatment successfully. In 2 of 6 patients who were additionally treated with anti-TNF therapy to achieve clinical remission, zero levels were found.

These data were later retrospectively collected and show that in daily clinical practice apparently alternative therapies, such as systemic or topical steroids or expensive anti-TNF therapy, are preferred over dose-adjustment based on thiopurine metabolite levels. This again demonstrates that the therapeutic potential of thiopurines certainly has not been fully utilized.

In thiopurine optimizing strategies many confounding factors are involved, like the large inter- and intra-individual variation of thiopurine metabolism (also during therapy), the slow therapeutic onset of thiopurines, the natural relapsing and remitting character of IBD, thiopurine resistance among IBD patients and the fact that thiopurine metabolite levels in erythrocytes actually are a surrogate marker for the target cells, the leucocytes.1,44,45

Therefore, to clarify the role of TDM in thiopurine optimizing strategies there is a need for larger long-term, prospective, randomized studies which follow patients on thiopurine treatment directly from the start of therapy, examining failure of treatment, efficacy and toxicity at several time points, comparing conventional follow up with dose-optimization guided by TDM.

The clinical relevance of our study results is as follows: in an individual IBD patient with an exacerbation or therapy failure, measurement of 6-TGN metabolite levels should be undertaken to exclude non-compliance or underdosing. In case of non-compliance, the patient should be convinced to take thiopurines properly. When TDM shows 6-TGN levels <235 pmol/8×10^8 RBC and 6-MMPR ≪5700 pmol/8×10^8 RBC, an attempt to dose adjustment should be undertaken. When TDM shows 6-TGN levels <235 pmol/8×10^8 RBC and 6-MMPR ≫5700 pmol/8×10^8 RBC, a preferential 6-MMPR phenotype is revealed, possibly leading to poor response and hepatotoxicity. In these cases, the clinician may consider the addition of mesalazine or low-dose allopurinol (along with thiopurine dose reduction to 25–33% of the original dose) to shift thiopurine metabolism towards the active 6-TGN and optimize thiopurine efficacy. Also, a switch to the alternative thiopurine, 6-thioguanine, should be considered.44

We conclude that TDM of thiopurine metabolites in a random IBD population reveals that non-compliance occurs frequently. IBD patients with sub-therapeutic 6-TGN levels have a significant higher chance of having active disease, whereas IBD patients with therapeutic 6-TGN levels have a significant higher chance of being in remission. These data support the role of TDM in thiopurine maintenance therapy in IBD to reveal non-compliance and underdosing and can be used as a practical tool to optimize thiopurine therapy, especially in case of thiopurine non-response.

Contribution of the authors to the manuscript

LG was leading in the design of the study, collected data, performed the statistical analysis, and drafted a great part of the manuscript.

DW participated in the study design and coordination, collected thiopurine samples, carried out the thiopurine and data analysis and drafted a great part of the manuscript.
LE was leading in the study design, collected data and helped to draft the manuscript.

JB participated in the design of the study regarding TPMT enzyme analyses and took care of the TPMT enzyme activity analysis and helped to draft the manuscript.

JAB participated in the design of the study regarding TPMT enzyme analyses, took care of the TPMT enzyme activity analysis and helped to draft the manuscript.

AP participated in the design of the study regarding TPMT enzyme analyses and took care of the TPMT enzyme genotyping.

MR participated in the design of the study and collected data.

AS collected data and helped to draft the manuscript.

PB collected data and helped to draft the manuscript.

LB participated in the design of the study and collected data.

PH participated in the design of the study, helped to draft the manuscript and provided the main authors significant advice concerning intellectual content.

RS participated in designing the study, helped to draft the manuscript and provided the main authors significant advice concerning intellectual content.

CN helped to draft the manuscript and provided the main authors significant advice concerning intellectual content.

AM provided the main authors significant advice and revised it critically for important intellectual content.

Conflict of interest statement

All authors declare that they have no conflicts of interest.
The study participants do not have an investigator’s conflict of interest.

Acknowledgments

The authors thank A. Voogd, PhD, for statistical advice; J. van Spreeuwel, MD, PhD and E. Schoon, MD, PhD, gastroenterologists from the Catharina Hospital Eindhoven, for including patients; the laboratory technicians from the Pharmacological and Toxicological Laboratory of the Maasland Hospital, Sittard and the Laboratory of Biochemical Genetics, Department of Clinical Genetics of the University Hospital Maastricht for their skillful technical assistance.

No financial supports from sponsors are involved in this study.

All authors read and approved the final manuscript.

References


