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Novel assay to improve therapeutic drug monitoring of thiopurines in inflammatory bowel disease

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Abstract

Background and aims: The thiopurines are widely used in the treatment of inflammatory bowel disease, but are limited by poor dose–effect relationship. The objective was to assess the ability of a novel assay, determining the mono-, di-, and triphosphates, of thioguanine as well as methylthioinosine as individual metabolites in erythrocytes, to predict clinical outcome compared to a routine assay, determining metabolites as sums.
Methods: Samples from 79 patients with Crohn's disease or ulcerative colitis treated with azathioprine or mercaptopurine were analysed by both assays. Clinical status was determined by the Harvey–Bradshaw and Walmsley indices. The genotypes of thiopurine methyltransferase (TPMT) and inosine triphosphatase were determined.
Results: TPMT wild-type patients with thioguanine nucleotide (TGN) levels below the cut-off level were more likely to have active disease when TGN was measured by the novel assay (p = 0.02), and when thioguanosine triphosphate (TGTP) was measured separately (p = 0.01). When TGN was measured by the routine assay the correlation was not evident (p = 0.12). Neither TGN levels nor TGTP correlated to disease activity in TPMT deficient patients. Patients with methyl thioinosine

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nucleotide (meTIN) levels above 1500 pmol/8 × 10^8 RBCs were more likely to have active disease (p = 0.07). We observed good correlations between the mono-, di-, and triphosphates and their respective sums (R^2 > 0.88).

Conclusions: The novel TGN assay was better in predicting clinical outcome compared to the routine assay, while determination of TGTP had no clinical advantage and TGTP ratio was not correlated to disease activity.

1. Introduction

Thiopurines constitute the mainstay of immunosuppression in inflammatory bowel disease (IBD). There are three different thiopurines in clinical use today, azathioprine (AZA), mercaptopurine (6-MP), and thioguanine (6-TG). They are pro-drugs believed to be active through the formation of thioguanine nucleotides (TGN) and methyl-thioinosine nucleotides (meTIN).[1] The metabolism of the different thiopurines can be seen in Fig. 1. The complexity of thiopurine metabolism is illustrated by the fact that it is common that patients intolerant to one thiopurine can tolerate another.[2] The pharmacological explanation for this is not yet completely understood.

The pharmacokinetics of thiopurines not only is complex, but also shows extensive inter-individual variability. One cause of variability is the polymorphism of thiopurine methyltransferase (TPMT).[3] However, even when taking TPMT activity into consideration, inter-patient variability is high. In theory, concentration measurements of active metabolites could be used to tackle this variability but in reality a definite place for therapeutic drug monitoring (TDM) of thiopurines has been difficult to establish, possibly for methodological reasons.

Two assays are commonly used in the TDM of thiopurines,[4,5] both measuring the hydrolysis products of TGN and meTIN in red blood cells (RBCs). In these assays, the nucleotides are hydrolysed back to nucleic bases and then analysed by liquid chromatography. These assays cannot distinguish between nucleotides, ribosides, and their deoxy analogues,[6] and co-determine these as sums. Thus, information about the metabolite distribution is lost. These assays are used for practical reasons, as nucleic bases are easier to analyse than nucleotides and RBCs are by far more abundant than the white
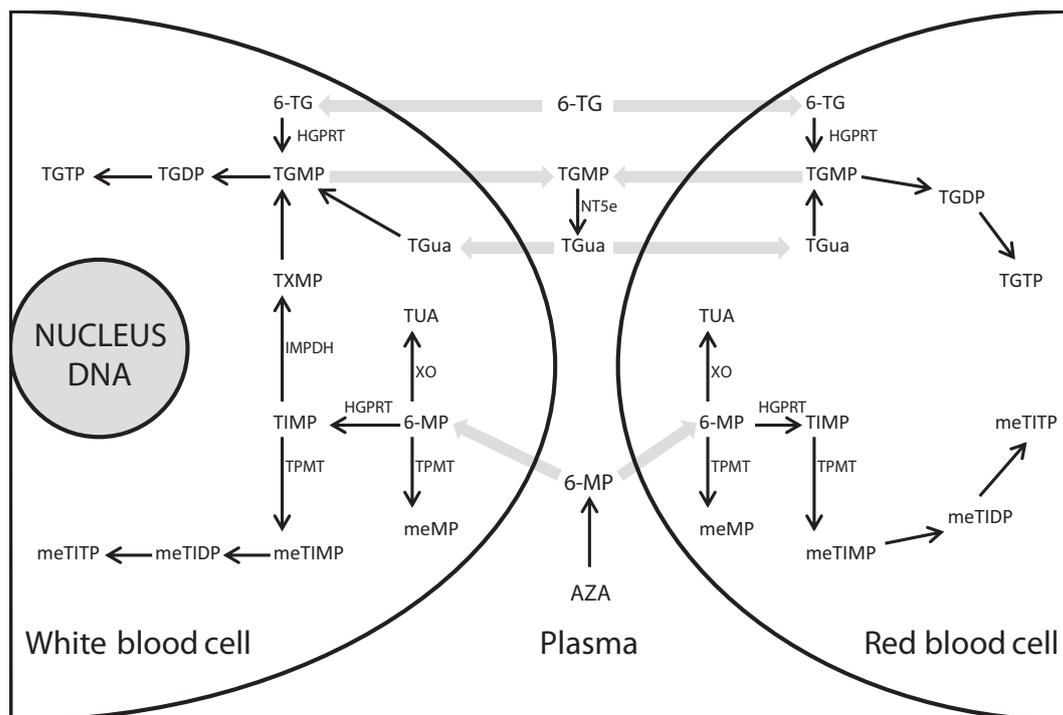
Figure 1   Simplified scheme of thiopurine metabolism. 6-MP, 6-mercaptopurine; 6-TG, thioguanine; ABCC4, multidrug resistance-associated protein 4, HGPRT, hypoxanthine-guanine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; meMP, methylmercaptopurine; meTIDP, methylthioinosine diphosphate; meTIMP, methylthioinosine monophosphate; meTITP, methyl-thioinosine triphosphate; NT5e, ecto-5-nucleotidase; TGDP, thioguanosine diphosphate; TGMP, thioguanosine monophosphate; TGTP, thioguanosine triphosphate; TIMP, thioinosine monophosphate; TGua, thioguanosine; TPMT, thiopurine methyltransferase, TUA, thiouric acid; TXMP, thioxanthine monophosphate; XO, xanthine oxidase.

blood cells (WBCs), containing the active sites of thiopurine therapy. However, interpretation relies on the assumptions (1) that the relative distribution of mono-, di-, and triphosphates is similar in all patients, and (2) that concentrations in the RBCs correlate well with concentrations at the active sites. Other proposed reasons for a lack of success in thiopurine TDM are the low precision of some of the assays used, and the possibility that metabolites other than the TGN, such as meTIN, are important for the effect of thiopurine treatment as well as the adverse events.[6] In either case, this calls for new methodology in the thiopurine TDM setting.

Measuring the different nucleotides, especially the triphosphates, separately as well as measuring in a different matrix such as WBCs or bases incorporated in the DNA have been suggested as alternative possible strategies.[7–9] A few novel assays have been published, especially in the area of measuring the triphosphates separately.[9–12] We recently presented a novel assay capable of measuring the mono-, di-, and triphosphates of TGN and meTIN separately.[13] Especially measuring the triphosphates separately is of interest as they are the end metabolites used for incorporation in RNA and DNA.

The first aim of this study was to investigate the ability of our novel assay to predict clinical outcome during thiopurine treatment in IBD. Secondary aims included (i) an estimate of threshold concentrations for the different metabolites as well as (ii) an investigation of the relative distribution of thiopurine nucleotides in RBCs.

2. Materials and methods

This was a cross-sectional study involving gastroenterological outpatients at Linköping and Lund University hospitals. Inclusion criteria were (i) established Crohn's disease (CD) or ulcerative colitis (UC) according to conventional criteria and (ii) ongoing therapy with AZA or 6-MP on a stable dose for at least eight weeks. To estimate 6-MP equivalent doses, AZA doses were divided by 2.08, accounting for differences in molecular weight (277.3, and 152.2 for AZA and 6-MP, respectively), as well as 88% efficiency in the conversion of AZA into 6-MP.[14]

A case record form (CRF) was completed by one experienced IBD-clinician at each site, containing information about sex, age, disease history, treatment history and disease activity. Disease activity was assessed at patient visits before any assay results were available. We used the Harvey–Bradshaw (HBI)[15] and Walmsley[16] indices, for CD and UC, respectively, to provide a score of activity. Remission was defined as a score less than 5 points.

For metabolite measurements and genetic analysis two EDTA-tubes were drawn from an antecubital vein. One tube was used for the routine assay, TPMT phenotyping, and to extract DNA for genotyping and the second tube was used for the novel assay. The routine assay measured TGN and meTIN in RBCs as sums, while the novel assay measured them separately.

The routine assay has previously been described[12] and measured TGN as 6-TG and meTIN as AMTCI.[17] Briefly, washed and frozen RBCs were diluted and the nucleotides hydrolysed back to nucleic bases using sulphuric acid. Adducts were formed by the addition of phenyl mercury acetate and extracted into toluene. The pH was lowered

using hydrochloric acid, breaking the adducts and extracting the bases back into the aqueous phase. Analytes were separated by liquid chromatography, using triethylamine as an ion-pairing agent, and detected by UV absorption.

The novel assay[13] measured thioguanosine monophosphate (TGMP), thioguanosine diphosphate (TGDP), TGTP, as well as methylthioinosine monophosphate (meTIMP), diphosphate (meTIDP), and triphosphate (meTITP), separately in RBCs. Briefly, RBCs washed and frozen within 6 h of sampling were precipitated with methanol and dichloromethane followed by derivatisation with potassium permanganate and hydrogen peroxide. Analytes were separated by liquid chromatography, using tetrabutyl ammonium as an ion-pairing agent, and detected by UV absorption and fluorescence.

Genotyping for TPMT *2, *3A, *3B, *3C as well as inosine triphosphatase (ITPA) 94C>A was carried out according to previously published methods.[18,19] TPMT activity in RBC was measured according to a previously published radiochemical assay.[20,21]

Informed consent was collected from all subjects and the study was approved by the local ethics committee (Dnr 01-016).

2.1. Statistics

The correlation between treatment response and metabolite levels was investigated by dividing the material into quartiles based on the metabolite concentrations (quartile analysis). The percentage of active disease was then calculated for each quartile. Furthermore, receiver operated characteristic (ROC) curves were constructed by plotting the rate of true positives (sensitivity) against the rate of false positives (1 − specificity) at different cut-off values in order to define threshold concentrations for the metabolites to predict active disease. Treatment response was also investigated by Fisher's exact test (two-sided) based on these threshold concentrations. Correlation between metabolite levels and thiopurine dose was investigated using scatter plots and linear regression. Differences in mean concentrations were tested by Student's t-test (two-sided, equal variance).

3. Results

Characteristics of the 79 patients are shown in Table 1. The only statistically significant difference between the patients in remission and with active disease was that biological treatment and steroids were more common among the patients with active disease (p < 0.05). No differences were observed between patients with CD and UC, nor between patients sampled in Lund and Linköping, with regard to all findings reported below.

The genotype of TPMT was determined in all patients. 74 patients had wild type TPMT activity (*1/*1, five identified by wild type activity when lacking genotyping data), four patients had intermediate TPMT activity (*1/*3A) and one patient had low TPMT activity (*3A/*3C). 59 patients were genotyped for ITPA 94C>A, while suitable material was not available in the remaining 20. Of these, 42 patients had the C/C genotype, 16 had the C/A genotype and one had the A/A genotype.

TGN concentrations with the novel assay ranged from 24 to 580 pmol/30 mg Hb (median 104), and meTIN concentrations

Table 1 Characteristics of the 79 patients included in the study. Median, minimum and maximum values are given. 6-MP, 6-mercaptopurine; BW, body weight; Hb, haemoglobin; TGN, thioguanine nucleotides.

	Remission	Active
n	55	24
% male	56% (31/55)	50% (12/24)
Age (median)	38 (19–79)	33 (18–73)
% Crohn's disease	47% (26/55)	67% (16/24)
% ulcerative colitis	53% (29/55)	33% (8/24)
% azathioprine	82% (45/55)	79% (19/24)
% 6-mercaptopurine	18% (10/55)	21% (5/24)
Dose (mg 6-MP eq/kg BW, median)	0.91 (0.29–1.50) [a]	0.96 (0.05–1.39)
TGN (pmol/30 mg Hb, median)	105 (24–355)	93 (42–575)
Years after diagnosis (median)	8 (1–43)	8 (1–31) [b]
Concomitant medication		
Biologicals [c]	2% (1/55)	17% (4/24)
Steroids	7% (4/55)	25% (6/24)
Other immunomodulators	0% (0/55)	0% (0/24)
Nutritional support	0% (0/54)	0% (0/24)
HBI score (Crohn's disease only) [c]	2 (0–4)	9 (5–20)
Walmsley score (ulcerative colitis only) [c]	2 (0–4)	10 (5–19)

[a] n = 54.
[b] n = 23.
[c] p < 0.05.

from below 100 up to 13,000 pmol/30 mg Hb (median 1870). The relative fractions of mono-, di-, and triphosphates were calculated, Fig. 2. Monophosphates were more abundant for meTIN than for TGN, while the fraction of nucleotides found as diphosphates was smaller for meTIN than for TGN. Good correlations between the different mono-, di-, and triphosphates and their respective sums were observed (all $R^2 > 0.88$), Fig. 3. Correlations between the novel and the routine assay are presented in Fig. 4. Neither TGN nor meTIN concentrations correlated with the thiopurine dose,
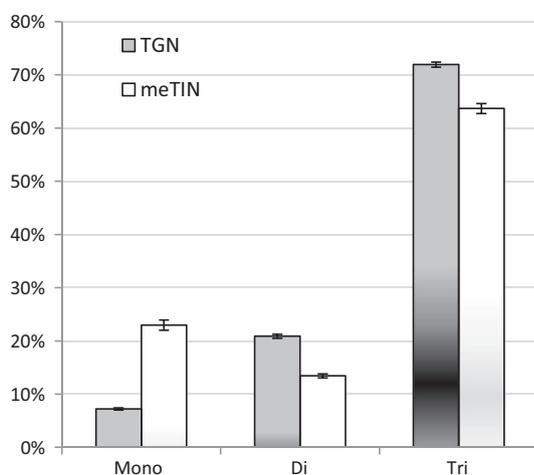

Figure 2 Relative fractions of mono-, di-, and triphosphates. Relative fractions of mono-, di-, and triphosphates for TGN and meTIN in samples where all three nucleotides for either TGN (n = 79) or meTIN (n = 62) were quantifiable. Error bars represent standard error of the mean. meTIN, methylthioinosine nucleotides; TGN, thioguanine nucleotides.

determined as 6-MP equivalent does (see Materials and methods above).

The proportion of patients with active disease in quartiles based on TGN, TGTP, or TGN as measured by the routine assay (R-TGN), in TPMT wild type patients, as well as an ROC-curve for active disease as a function of TGN (novel assay), is presented in Fig. 5. ROC-curves for TGTP and R-TGN were similar to that of TGN (data not shown). The proportion of patients with active disease was higher below threshold levels, based on ROC analysis, for the novel TGN assay and the TGTP metabolite, but not for the routine TGN assay (Table 2). All TPMT-deficient (intermediate or low activity) patients (n = 5) had TGN levels above 120 pmol/30 mg Hb and 60% (3/5) had active disease.

TPMT wild type patients with meTIN levels above 1500 pmol/30 mg Hb were more likely to have active disease than those below (36%, 16/44, versus 17%, 5/30, p = 0.07). 14 (18%) patients had a skewed metabolism with a ratio between R-meTIN and R-TGN above 20 in conjunction with an R-meTIN level above 5000 pmol/8 × 10^8 RBCs and an R-TGN level below 250 pmol/8 × 10^8 RBCs.[22] Eight (57%) of these had active disease.

The TGTP ratio (mean 77.8%, standard deviation 3.8%, n = 74) defined as TGTP/(TGDP + TGTP) was evaluated in TPMT wild type patients. No difference in TGTP ratio was observed between patients with active and quiescent diseases, nor between patients with high (≥80 pmol/30 mg Hb) and low (<80 pmol/30 mg Hb) TGN levels according to the novel assay. Lower TGTP ratios were observed in TPMT wild-type patients with the ITPA 94C>A genotype than in patients with the C/C genotype (76.5%, n = 15, compared to 78.5%, n = 40, p = 0.02). This was also true for the corresponding ratio for meTITP (81.2%, n = 13, compared to 84.0%, n = 35, p = 0.02).
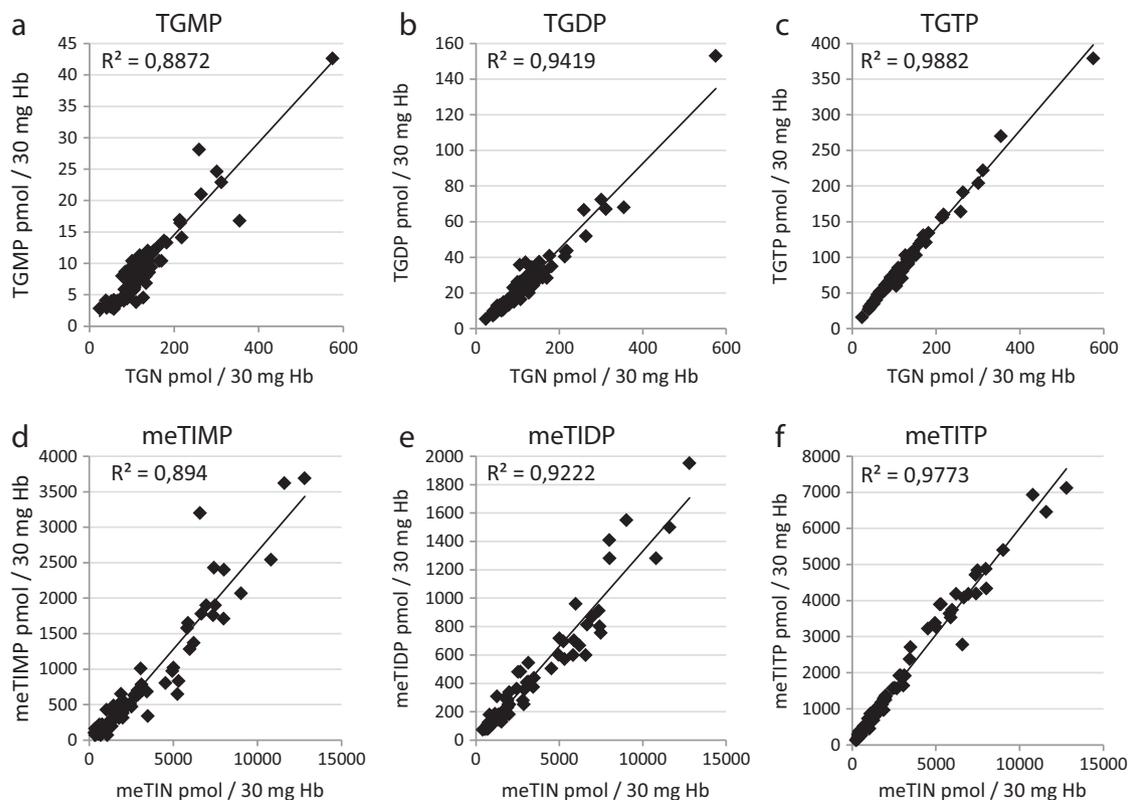
Figure 3 Correlation between individual nucleotides and their respective sums using the novel assay. TGMP (a), TGDP (b), and TGTP (c) correlated to TGN, and meTIMP (d), meTIDP (e), and meTITP (f) correlated to meTIN. meTIDP, methylthioinosine diphosphate; meTIMP, methylthioinosine monophosphate; meTIN, methylthioinosine nucleotides; meTITP, methylthioinosine triphosphate; TGDP, thioguanosine diphosphate; TGMP, thioguanosine monophosphate; TGN, thioguanine nucleotides; TGTP, thioguanosine triphosphate.

4. Discussion

The aim of this cross-sectional study was to investigate the value of TDM in patients with IBD using an assay capable of determining phosphorylated and methylated metabolites individually. One limitation of the cross-sectional design is that the natural disease course may vary over time. However, the way thiopurine metabolite measurements are used in clinical routine is often as a single measurement to define if the patient lies within 'therapeutic ranges' and/or has potentially toxic metabolite levels. If the values obtained are within the therapeutic range, most often no follow-up
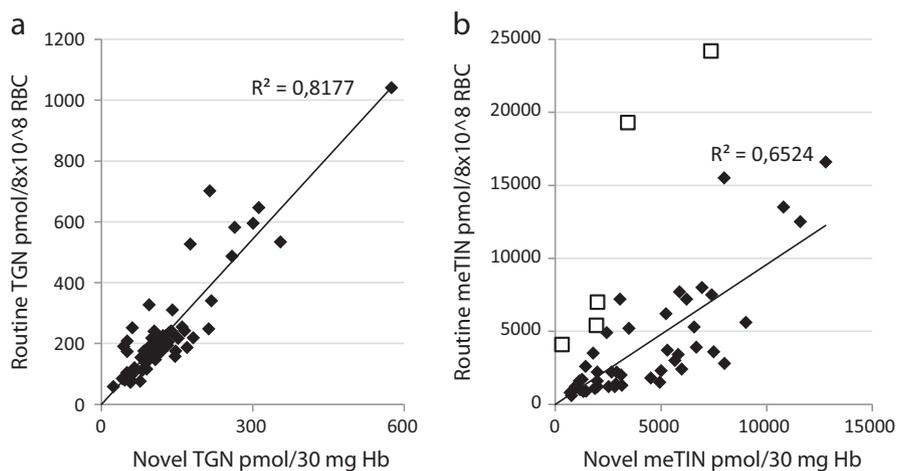
Figure 4 Correlation between routine and novel assays for TGN (a) and meTIN (b). Unfilled squares represent samples were the ratio between the routine and novel assays were more than 3-fold higher than the median ratio of all samples and were not used to calculate R^2. Hb, haemoglobin; meTIN, methylthioinosine nucleotides; RBCs, red blood cells; TGN, thioguanine nucleotides.
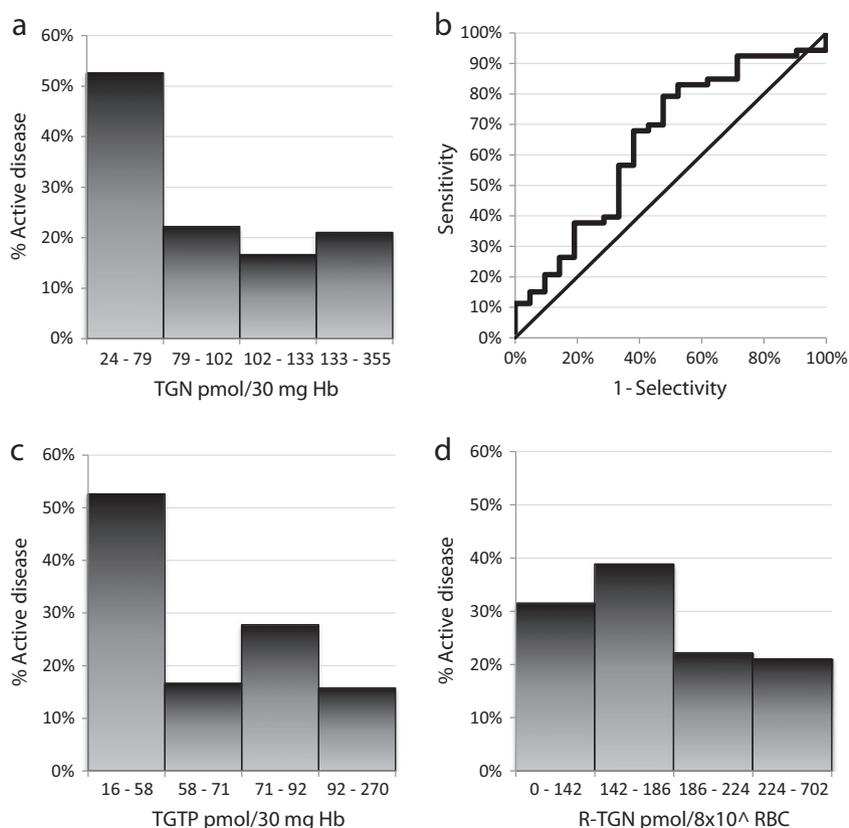
Figure 5 The proportion of patients with active disease in quartiles. The proportion of patients with active disease in quartiles based on TGN (a), TGTP (c), or R-TGN (d) in TPMT wild type patients (n = 74), and an ROC-curve for active disease as a function of TGN determined with the novel method (AUC = 0.65) (b). Hb, haemoglobin; RBCs, red blood cells; R-TGN, thioguanine nucleotides as measured by the routine assay; TGN, thioguanine nucleotides; TGTP, thioguanosine triphosphate.

measurements are done. We therefore consider that our study design represents a 'real life' scenario.

In this study, both high TGN levels and high TGTP levels measured with a novel assay were associated with disease remission. In fact, using the novel assay TGTP measured separately and TGN measured as a sum provided similar results. In contrast, high TGN levels according to the routine assay were not associated with disease remission. This fact illustrates the choice of assay used for TDM monitoring and more specifically the accuracy of the assay used. Due to the strong correlation between TGN and TGTP concentrations, measuring the latter separately provided no clinical benefits. As it is technically more demanding and costly to

measure TGTP separately, our results defer a role for measuring TGTP in the monitoring of thiopurine effects in an IBD population.

The correlation between TGN measurements with the novel and the routine assays (Fig. 4) was in line with what could be expected based on previously reported assay inter-batch imprecisions, below 10%[13] and 20%,[12] for the novel and the routine assays, respectively. The difference in accuracy between the assays was attributed to differences in the calibration procedure, recovery, and standardization unit (Hb vs RBC counts). TGTP or TGN measured by the novel assay predicted remission equally well while the correlation to remission using the routine assay was not significant. This

Table 2 Percentage of active disease above and below cut-off concentrations for TGN, TGTP, and R-TGN in TPMT wild type patients (n = 76). Hb, haemoglobin; RBCs, red blood cells; R-TGN, thioguanine nucleotides as measured by the routine assay; TGN, thioguanine nucleotides; TGTP, thioguanosine triphosphate.

Analyte	Cut-off pmol/30 mg Hb	Percentage active disease		Prediction of remission		
		Below cut-off	Above cut-off	Sensitivity	Selectivity	p-Value
TGN	80	53% (10/19)	20% (11/55)	0.83	0.48	0.02
TGTP	64	48% (12/25)	18% (9/49)	0.75	0.57	0.01
R-TGN	175.5 [a]	38% (13/34)	20% (8/40)	0.60	0.62	0.12

[a] pmol/8 × 10^8 RBCs.

was probably an effect caused by the improved accuracy of the novel assay. 14% of all measurements by the novel assay (CV 10%) were within one standard deviation from the cut-off, compared to 47% for the routine assay (CV 20%). In other words, due to measurement uncertainty in the routine assay we cannot determine if the true concentration is below or above the cut-off for around half of the samples, reducing our ability to predict remission. The difference in imprecision between the assays might be due to a variable recovery in the conversion of the TGN into free TG, a step present in the routine assay but not in the novel assay.

We found that the TGTP ratio could not predict the clinical outcome and this was due to the high correlations between mono-, di- and triphosphates and their respective sums. Our findings are in contrast to the findings by Neurath et al.,[9] who reported that the TGTP ratio was predictive of response to thiopurine therapy. Direct comparisons of TGN values or TGTP ratios in our study and in theirs are not possible as different assays with assay specific results were used. The correlation between TGTP and TGN in our study was above 0.98 and it is likely that a majority of the variability still observed is due to assay imprecision rather than biological differences. The correlation was much higher than in the study by Neurath et al.[9] who also reported larger variations in observed TGTP ratios. This difference might be explained by more stringent sample handling in our study and the higher precision of the novel assay. A stringent sample handling is absolutely necessary due to the limited stability of thiopurine metabolites in blood.[13,23]

More TPMT deficient patients had TGN levels above 80 pmol/30 mg Hb even in active disease compared to patients with normal TPMT-activity. One possible explanation could be the lower meTIN levels observed in TPMT deficient patients and that these patients thus need higher TGN levels to achieve remission. As the proposed cut-off levels for TGN, associated with clinical remission, were based on patients with normal TPMT-activity they might not be applicable to TPMT-deficient patients.

The higher meTIN levels observed in patients with active disease are explained by skewed metabolism, since 33% of the patients with active disease had metabolite measurements consistent with this phenomenon.[22] A skewed metabolism is caused by preferential metabolism to meTIN with frequently low and possibly sub-therapeutic TGN levels. This phenomenon is clinically associated with lack of response as well as hepato- and myelotoxicity.[24]

The distribution of mono-, di-, and triphosphates differed between TGN and meTIN. While, 24% of meTIN was found as meTIMP, only 7% of TGN was found as TGMP, indicating a difference in the mechanism of regulation between these two phosphate compounds. One enzyme that could account for the difference is ITPA, an enzyme converting ITP into IMP. However, it is unlikely that ITPA is responsible for observed differences as the effects of the C/C and C/A genotypes on the TGTP and meTITP ratios were similar.

4.1. Conclusions

TGN levels in RBC, as measured by the novel assay, were predictive of clinical remission in TPMT wild type patients while the correlation between TGN measured by the routine assay and remission was not significant. These findings could not be generalised to TPMT deficient patients.

No correlation between TGTP ratio and active disease was observed, and TGTP measured separately did not offer better prediction of remission than TGN measured as a sum. We conclude that measuring the mono-, di-, and triphosphates separately is not beneficial in predicting remission, mainly due to the strong correlations between individual phosphates and the TGN and meTIN sums.

We still need more data in order to develop more useful guidelines for thiopurine monitoring than those available today. In such studies assays measuring the TGN as a sum could be used.

Conflict of interest

None.

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