Thiopurine methyltransferase and thiopurine metabolite testing in patients with inflammatory bowel disease who are taking thiopurine drugs

Thiopurine methyltransferase genotyping and thiopurine metabolite testing has been established as an adjunct to monitoring patients taking thiopurine drugs. This special report describes the clinical implications for this type of testing for patients with inflammatory bowel disease who are taking thiopurine drugs. A total of 10% of patients were found to be intermediate metabolizers and the mean dosage (in mg/kg equivalent) was lower in intermediate metabolizers than extensive metabolizers. The metabolite levels did not correlate with scores measuring clinical severity but levels of 6-methylmercaptopurine were related to the dosage of the drugs. Despite considerable study of thiopurine methyltransferase testing in the literature, it is still not widely used in many geographical areas. This study adds to the evidence about using such testing as well as expanding the role of simultaneously measuring thiopurine metabolites. Further work is planned to evaluate the uptake when such testing becomes available locally as a clinical service.

KEYWORDS: 6-mercaptopurine ■ 6-methylmercaptopurine ■ 6-thioguanine nucleotides ■ azathioprine ■ dosage ■ extensive metabolizer ■ intermediate metabolizer ■ thiopurine drugs ■ thiopurine methyltransferase testing ■ TPMT

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The thiopurine drugs, azathioprine and 6-mercaptopurine, are widely used as a treatment for inflammatory bowel disease (IBD), as well as childhood acute lymphoblastic leukemia, rheumatic diseases and dermatology [1–4]. Azathioprine is converted to 6-mercaptopurine and in a series of steps and eventually metabolized to 6-thioguanine nucleotides (6-TGNs), the active metabolites, which are toxic to the cells. A schema of the pathway is shown in Figure 1. Normally only a small percentage of the parent drug results in the production of 6-TGNs because the majority is broken down through this pathway, especially by the enzyme thiopurine methyltransferase (TPMT) [2,3]. Heterozygotes and homozygotes of TPMT deficiency have an increased risk of myelotoxicity and consequently require a lower dosage [2,3,4]. Heterozygotes are classified as intermediate metabolizers (IMs) [1,3,5]. Measurement of TPMT in red blood cells and/or genetic testing for the common genetic deficient variants in *TPMT* has become a method of monitoring and guiding dosage for the prescription of these drugs in many clinical settings, but is not yet universally used [6,7]. More recently it has been shown that measuring 6-TGN directly, and also another metabolite, 6-methylmercaptopurine (6-MMP), may be an aid in determining dosage or identifying potential toxicity [1,2,8].

Whilst the use of TPMT testing for patients on thiopurine drugs is well established in some locations, it is still not widely used in Europe and Australasia [6,7]. A survey carried out by one of the authors in a Melbourne teaching hospital showed that TPMT testing was only carried out in 6% of cases prescribed thiopurine drugs across all departments in the hospital over a 2 year period [9]. At that time there was no local testing for TPMT and the specimen had to be sent interstate for biochemical testing (phenotyping). Both a local and a European study have shown that there are many reasons why doctors do not request pharmacogenomic testing, including concern regarding who pays for the test, lack of knowledge of how to order it and the lack of easily available sources regarding interpretation [6,10]. As a consequence of no local testing services being available it was decided to establish a TPMT metabolite testing service, which was possible using local resources and was judged to be more feasible than a TPMT biochemical assay. It was also decided to link this with a *TPMT* genotyping service using an experienced molecular genetic diagnostic laboratory. This new combined approach was established in order to provide additional information for clinicians, such as identifying TPMT heterozygotes and using a metabolite assay to monitor dose adjustments. This study

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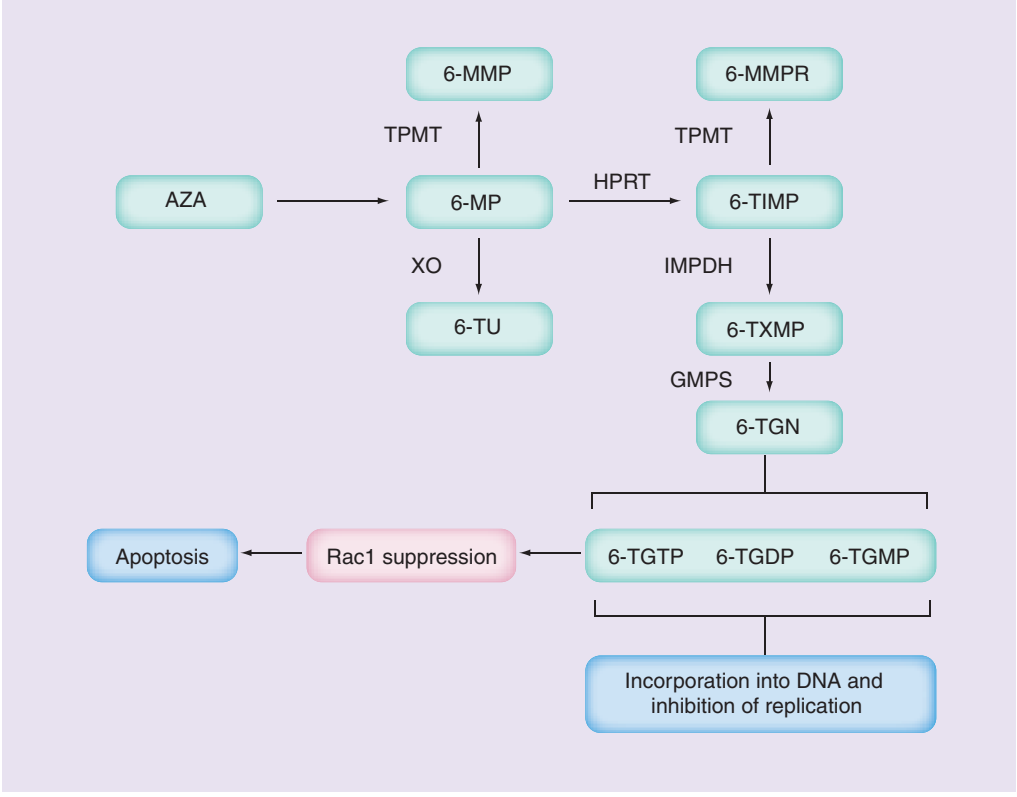

Figure 1. Metabolism of AZA and 6-MMP. 6-MMP: 6-methylmercaptopurine; 6-MMPR: 6-methylmercaptopurine ribonucleotides; 6-MP: 6-mercaptopurine; 6-TGDP: 6-thioguanine di-phosphate; 6-TGMP: 6-thioguanine monophosphate; 6-TGN: 6-thioguanine nucleotide; 6-TGTP: 6-thioguanine tri-phosphate; 6-TIMP: 6-thiosine 5′-monophosphate; 6-TU: 6-thiouric acid; 6-TXMP: 6-thioxanthosine monophosphate; AZA: Azathioprine; GMPS: Guanosine monophosphate synthetase; HPRT: Hypoxanthine phosphoribosyltransferase; IMPDH: Inosine monophosphate dehydrogenase; TPMT: Thiopurine methyltransferase; XO: Xanthine oxidase.

describes the process of using these new local tests and documents the results in the first 132 cases.

Materials & methods

Patients

Patients were recruited from patients attending IBD or gastroenterology clinics of three metropolitan teaching hospitals in Melbourne, Australia. Patients aged between 18–80 years were eligible for inclusion if they were currently taking a thiopurine drug and had been on therapy for at least 3 months and were on a stable dose for at least 4 weeks. The research protocol was passed by the local institutional ethics committee. Details of clinical variables relating to general heath, and the drug taken and the dosage were collected. The azathioprine dose was calculated as mg/kg and for patients on 6-mercaptopurine the dose was converted to azathioprine equivalents by multiplying by 2.08 [11]. The Harvey Bradshaw Index (HBI) was computed from clinical symptoms for patients with Crohn's disease and the Simple Clinical Colitis Activity Index (SCCAI) was computed for patients with ulcerative colitis [12,13]. Scores of HBI $\leqslant$ 4 and SCCAI $\leqslant$ 2 were taken as evidence of clinical remission. Blood was taken for genotyping common *TPMT* variants and measuring the metabolites 6-TGN and 6-MMP. Results were issued with interpretation and clinical guidance for the clinicians. Tests done as part of clinical care were recorded, such as alanine transaminase (ALT) albumen levels, hemoglobin, white blood and lymphocyte count. Because of the increased frequency of *TPMT*3C* in Southeast Asians the ethnic group of each patient was recorded as Caucasian, Southeast Asian or other.

Genetic testing

A single base extension multiplex reaction was developed using an ABI 3130 Genetic Analyzer (Applied Biosystems, CA, USA) to identify *TPMT*2*, **3A* and **3C* alleles. This is expected to detect approximately 80–95% of deficient alleles [3]. Those patients without a demonstrated mutation were classified as extensive metabolizers

(EMs) (normal). Those with one deficient allele were classified as IMs as they correspond with intermediate red blood cell TPMT activity [3].

Phenotyping

Patients who were found to be heterozygotes for *TPMT* deficient alleles were batched and assayed for red blood cell TPMT levels. TPMT levels were assayed as previously described by a radiochemical method [14]. The purpose of phenotyping these samples was to ensure that heterozygotes were in fact not TPMT-deficient due to a nontested allele. Those with 6-TGN levels greater than 450pmol/8 × 10^8 red blood cells were similarly assayed for similar reasons. Those with raised 6-MMP values greater than 7500 were also assayed as an exploration of all reasons for the high metabolite result. Our samples were prepared by the protocol used by the phenotyping laboratory but then frozen and assayed as a batch. This is different from the usual protocol used by this laboratory.

Metabolites

6-thioguanine nucleotide levels and 6-MMP levels were assayed by a previously described method using HPLC method [2]. Each patient was tested for both of these metabolites. The red blood cells were washed in saline and counted to normalize at 8 × 10^8 red blood cells. The reference target range used for 6-TGN was 235–450 pmol/8 × 10^8 red blood cells and was less than 7500 pmol/8 × 10^8 red blood cells for 6-MMP.

Statistical analysis

The data was entered into a spreadsheet and checked for inconsistencies. The data was analyzed using Stata version 9 [15]. The primary outcome being investigated statistically was the average dosage that the heterozygotes were stabilized on. Correlations were looked at by the method of multiple linear regression where the outcome variable was logarithm (ln) transformed, the transformation being necessary to make the outcome variable normally distributed. Other assumptions of the linear regression model that were tested were the normal deviation of residuals and random scatter of residuals in a residual versus fitted plot. The primary hypothesis regarding dosage differences between heterozygotes and normal homozygotes was tested for the significance level set at 0.05.

An additional 10 possible statistical associations were examined and so a Bonferroni correction was used to correct for multiple testing. Starting with a type 1 error rate of 0.05, the Bonferroni correction suggested that the new significance level to take should be 0.005. This approach is quite a conservative one [16].

Results

There were 13 heterozygotes out of 132 patients (9.8%), matching the expected 10%. Table 1 shows the distribution of 6-TGN and 6-MMP levels in IMs and EMs. The mean 6-TGN levels were higher in IM (heterozygotes) than EM (normal homozygotes) and these reached the adjusted statistically significant levels (p = 0.002). Figure 2 shows the distribution of 6-TGN and metabolizer status and Figure 3 shows the 6-MMP levels. Table 1 shows the mean 6-MMP is lower in IMs than normal, the difference being statistically significant (p = 0.001). However this is probably explained by all eight high 6-MMP values being found in the normal homozygotes.

The dosage expressed in mg/kg dose equivalents was 1.58 mg/kg in IM versus the 2.02 mg/kg in EMs shown in Table 2. This difference was judged to be statistically significant as it was the primary hypothesis of the study.

Dosage differences were found when the patients were categorized for being in the reference range for 6-MMP or above this range (Table 3). The mean dosage of those with values in the reference range was 1.89 mg/kg compared with those above the reference range who had had a near-double dose in mg/kg equivalent (Table 3). The dosage difference for 6-TGN was also numerically different

Table 1. Levels of 6-TGN and 6-MMP in intermediate and extensive metabolizers (pmol/8 × 10^8 red blood cells).

Group	Mean 6-TGN (median)	Mean 6-MMP (median)	Numbers
Intermediate metabolizers (heterozygotes)	624.9 (543)	384.8 (359)	13
Extensive metabolizers	341.5 (262)	2025 (1105)	113

Logarithm transformed values for 6-TGN and 6-MMP were used to calculate the differences between intermediate metabolizers and extensive metabolizers. For both 6-TGN and 6-MMP the difference between the means was statistically significant. For mean 6-TGN, t = 3.1 (p = 0.002) and for mean 6-MMP, t = -3.41 (p = 0.001). The statistical significance level reached the level corrected for multiple testing both for 6-TGN and 6-MMP.
6-MMP: 6-methylmercaptopurine; 6-TGN: 6-thioguanine nucleotide.

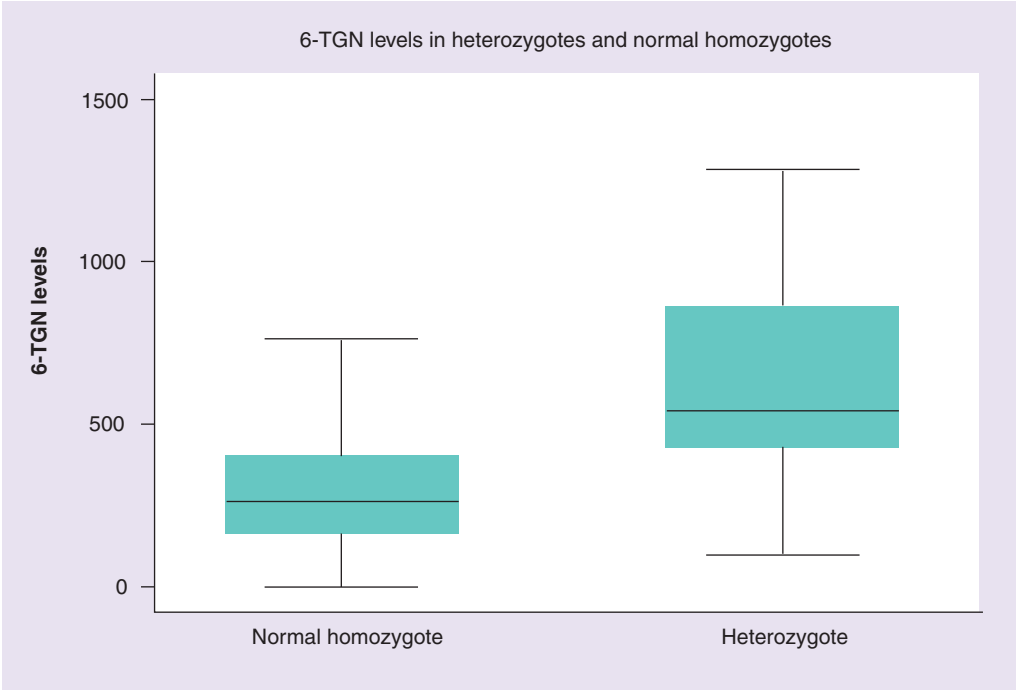

Figure 2. Box whisker plot showing the distribution of the actual 6-thioguanine nucleotide levels in pmol/8 × 10^8 red blood cells. The outer lines show the range, the shaded area has the 25th and 75th percentile and the middle line is the median.
6-TGN: 6-thioguanine nucleotide.

in the two groups but did not reach the adjusted significance value. However a trend for increasing dosage was observed in the categories starting with low 6-TGN levels, increasing through the reference range and further increasing with those above the reference range (Table 4). There were three heterozygotes with 6-TGN values in the therapeutic range and one in the subtherapeutic range.

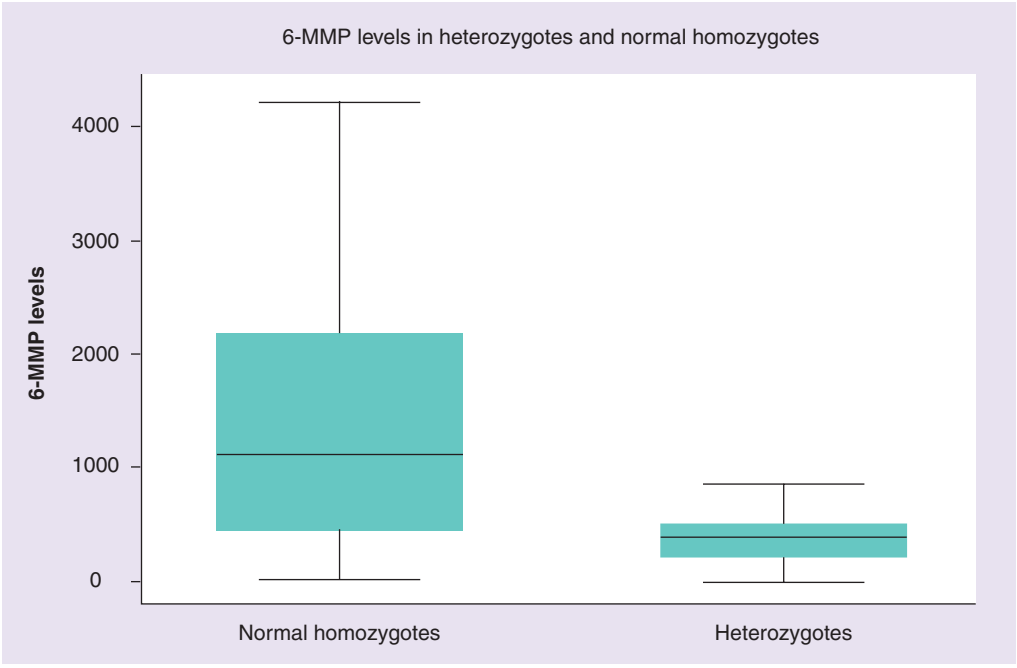

Figure 3. Box whisker plot showing the distribution of the actual 6-MMP levels in pmol/8 × 10^8 red blood cells. The outer lines show the range, the shaded area has the 25th and 75th percentile and the middle line is the median.
6-MMP: 6-methylmercaptopurine.

Table 2. Dosage equivalents in mg/kg for intermediate and extensive metabolizers.

Group	Mean dose (mg/kg)	Numbers
Intermediate metabolizers	1.58	11
Extensive metabolizers	2.02	113

The mean dosage equivalents appeared lower in intermediate metabolizers than extensive metabolizers (t = -2.11 p = 0.037) but did not reach the statistical significance level adjusted for multiple significance testing. However the relationship between intermediate metabolizers and dose was the prime purpose of the study so the corrected significance level is probably too conservative for this.

Tables 3 & 4 were also analyzed individually using a regression model and this regression model was extended by adding two further variables of smoking status and ALT values. Table 5 shows the statistics for a linear regression of ln 6-MMP versus metabolizer status, dosage, never smoked and ALT values. All the latter were found to be significant at conventional significance values but only dosage reached the adjusted significance level of less than 0.005.

All those who were shown to be an IM or had an increased 6-TGN level or 6-MMP were assayed for red blood cell TPMT activity. A total of 40 samples were thus assayed. The laboratory protocol performs this TPMT enzyme assay as its initial screen. Any value of TPMT activity of less than 12 U/ml is representative of a TPMT heterozygote or deficient homozygote status and is then referred for genotyping. This strategy would have detected all except one of the carriers. Conversely, of all the 27 EMs assayed due to high 6-TGNs levels or high 6-MMPs levels, 18 would have been suspected heterozygotes as they were below the laboratory cut-off (<12 U/ml) and one would have been a suspected deficient homozygote. The laboratory protocol would have sent them for genotyping, which demonstrates that phenotyping can be imprecise as they were all normal homozygotes on genotyping. The high number below the laboratory cut-off value may be higher than expected because of the freezing procedure and batching of the samples, which may have lowered the range of values seen by an unknown amount.

The clinical severity for patients with Crohn's disease is assessed by the HBI. Those patients who score 4 or less are judged to be in clinical remission. There was no statistical difference between those patients with low or normal 6-TGN levels and those with high 6-TGN levels in regard to the number in clinical remission (Table 6). The amount of pack years of smoking increased the HBI score. ($r^2 = 0.07$, $p = 0.018$). For patients with ulcerative colitis the equivalent clinical score is the SCCAI and those that score 2 or less are regarded as in clinical remission. However none of these differences were statistically significant as judged by a regression of SCCAI scores or HBI scores against ln 6-TGN or ln 6-MMP. The number of smoking pack years did not correlate with SCCAI scores

When ln 6-MMP was regressed against the multiple variables of metabolizer status, dosage, nonsmoker and ALT value, only the coefficients of dosage reached the adjusted significance level, although the other variables reach conventional significance levels (Table 5). The direction of the changes were that heterozygotes had lower 6-MMP levels, a rise in dosage was reflected by rises in 6-MMP and people who never smoked or with increasing ALT had higher 6-MMP. None of these changes were seen for 6-TGN.

There were no significant correlations between albumin levels and SCCAI scores or HBI scores. None of the blood indices predicted 6-TGN or 6-MMP or the SCCAI or HBI score.

Discussion

Choosing the optimal dosage of thiopurine medication in patients with IBD is important in order to improve the chances of achieving clinical remission whilst minimizing the risk of

Table 3. Categorization of 6-MMP levels and mg/kg dosage.

Levels of 6-MMP	Mean mg/kg dose*	Statistical significance
Reference range	1.89 (113)	t = -5.37, p < 0.001
Greater than reference range	3.05 (8)	

**Dosages of 6-MMP were converted to an equivalent of azathioprine by multiplying by 2.08. There was a statistically significant lower dosage equivalent expressed in mg/kg in those testing higher than the reference range and the mean was higher than the desired 2–2.5 mg/kg range.*
6-MMP: 6-methylmercaptopurine.

Table 4. Categorization of 6-TGN levels and mg/kg dosage.

Levels of 6-TGN	Mean dosage in mg/kg (n)	Statistical significance
Low 6-TGN	1.74 (44)	F = 5.07, p = 0.008
Normal 6-TGN	2.03 (47)	
High 6-TGN	2.20 (30)	

There appeared to be a correlation between increasing TGN metabolite level and mean dosage equivalents. However, it did not reach the corrected significance level.
6-TGN: 6-thioguanine nucleotide.

developing side effects. It has been well shown that those patients who are heterozygotes or deficient homozygotes have a higher risk of myelotoxicity [2,3,17]. Traditional fixed dose or weight-based dosing has significant limitations, and the role of metabolite testing to optimize dosing is still not fully understood. This study advances the knowledge in this area by comparing clinical and biochemical parameters from patients with IBD with the results of metabolite and genotype testing.

The percentage of heterozygotes was approximately 10% matching the expected 10% frequency in the population [2]. This suggests that most of the heterozygotes in our population were detected. The mean dosage was significantly lower in heterozygotes than normal homozygotes, which has been found previously in IBD [18]. In patients with acute lymphatic leukemia, on a fixed regime of 6-mercaptopurine, heterozygotes were found to have a 15% reduction in dosage as compared with the wild-type [19]. Table 2 shows that in the current study there was a reduction of 22% in heterozygotes. Sanderson *et al.* suggested the dose in heterozygotes should be approximately 50% of the standard dose because the enzyme level is 50% and Ansari *et al.* found that heterozygotes tolerated 1 mg/kg well but not 2 mg/kg [20,21]. A recent study showed that individuals who were normal homozygotes ended up with a dose that was twofold higher than heterozygotes after 9 months of dose adjustments, but this study studied dose adjustment and so had the opportunity to titrate the dose against the TMPT activity over a 9 month period. [18] The overall dosage in the heterozygotes was 0.9 mg/kg [18]. However the length of duration of therapy also influences the amount of time a heterozygote can tolerate therapy [21]. The current study had entry requirements that were only a month at a stable dosage so it is likely that with time more heterozygotes would develop signs of myelotoxicity. This would result in reduction of their dose and so it is probable that the optimum dose for heterozygotes is lower than we found. Thus a 50% reduction seems a reasonable figure [20,21].

Our study is unique because it was performed in patients whose dose titration was based purely on clinical grounds without prospective knowledge of genetic status by genotype or phenotype.

There were no cases of homozygote *TPMT*3/*3* in this study and this may be both reflective of the patients included in the study (stabilized on thiopurine drug therapy) and the total numbers in the study (132). If the patient was intolerant of the thiopurine drug they may have been removed from the study. A similar result was observed in a prospective study where heterozygotes were withdrawn with time because of symptoms [21].

Levels of 6-MMP were found to be significantly related to increasing dose equivalents. The correlation with 6-TGN and dose equivalents did not reach the adjusted statistical levels and is similar to a weak correlation found in two

Table 5. Regression of 6-MMP values against metabolizer status, mg/kg equivalent dosage, nonsmoking status and alanine transaminase.

Logarithm(6-MMP)	Coefficient	Standard error	t	p-value	Lower 95% CI	Upper 95% CI
Heterozygote	-0.80	0.34	-2.32	0.02	-1.48	-0.12
Dosage	0.82	0.17	4.84	<0.001	0.49	1.16
Never smoked	0.44	0.19	2.27	0.03	0.05	0.82
ALT	0.01	0.01	2.18	0.03	0.00	0.02
Constant	4.77	0.37	12.83	<0.001	4.03	5.51

All coefficients reached standard statistical significance but only dosage reached the adjusted statistical level. It was concluded that raised 6-MMP is correlated with increasing dosage.
6-MMP: 6-methylmercaptopurine; ALT: Alanine transaminase.

Table 6. Relationship between metabolite levels and HBI or SCCAI score.

Categorization of metabolite	Mean HBI score	Number of samples (HBI score ≤ 4)	Mean SCCAI score	Number of samples (SCCAI score ≤ 2)
Low or reference range 6-TGN	3.56	62(45)	3.1	30 (4)
High 6-TGN	3.42	17 (11)	2.78	14 (7)
Normal 6-MMP	3.55	76 (54)	2.62	39 (22)
High 6-MMP	3.0	3 (2)	6.0	5 (1)

This table was evaluated by a linear regression of HBI scores against whether the subject was in clinical remission or not and a similar regression for SCCAI scores. These regressions showed there was no statistical relationship for either scoring system.
6-MMP: 6-methylmercaptopurine; 6-TGN: 6-thioguanine nucleotide; HBI: Harvey Bradshaw Index; SCCAI: Simple Clinical Colitis Activity Index.

other studies [2,18]. The correlation for 6-MMP was 0.37, which is a similar value to that found in the latter study [18].

It was initially hypothesized that 6-TGN and 6-MMP levels might be associated with clinical remission as judged by the HBI and SCCAI scores [22–24]. This was not seen in this dataset.

This project has introduced the concept of *TPMT* testing into three hospitals in the management of IBD by genotyping *TPMT* common variants and measuring thiopurine metabolites. Only a subsection of the sample was phenotyped and it is possible that we may have not detected some IMs, (nondiagnosed heterozygotes) by not phenotyping everyone. However genotyping/phenotyping discrepancies are well described and both methods may miss cases [14,25,26]. In the current study genetically determined heterozygotes would have been missed if phenotyping was done alone.

There are six cost–benefit studies of TPMT testing in azathioprine users that have been recently reviewed and compared [27]. Many of the studies were based on expert opinion studies rather than clinical data. The latter authors make a case for more prospective clinical studies and trials. The current cross sectional study adds to the clinical literature relating to dosage in heterozygotes and the usefulness of metabolite testing. It is not possible to look at the testing uptake rate with the design of this study. This is the next phase of the study.

Future perspective

It is envisioned that the use of thiopurine drugs will be strictly monitored by DNA testing all mutations for *TPMT*, possibly with simultaneous mutation detection of other genes in the metabolic pathway of thiopurine drugs. The need for the additional TPMT enzyme assay will be clearer at this time. Thiopurine metabolites may also be measured. In this way the drugs will be used most effectively with minimal risk of adverse events. With more prospective studies and trials, more evidence based data will be collected so that the cost–benefit value of testing all patients on thiopurine drugs will be firmly established.

Acknowledgements

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Executive summary

- This study is a multicenter study involving the documentation of genetic changes in thiopurine methyltransferase and thiopurine metabolite testing in patients taking thiopurine drugs for inflammatory bowel disease.
- The study introduced such monitoring into 3 hospitals and looked at the clinical information gained regarding the patient with these new tests.

Method

- Genotyping common thiopurine methyltransferase genotypes and measuring thiopurine metabolites.

Results

- A total of 9.8% of the patients were found to be intermediate metabolizers and they had stabilized to a statistically significant lower dosage than the remaining extensive metabolizers.
- 6-methylmercaptopurine levels reflected the dose that the patient was on and whether they were intermediate metabolizers or not.
- The metabolite levels did not correlate with scales that measure clinical remission.

Discussion

- Measuring metabolizer status by genotype and thiopurine metabolites yielded important information that could be used in the clinical management of the patient.
- This study adds to the literature evidence that genotyping and metabolite measurement are useful measures in the management of every patient taking thiopurine drugs.

Financial & competing interests disclosure

LJS has a pharmacogenomic information company (GenesFX Health Pty Ltd) that interprets pharmacogenomics tests as a research and noncommercial service currently but will also start to offer some interetations commercially later in the year. MS is a gastroenterologist supervising a laboratory in an academic institution that assays thiopurine metabolites. KB is the scientist supervising a commercial pathology molecular genetics laboratory and has started offering TPMT testing. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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