Thiopurine S-methyltransferase (TPMT) assessment prior to starting thiopurine drug treatment; a pharmacogenomic test whose time has come

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ABSTRACT
Thiopurine S-methyltransferase (TPMT) is involved in the metabolism of thiopurine drugs. Patients that due to genetic variation lack this enzyme or have lower levels than normal, can be adversely affected if normal doses of thiopurines are prescribed. The evidence for measuring TPMT prior to starting patients on thiopurine drug therapy has been reviewed and the various approaches to establishing a service considered. Until recently clinical guidelines on the use of the TPMT varied by medical specialty. This has now changed, with clear guidance encouraging clinicians to use the TPMT test prior to starting any patient on thiopurine therapy. The TPMT test is the first pharmacogenomic test that has crossed from research to routine use. Several analytical approaches can be taken to assess TPMT status. The use of phenotyping supported with genotyping on selected samples has emerged as the analytical model that has enabled national referral services to be developed to a high level in the UK. The National Health Service now has access to cost-effective and timely TPMT assay services, with two laboratories undertaking the majority of the work at national level and with several local services developing. There appears to be adequate capacity and an appropriate internal market to ensure that TPMT assay services are commensurate with the clinical demand.

INTRODUCTION
Pharmacogenomics, the study of how genetic variation relates to drug response, has the potential to revolutionise patient treatment. Individualising drug therapy aims to ensure patients are given the right dose at the outset, with the potential to improve outcome, reduce wastage and limit adverse reactions. In 2006, of the 16 million hospital admissions in the UK, 6.5% were as a result of adverse reactions to drugs.1 According to the centre-left think-tank Compass, the annual cost to the National Health Service (NHS) to treat these patients was nearly £2 billion.2 In 2003, the Department of Health (DH) published a white paper entitled ‘Our inheritance, our future: realising the potential of genetics in the NHS’, in which it pledged to invest £4 billion in pharmacogenomic research.3 In 2009, together with the Wellcome Trust, the DH set up the Health Innovation Challenge Fund (HICF), with a further £20 million towards proposals that help convert genetic discoveries into improved care.4 Despite these initiatives and other investment, the crossover of pharmacogenomic testing into routine clinical use in the UK remains limited. Barriers have included high test costs, limited availability and slow turnaround of results. The complexity of the systems and poor awareness often means clinicians lack the necessary knowledge to apply pharmacogenomics to clinical practice.5–7 One of the few pharmacogenomic tests that has successfully made the transition from research into clinical use is thiopurine S-methyltransferase (TPMT). Here we review the current evidence supporting routine TPMT screening, how it has improved patient care, the challenges of providing a service and what lessons can be learnt for introducing other pharmacogenomic tests in the UK.

TPMT AND THIOPURINE DRUGS
TPMT (EC 2.1.1.67) is part of a cascade of enzymes responsible for the metabolism of thiopurine drugs including azathioprine (AZA), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG). TPMT is expressed in many cells, with highest levels in the liver and lowest levels in the brain and lung.8–9 No endogenous substrate has been identified and the biological role of TPMT is currently unknown.

Thiopurine drugs are in wide use by a number of medical specialties as steroid sparing agents to treat chronic inflammatory and autoimmune disease. They are also used to treat leukaemia and to prevent post-transplant organ rejection.10–14 Despite high efficacy, adverse reactions occur in 10–25% of patients, including gastrointestinal intolerance, pancreatitis, hypersensitivity, and life threatening bone marrow suppression, which often results in the withdrawal of treatment.15–19

THIOPURINE DRUG METABOLISM
Thiopurines are prodrugs and must be converted intracellularly to cytotoxic 6-thioguanine nucleotides (TGNs) to have a therapeutic effect. Following oral administration, AZA is rapidly absorbed from the gut and converted to 6-MP by a non-enzymatic process. As figure 1 shows, three pathways compete to metabolise 6-MP, TPMT, hypoxanthine guanine phosphoribosyltransferase (HGPRT), and xanthine oxidase (XO). The low bioavailability suggests metabolism of 6-MP mostly occurs in the liver.20

The therapeutic action of TGNs is a result of their incorporation into DNA and inducing T cell apoptosis by modulating Rac 1 signalling.21–23 S-methyl-thioinosine 5'-monophosphate (MeTMP), which is created by TPMT methylation of an intermediate of 6-MP metabolism (6-thioinosine monophosphate, 6-TIMP), may also contribute
to thiopurine cytotoxicity, by inhibiting purine de novo synthesis.  

TPMT PHENOTYPE
The side effects of thiopurine drugs have been attributed to the accumulation of high levels of TGNs.  

TPMT GENOTYPE
The trimodal frequency distribution of TPMT activity is a result of genetic polymorphism. The TPMT gene is located on chromosome 6 (6p22) and is approximately 34 kb in length. It consists of 9 introns and 10 exons, 8 of which encode for a 245 amino acid long protein.  

UTILITY OF TPMT SCREENING
Patients with deficient TPMT activity (homozygous for mutant TPMT alleles) are at severe risk of developing myelosuppression if treated with standard doses of thiopurine drugs, and deaths
have occurred. Patients usually present with leucopenia within days after taking the first dose, but it may take longer (1–2 months). According to the Medicines and Healthcare products Regulatory Agency (MHRA) Drug Analysis Print (DAP), which is a listing of all suspected adverse reactions reported by healthcare professionals and patients via the yellow card scheme in the UK, from 1963 to 2009 there have been 138 fatal reports for AZA, 7 for 6-MP, and up to 2008, 3 fatal reports for 6-TG. Identifying individuals with deficient TPMT activity and preventing the most severe reactions to thiopurines is the primary purpose for TPMT screening.

Patients with low TPMT activity and who are heterozygous for mutant TPMT alleles, have a 30–40% risk of developing adverse reactions when treated with a standard dose of thiopurine drug, and effectively these patients have received an overdose. Individuals with very high TPMT Activity may not respond to standard doses of thiopurine drugs and are at risk of hepatotoxicity from the increased production of methylated thiopurine metabolites, such as methyl-mercaptopurine (6-MMP). TPMT screening may also reduce adverse reactions in patients with low TPMT activity and improve drug efficacy in patients with ultra-high TPMT activity by guiding adjustment of thiopurine dose.

**FURTHER FACTORS AFFECTING THIOPURINE DRUG METABOLISM**

TPMT only accounts for 5–10% of adverse reactions to thiopurine drugs, and TPMT deficiency about 30% cases of neutropenia. Non-genetic factors such as diet, drug interactions, and genetic variation of other enzymes involved in thiopurine drug metabolism have also been implicated.

**Xanthine oxidase (EC 1.2.3.2)**

XO deficiency increases 6-MP metabolism by TPMT, resulting in higher levels of TGNs and toxicity. Although rare, XO deficiency has been implicated in a patient developing severe leucopenia after commencing AZA following renal transplantation. Allopurinol inhibits XO activity and co-administration with thiopurine drugs is associated with an increased risk of myelosuppression. Population studies have shown a 10-fold variation in XO activity between individuals. Although XO activity can easily be determined by measuring its major metabolite 6-thiouric acid (6-TU) in urine, the effect this variation has on thiopurine drug efficacy remains to be determined, and so far has no routine clinical application.

**Inosine triphosphate pyrophosphohydrolase (EC 3.6.1.19)**

Inosine triphosphate pyrophosphohydrolase (ITPase) catalyses the pyrophosphohydrolysis of inosine triphosphate (ITP) to monophosphate (IMP), Hypoxanthine guanine phosphoribosyltransferase (HGPRT) converts 6-MP to a 6-thio-IMP intermediate (6-TIMP), which is either transformed to TGNs by a cascade of enzymes, MeTTP by TPMT, or phosphorylated to 6-thio-ITP (6-TITP). Patients with reduced or deficient ITPase activity are unable to convert 6-TITP back to 6-TIMP, resulting in toxic accumulation of 6-TITP. Side effects are similar to TPMT deficiency and include myelosuppression. So far two mutations resulting in deficient ITPase activity have been identified, 94C→A and IVS2+A21C. ITPase deficiency is more common in some populations than TPMT, for example Asians.

Currently there is insufficient evidence to support introduction of ITPase screening. Not all studies have found a correlation between ITPase deficiency and risk of adverse reactions to thiopurine drugs. However, most ITPase studies are small, retrospective, and carried out over different durations with variable outcome measures. Thiopurine drug treatment is not always guided by TPMT activity, so in some studies TPMT activity is the predominant cause of toxicity, whereas in others, with thiopurine drug adjustment, ITPase activity is more significant.

**TGNs and 6-MMP**

The majority of studies show a correlation between RBC TGNs, disease remission and risk of adverse reactions to thiopurine drugs. TGN monitoring may therefore be of benefit to guide further dosing decisions for some patients once thiopurine drug treatment has started. This includes patients with low TPMT activity, those on allopurinol or drugs that inhibit TPMT activity, or those that are resistant to therapy. In patients with inflammatory bowel disease it has been suggested RBC TGN concentrations greater than 255 pmol/8×10^6 cells are associated with maximum drug efficacy, whereas lower levels suggest non-compliance or sub-optimal dosing. Higher levels are associated with an increased risk of adverse reactions.

There is a large intra-patient variability in TGN production and repeat measurements are required. Higher TGN concentrations have been found in patients treated with branded AZA compared to the generic version of the drug, suggesting there is a difference in bioavailability, and therefore drug efficacy.

An increase in AZA dose does not always result in therapeutic levels of TGN, with some patients preferentially metabolising AZA to 6-MMP. High concentrations of RBC 6-MMP (>5700 pmol/8×10^6 cells) are associated with hepatotoxicity, which in some patients with very high levels has resulted in liver failure. Measurement of 6-MMP is also useful in identifying patients resistant to thiopurine drug therapy. Approximately 9% of patients are unable to achieve therapeutic levels of TGNs despite increased thiopurine dosage, and instead accumulate hepatotoxic 6-MMP. A 6-MMP/TGN ratio of >30:1 indicates resistance to thiopurine drug therapy. Since only 2% of patients have ultra-high TPMT activity,卉other causes of thiopurine resistance have been sought. So far genetic variation of inosine-5-monophosphatase dehydrogenase (IMPDH, EC 2.1.1.67), one of the cascade of enzymes responsible for transforming 6-TIMP to TGN, has been identified as a possible cause of resistance, but only in a few individuals.

**TPMT GUIDED TREATMENT**

TPMT screening should ideally be performed prior to treatment, since it is not predictive of clinical response or drug toxicity in patients already established on thiopurine drugs. Treatment is contraindicated where thiopurine treatment has already commenced, monitoring TGN concentration provides useful information on dose adjustment.

Various authors have published recommendations for TPMT guided thiopurine treatment. The consensus opinion is that patients with normal TPMT activity can be administered a full dose of thiopurine drug at the outset. Patients with low TPMT activity can be treated with fewer side effects by reducing the standard dose by 50–67%, Treatment is contraindicated for patients with deficient TPMT activity, although there are reports of patients being successfully treated, by reducing the standard dose to less than 20% normal. Where allopurinol is co-prescribed, it is recommended that thiopurine dose is reduced to 25% of normal. Since the majority of adverse reactions to thiopurine drugs, including myelosuppression, are not explained by TPMT, regular monitoring of full blood count and liver function tests is still essential.
TPMT PHARMACOECONOMICS

Pharmacist testing promises significant cost savings, and demonstration of cost effectiveness would be a major driver for routine clinical use. The cost of thiopurine drugs in recent years has dropped, with a generic pack of AZA costing approximately £7.00 for 28×25 mg tablets. Imuran (GlaxoSmithKline) costs £10.99 for 100×25 mg tablets. With a typical daily dose of 100 mg, the cost of thiopurine treatment per patient is approximately £1 per day. According to NHS prescription cost analysis data for England in 2008, of the total £10.1 million spent on drugs affecting the immune response (British National Formulary (BNF), section 8.292), AZA accounted for only 8% of costs despite being responsible for 58% of these prescriptions. The cost of TPMT screening has been cited as a barrier to testing, even though it is a one-off cost. At around £50, TPMT screening in the UK is low-cost compared to other countries. With the number of new starters estimated at between 45,000 and 60,000 a year, the potential annual cost of TPMT screening to the NHS is £1.4–1.8 million if all patients were screened prior to treatment.

Assessment of cost-effectiveness TPMT requires accurate data on how many adverse reactions are prevented by screening, compared to the cost of treating each episode. All published economic evaluations have so far concluded that TPMT screening is a cost-effective use of healthcare resources. Even when a test cost in excess of £100 is used, the cost per life-year gained at <£2000 is well below the quality-adjusted life-year (QALY) threshold limit of £20–50,000 recommended by NICE to assess cost-effectiveness. However, design of these studies has been criticised for over reliance on expert opinion rather than clinical trial data, for example, for determining the prevalence of neutropenia, length of hospitalisation, clinical outcome and clinical effectiveness.

To address the limitations of previous studies, a DH funded randomised controlled trial, ‘TPMT AZA response to genotyping and enzyme testing (TARGET)’, has studied the clinical utility and relative cost effectiveness of TPMT screening. Results of this trial are due to be published soon.

CURRENT RECOMMENDATIONS FOR TPMT SCREENING IN THE UK

A comparison of the published guidance on AZA treatment in the UK shows variation in recommendations for dosage and monitoring. Only dermatology guidelines recommend pre-screening for TPMT and dose adjustment according to TPMT status. Hilton and Palace state: ‘If the assay is available then it is sensible to use it’. In the guidelines for management of inflammatory bowel disease in adults, TPMT screening was not recommended ‘because decades of experience has shown clinical AZA to be safe in ulcerative colitis and Crohn’s disease’. There is no specific recommendation for TPMT screening in the BNF, but it does state: ‘the risk of myelosuppression is increased in those with low activity of enzyme, particularly in the very few individuals who are homozygous for low TPMT activity’, and gives hypersensitivity to AZA or 6-MP as contraindications.

A recent national survey found that 67% of UK consultants were testing patients prior to AZA treatment. Uptake was highest among dermatologists (94%), compared to gastroenterologists (60%) and rheumatologists (47%). The same survey also found that AZA was most commonly prescribed for Crohn’s disease and systemic lupus erythematosus. This would suggest that in the UK only about 50% of patients are currently screened for TPMT prior to starting therapy.

Recently, guidance free from clinical specialty has been published in the Drugs and Therapeutics Bulletin, which, pending the publication of the TARGET trial, recommends as a sensible precaution that all patients starting on azathioprine are tested for TPMT enzyme activity.

METHODS FOR TPMT SCREENING

There are two strategies for determining TPMT status, phenotyping and genotyping. Both testing strategies have disadvantages, and are technically demanding. There are few commercial kits available, and TPMT screening services in the UK currently rely on in-house methods.

Phenotyping

Erythrocyte TPMT activity has been shown to correlate with that in other tissues, including the liver. For phenotyping, TPMT activity can therefore be readily determined by incubating a whole blood or RBC lysate with either 6-MP or 6-TG with the methyl donor S-adenosyl-L-methionine (SAM) to produce the methylated products 6-MMP or 6-methylthioguanine (6-MTG). These methylated products can be detected in a number of different ways, including radiochemical assay, mass spectrometry, or high-performance liquid chromatography (HPLC) with absorbance or fluorescence detection. A TPMT activity ELISA kit is now available.

The units of TPMT measurement are variable and this has led to different reference ranges. TPMT activity has classically been related to RBC count or haemoglobin concentration in the lysate, but commercial products do not include this component. TPMT activity accurately predicts myelosuppression in patients, but is less able to identify individuals with low TPMT activity. Pre-analytical factors such as sample age can affect measured TPMT activity; for example, TPMT activity is stable in whole blood for about a week at room temperature. The assays themselves can be difficult to control, with many factors affecting the enzyme reaction, such as the concentration and source of SAM, incubation temperature and duration. This may result in over-reporting of patients with low TPMT activity status. It is unclear if the affect of non-genetic factors such as drug inhibition and disease on TPMT activity is universal or organ specific. Typically preparation of RBC for TPMT analysis involves washing steps, which could remove any inhibiting drugs. In either case this could result in a loss of correlation between measured RBC TPMT activity and that in other organs, in particular the liver.

Since TPMT activity is measured using RBCs, it is not possible to determine reliably the TPMT activity status of patients receiving a blood transfusion, due to interference from the donated blood. Theoretically TPMT activity cannot be accurately measured until at least 120 days later, the average life span of RBCs.

Genotyping

Strategies to detect mutations of the TPMT gene include restriction digest, single strand conformational polymorphism (SSCP), amplification refractory mutation system (ARMS), denaturing HPLC, pyrosequencing, and fluorescence hybridisation probe assays using LightCycler Instruments. Qiagen supply a real time artus TPMT LC PCR kit for detection of TPMT, *3A, *3B and *3C for use on LightCycler (24 reactions).

Care must be taken when designing PCR primers for TPMT analysis due to the presence of an inactive TPMT pseudogene located on chromosome 18, which has 96% homology to the TPMT gene. Since this inactive TPMT pseudogene is
intronless, ensuring at least one primer is complementary to the TPMT intron sequence overcomes this potential interference.

Unlike TPMT phenotyping, genotyping is not affected by blood transfusions and is less affected by pre-analytical factors, although results for some methods are very dependent on the quality of DNA used, and not well suited for high throughput, for example, restriction digest. Restriction digest is a popular method choice since it is easy to perform and requires only basic equipment. However, the results are not always reliable. In early studies, incomplete digest by Acc 1 of the cutting site created by the presence of the TPMT*3C mutation resulted in over-reporting of rare TPMT*B mutant alleles, rather than the more common TPMT*5A double mutant allele. A major disadvantage of most TPMT genotyping methods is that they do not identify on which allele a mutation lies. Hypothetically, a patient with a homozygous deficient TPMT activity genotype could be missed; for example, a TPMT*1/*3A heterozygote with low activity cannot be distinguished from a compound TPMT*2C/*3B heterozygote resulting in deficient TPMT activity. This can be overcome by using RNA methods such as multiplex induced heteroduplex analysis, but only when DNA is completely absent and this is difficult to achieve.

Screening the whole TPMT gene for all 29 different TPMT alleles routinely, would be technically demanding and time consuming. Also it has yet to be demonstrated whether some variant alleles result in deficient TPMT activity. Since TPMT*2, TPMT*5A, and TPMT*3C make up between 60% and 95% of mutant alleles for deficient TPMT activity in most populations, TPMT genotyping is usually performed for these mutations only. Consequently, patients with new mutations or from certain ethnic populations with rare TPMT deficient alleles may be missed. Genotyping, unlike phenotyping, is unaffected by disease activity and drugs.

While it may seem a contradiction for a pharmacogenomic test, in the UK phenotyping is the preferred method for routine TPMT screening as apart from patients recently transfused, a well controlled TPMT phenotyping assay always identifies patients with deficient TPMT activity, which is accepted as the primary reason for testing. In our opinion a combination of phenotyping with supportive genotyping on selected samples overcomes the limitations of both tests, and is currently the best model for any TPMT screening service.

**TPMT PATENTS**

The situation regarding US and EU patenting of TPMT phenotyping and genotyping is confusing. In the USA, Prometheus Laboratories Ltd hold patents for PCR methods for TPMT phenotyping, detection of common TPMT mutations and monitoring thiopurine drug metabolites for patients with inflammatory bowel disease (IBD). It is difficult to see how this situation has arisen as all of the Prometheus patents appear to originate from already published research conducted by other groups.

How enforceable TPMT patents are in the UK remains to be determined. So far they have only influenced testing in the USA. In the UK, all TPMT screening services use phenotypic methods that differ from the patents, and are intended for testing all patients, not just those with IBD. While TPMT genotyping is also available in the UK, its use is limited to specialist investigations in support of phenotyping and research.

**TPMT screening in the UK**

Historically, the availability of TPMT testing has often been cited as a major factor limiting the uptake of screening. To determine the ideal number of UK screening services requires the primary reason for testing to be taken into account, as well as the requirements of users. These are first to always identify patients with deficient TPMT activity, and second do so in a clinically relevant time frame. TPMT phenotyping is an inherently complex assay which is not well suited to low sample throughput. In the UK there are 391 hospital trusts, each of which generates only a handful of requests for TPMT screening each week. As with other screening programmes, one can advocate that the detection of TPMT deficient results should occur frequently enough to ensure the continuing quality of the method. With a prevalence of 1:500, in order to detect a deficient result at least once a month would require a minimum workload of 5600 samples per annum. With the technical challenges and costs needed to maintain a routine TPMT service, as well as the scientific expertise to support clinicians, regional or national services are the logical TPMT service model. This is what has already happened in countries where routine TPMT screening has been established for longer, such as New Zealand, and a similar picture has now emerged in the UK.

Currently in the UK the majority of routine TPMT testing is performed by two national services, the Purine Research Laboratory in Guy’s Hospital London and Clinical Biochemistry, City Hospital, Birmingham. Both services provide TPMT phenotyping for routine analysis. Turnaround of results is rapid, with published times of 1–2 working days. Due to an ongoing national clinical trial for leukaemia, UK patients with acute lymphoblastic leukaemia (ALL) are tested at Sheffield, using a TPMT phenotyping assay. There are several other NHS laboratories in the UK providing smaller, local TPMT services.

**Ways forward**

Neither a phenotype nor genotype approach is ideal in all situations. A service model using phenotyping supported by genotyping is a sensible strategy at the present time. A clear focus on

**Take-home messages**

- The use of thiopurine S-methyltransferase (TPMT) in the pre-assessment of patients starting on thiopurine drugs is now clearly established as a routine test and readily available.
- The harmful effects of treating homozygous patients are not specialty dependent. It has been anomalous that clinical disciplines have offered very different advice on use of TPMT. This has recently been clarified with the recommendation that all patients should be screened.
- Phenotyping offers a convenient way of screening large numbers of patients at moderate cost, with genetic confirmation also having a role in certain patients.
- Considering the low numbers of patients starting on thiopurine drugs in any one locality, the complexity of TPMT analysis together with the high level interpretative support required, regional and national TPMT services with acceptably fast turnaround times are the sensible way of providing a service. In the UK, two national services are clearly established which meet these criteria.
- In heterozygous patients and in other situations where there is concern on whether the levels of active metabolites are in the therapeutic range, the measurement of 6-thioguanine nucleotides (TGNs) clearly has a role to play. One can expect routine TGN analysis to be increasingly requested as this is appreciated by clinicians treating patients with thiopurine drugs.
turn-round, clinical support and service development along with economy of scale has meant that the cost of these services offers excellent value to the NHS. Development of local services for such a complex test is neither scientifically sensible nor cost-effective and is likely to lead to poor quality results. The availability of TPMT analytical services should reduce the risk of thiouracil induced myelosuppression in TPMT deficient patients and also facilitate optimum dose adjustment according to TPMT status.

For patients heterozygous for TPMT, reduced-dose treatment strategies are increasingly accepted and it is in such patients that TGNs and 6-MMP measurement may be of use. In the USA and New Zealand, TGNs and 6-MMP testing is more established, but despite routine services being offered by both UK national services, so far clinical interest has been limited. One of the main reasons for this is a lack of awareness among clinicians that this service is readily available.

**Competing interests** The authors work in a laboratory that offers a national service for TPMT assessment to the NHS and other healthcare organisations.

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