Thiopurine methyltransferase Phenotyping and Genotyping in Clinical Practice

by

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ABSTRACT

Thiopurine methyltransferase (TPMT) metabolises the widely prescribed thiopurine drugs. The activity of this enzyme varies between individuals and this can influence treatment success. Rapid and accurate assays of TPMT and 6TGN, the active thiopurine metabolites, are increasingly recognised as valuable clinical tools, to guide dosing and treatment.

This work examines and develops methods for determining a patient's TPMT status and measuring 6TGN levels, adapting them for analysis using whole blood rather than washed red blood cell preparations. The advantages and limitations of these new assays in practice are investigated, which clearly shows that the more rapid new methods have increased clinical significance and utility. Experimental work and research provides an increased understanding of factors influencing determination and significance of a patient's TPMT status and thiopurine metabolite levels. This includes possible misclassification of TPMT status when expressing results in relation to patient haemoglobin and a longitudinal study of TPMT activity of patients commencing thiopurine therapy which demonstrates no enzyme induction.

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Ford L, Graham V, Berg J. (2006) **Ann Clin Biochem.**

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Appendix 6 **Poster Abstract**

Patients with high-normal TPMT activity identified during routine phenotypic testing: What is going on? Ford LT, Graham V, Berg JD (2006) **Ann Clin Chem**

Appendix 7 **Poster Abstract**

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LIST OF ABBREVIATIONS

6MMP	6-methylmercaptopurine
6MP	6-mercaptopurine
6MTG	6-methylthioguanine
6TG	6-thioguanine
6TGN	6-thioguanine nucleotides
ADR	Adverse drug reaction
ARMS	Amplification refractory mutation system
CE	Conformité européenne
DHPLC	Denaturing high-performance liquid chromatography
DNA	Deoxyribonucleic acid
DPK	Diphosphate kinases
DTT	DL-dithiothreitol
EDTA	Ethylenediaminetetraacetic
EQA	External quality assurance
FDA	Food and drug administration
FBC	Full blood count
FMEA	Failure modes and effect analysis
GMPS	Guanosine monophosphate synthase
Hb	Haemoglobin
HPLC	High performance liquid chromatography
HPRT	Hypoxanthine phosphoribosyltransferase
IMPDH	Inosine monophosphate dehydrogenase
IS	Internal Standard

ITPA	Inosine triphosphatate pyrophosphohydrolase
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight analysis
MCV	Mean cell volume
MPK	Monophosphate kinases
MTHFR	Methylenetetrahydrofolate reductase
NEQAS	National External Quality Assessment Service
NCBI	National centre for biotechnology information
PCR	Polymerase chain reaction
QC	Quality control
RBC	Red blood cells
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SAM	S-adenosyl methionine
SNP	Single nucleotide polymorphisms
SSCP	Single strand conformational polymorphism
TBE	Tris borate EDTA
TPMT	Thiopurine methyltransferase
UGT1a1	Uridine diphosphate glucuronosyltransferase 1A1
UV	Ultraviolet
WBC	White blood cells
XO	Xanthine oxidase

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1. INTRODUCTION

1.1. Pharmacogenetics

A patient's response to a drug can be influenced by the genetic make-up of the individual. Genetic variation affecting drug metabolism can result in an unpredictable outcome to treatment. Pharmacogenetics is the linking of differences in gene expression (genotype) to drug response (phenotype) and advances in this field are helping to better predict drug efficacy and toxicity. These developments have obvious major medical benefits as they enable optimization of drug dosage.

The field of pharmacogenetics dates back to the 1950s, when it was first proposed that inherited traits may account for individual variability in drug response (Motulsky 1957). Today, one of the main applications of pharmacogenetics is research into disease mechanisms and new drug discovery. Meanwhile, development and clinical application, help to improve drug efficacy and safety.

The possibility of therapy being more effective and better tolerated has enormous financial implications. Each year in the United Kingdom, adverse drug reactions (ADR) account for an estimated 6.5% of hospital admissions, at a cost to the National Health Service of approximately £466 million (Pirmohamed et al 2004). In the future, these medical costs may be compounded by claims for compensation, as patients may have a strong legal case, if their suffering could

have been averted. Many of the drugs responsible for ADR are metabolized by an enzyme with variant alleles (Zhou et al 2008). Pre-treatment screening of the enzyme phenotype or genotype could predict therapy outcome. Currently, the best clinically applied example of pharmacogenetics is the determination of thiopurine methyltransferase (TPMT) status prior to commencement of thiopurine drugs (Wang & Weinshilboum 2006), and it is this which forms the basis of this thesis.

1.2. **Clinical significance of TPMT**

TPMT (EC 2.1.1.6.7) is a cytosolic enzyme present in most cells of the body. It catalyses the S-methylation of aromatic and heterocyclic sulphydryl compounds (Weinshilboum 1989). TPMT is of clinical interest as it is involved in the metabolism of thiopurine drugs, for example azathioprine, 6-mercaptopurine (6MP) and 6-thioguanine (6TG). These drugs are largely used in the management of auto-immune diseases, in the disciplines of gastroenterology, dermatology and rheumatology. They are also used effectively in the management of acute lymphoblastic leukaemia and to prevent transplant organ rejection (Weinshilboum & Sladek 1980; Lennard & Lilleyman 1996; Anstey et al 2004; Ernest & Seidman 2003; Whisnat & Pelkay 1982).

It is estimated that approximately 60,000 patients per year commence thiopurine drug treatment in the UK. The most widely prescribed thiopurine drug is azathioprine, with the brand name IMURAN. This is now a generic drug, of

relatively low cost, having been at the centre of a cost reduction agreement between the Government and the pharmaceutical industry. It is used extensively as it provides an attractive alternative to steroid treatment. Although thiopurine drugs are widely used, gastrointestinal intolerance, pancreatitis, hypersensitivity and myelosuppression are observed in up to 30% of patients. These ADR often result in the withdrawal of treatment (Weinshilboum & Sladek 1980; Lennard & Lilleyman 1996; Clunie & Lennard 2004; Anstey et al 1992). In some instances the unexpected reaction to therapy can be fatal, for example in cases of severe myelosuppression (Slaner et al 2008). If this occurs, all cells produced in the bone marrow become suppressed. The reduction in leucocytes leaves the body susceptible to infection, while reduced platelets cause clotting abnormalities and red cell anaemia results.

Comprehensive evaluation of the clinical benefit and cost effectiveness of screening strategies for pharmacogenetic testing in the UK is incomplete, despite the Government published a strategy in 2003 for maximizing the potential of genetics in the NHS (Department of Health). So far evidence has indicated sufficient benefit to warrant informing prescribers, pharmacists and patients of the available tests and their possible role in the selection and dosing of these drugs. This has prompted the Food and Drug Administration (FDA), in the United States of America, to approve label changes for 6MP and Azathioprine.

1.3. **Thiopurine drug metabolism**

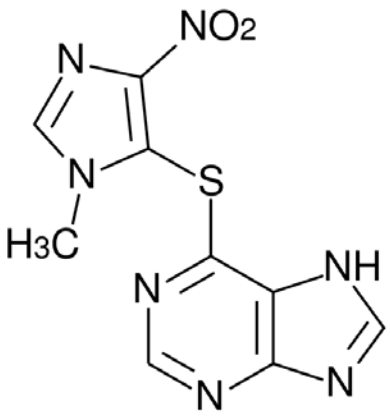


Figure 1.1 ***Azathioprine structure***

Azathioprine is the 1-methyl-4-nitro-imadazolyl of thioguanine. It is classed as a pro-drug which is converted by the sulphydryl compounds, glutathione and cysteine, in red blood cells (RBC) to 6-mercaptopurine (6MP). This metabolite undergoes activation, via a multi-step enzymatic pathway, into thioguanine nucleotides (6TGN) (Lennard & Maddocks 1983) as seen in Figure 1.2. 6TGN achieve therapeutic efficacy primarily by virtue of their cytotoxic action. They are incorporated as false bases into newly synthesized DNA, preventing proliferation of the target bone marrow and white blood cells, and also interfere with other biochemical pathways.

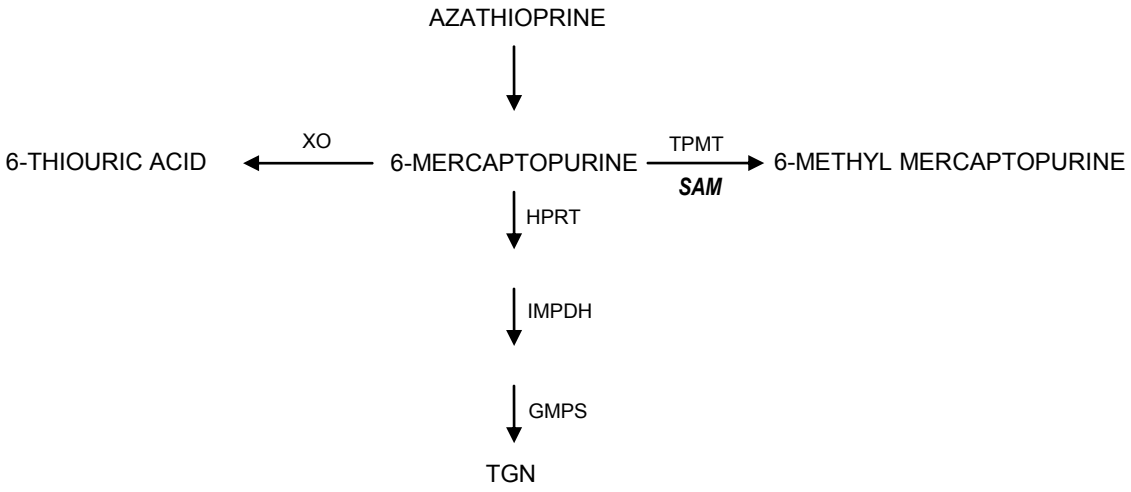


Figure 1.2 ***Simplified azathioprine and 6MP biotransformation***

HPRT= hypoxanthine phosphoribosyltransferase, XO = xanthine oxidase, TPMT= thiopurine methyltransferase, IMPDH = inosine monophosphate dehydrogenase, GMPS = guanosine monophosphate synthase , SAM = S-adenosyl methionine

Three enzymes, hypoxanthine phosphoribosyltransferase (HPRT), xanthine oxidase (XO) and thiopurine methyltransferase (TPMT) compete to break down 6MP.

The primary anabolic (HPRT) route, leads to pharmacologically active 6TGN. Normally, methylation by TPMT diverts a proportion of available substrate away from this pathway to form 6-methylmercaptopurine (6MMP). Therefore, a deficiency in TPMT activity will result in more substrate following the HPRT route and much higher concentration of 6TGN. Conversely, a high TPMT activity will result in more 6MMP but less 6TGN being produced, posing potential treatment

failure. The increased levels of 6MMP produced in this scenario can inhibit purine biosynthesis (Dever 2001) and lead to liver damage.

Xanthine oxidase (XO) metabolises 6MP, by oxidation of the purine ring, to inactive thiouric acid which is excreted from the body. This catabolic process takes place in the liver and intestinal mucosa after oral administration of the drug. It has been shown that there is a 4 to 10 fold inter-individual variation in XO activity, with a significant sex related difference, XO activity tending to be higher in men (Guerciolini et al 1991). XO activity is very difficult to measure. Consequently, the effect of this on thiopurine treatment is not, as yet, clearly defined.

1.4. Pharmacogenetics of TPMT

Activity and stability of TPMT are primarily governed by a common autosomal co-dominant polymorphism. This genetic variation gives rise to a trimodal distribution of TPMT activity, as shown in Figure 1.3, with approximately 89% of the population having normal TPMT activity, 0.3% with undetectable enzyme activity and a further 11% having low activity. (Weinshilboum & Sladek 1980).

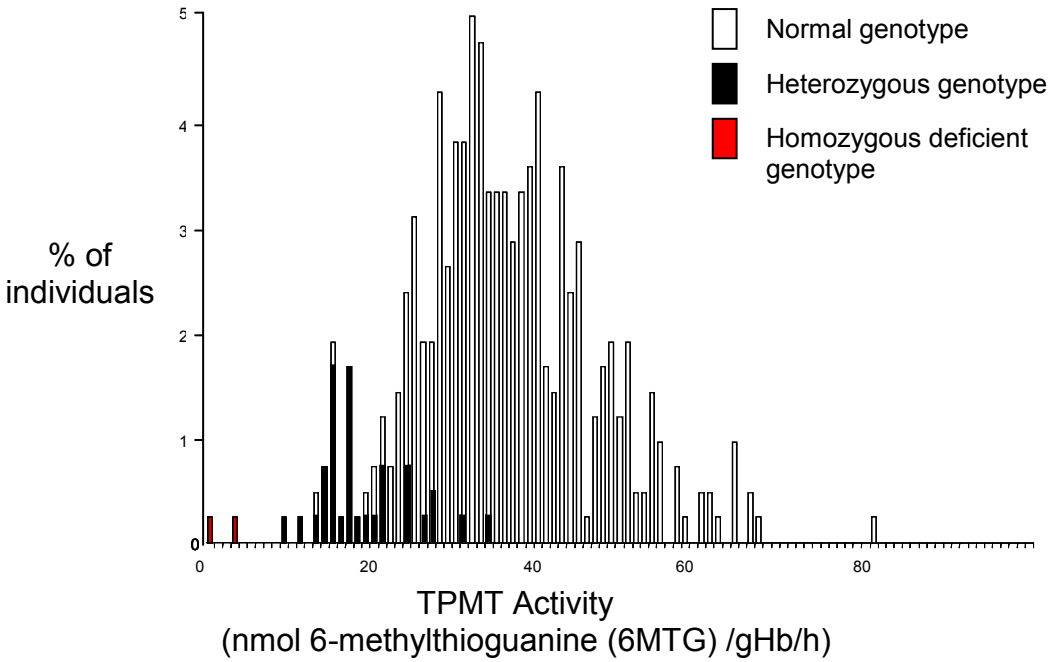


Figure 1.3 ***Distribution of TPMT activity in a 1000 individuals***
Ford LT, Cooper SC, Lewis MJV & Berg JD. Ann Clin Biochem 2004; 41: 303-8

Differences in mean TPMT activity between many ethnic groups have been observed. A local study by Cooper et al (2008) showed that Afro-Caribbeans have lower activity than Caucasians and south Asians. Gender differences have also been shown to exist, with slightly lower activity being observed among females (Klemetsdal et al 1993), especially South Asians (Cooper et al 2008).

The first description of a TPMT assay was at the Mayo Clinic in the USA (Szumlanski et al 1992). In the UK, one centre has offered an assay since 1990, initially setting up the assay to prove that adverse reactions were often due to treatment of unknown TPMT deficient patients (Holme et al 2002).

In recent years, it has gradually become established clinical practice to determine TPMT activity prior to treatment with azathioprine (Ernest & Seidman 2003). This

screening does not reduce the necessity for careful clinical monitoring. Other factors can influence individual degrees of tolerance and clinical guidelines appropriate for one disease are not necessarily applicable to other pathological states. Mutations in the inosine triphosphatate pyrophosphohydrolase (ITPA) gene may also cause intolerance to azathioprine (Marinaki et al 2004 a & b; Von Ahsen et al 2005; Marsh & van Boovan 2009). Deficiency in this enzyme could lead to potentially increased concentrations of thioinosine triphosphate metabolites. Resulting symptoms would be non-myelosuppressive, for example nausea and pancreatitis (Ansari 2008).

Thiopurine drugs have a relatively narrow therapeutic index, with little difference between therapeutic and toxic doses. Consequently, inappropriate dosing may result in ADR, at one end of the spectrum and treatment failure at the other.

Individuals with undetectable TPMT activity are those most at risk of severe ADR including haemopoietic toxicity, myelosuppression or even death, as grossly high levels of 6TGN can occur if they are given standard thiopurine medication (Clunie & Lennard 2004). Treatment with thiopurine drugs is contra-indicated for these patients, although there are reports of individuals with undetectable TPMT being treated successfully with <20% of normal thiopurine drug dosage (Kaskas et al 2003). Individuals with normal TPMT activity can be given the full dose of the thiopurine drug from the start. Those with low activity can be treated, with fewer adverse effects, using a reduced dose (Lennard & Lilleyman 1996; Sanderson et

al 2004; Gardiner et al 2006; Richard et al 2007). Studies, including our own, have provided evidence of a cohort, of approximately 1.8%, with high TPMT activity (Szumlanski et al 1996; Sanderson et al 2004; Coulthard et al 2002; Ford et al 2006b), which may be predicted by an unknown genetic polymorphism. These patients may not respond to standard doses of thiopurine drugs, and are at risk of hepatotoxicity from increased production of methylated thiopurine metabolites such as 6MMP (Dubinsky 2004).

1.5. Strategies for determining patient TPMT status

1.5.1. Phenotyping

Human TPMT phenotype is typically determined by measuring the enzyme activity in RBC. TPMT in these cells is easily accessible and demonstrates a level of enzyme activity and immunoreactive protein which is representative of that found in tissues, such as the liver, where the majority of thiopurine metabolism takes place. (Szumlanski et al 1992; Van Loon & Weinshilboum 1982; Woodson et al 1982).

The following methods are routinely used for TPMT phenotyping:

- **Radio-enzymatic methods** - these rely on the transfer of methyl groups from radio-labelled 14C-methyl-S-adenosyl-methionine to 6-mercaptopurine. The 14C-methyl thio-ether reaction product is then extracted into 20% iso-amyl alcohol in toluene. The radioactivity, which is directly proportional to TPMT activity, is measured using a scintillation

counter. (Weinshilboum et al 1978). These methods are unpopular as the use of radioactive isotopes has health and safety implications.

- **High Performance Liquid Chromatography (HPLC)** - this is the most widely used strategy in routine clinical use. In a typical assay, the products of a reaction in which rate is directly related to TPMT activity, are measured by HPLC with ultra-violet, fluorescence or mass spectrometry detection. The metabolites are injected into a stream of high pressure liquid. This mobile phase carries the sample into a column where separation takes place, as different components have a different affinity for the column packing. Those with least affinity elute first and are carried to the detector where they are quantified.

- **Immunoassays** - qualitative (Rapid Immunomigration) and quantitative (Enzyme Linked Immunosorbant) assays have been developed by BIOLOGIX Research Corporation. These utilize an antibody specific to the riboside of 6-methyl mercaptopurine. There is a kit on the market, but so far these methods have not found their way into routine use.

The advantages of phenotyping are the relatively low costs and ease of analysis. The result assesses the activity of TPMT, giving an idea of the extent of gene expression which can be of major benefit. The three to four fold variations in activity, seen amongst wild type individuals, can influence dosing

even among this group (Yip et al 2008). Phenotypic assays can also assess other factors that may affect enzyme activity, for instance presence of inhibitors such as aminosalicylates (Dewit et al 2002). Disadvantages of phenotyping include possible misclassification for recently transfused patients. This can have serious consequences as the deficient status of a patient can be masked by TPMT activity present in the patient's sample due to donor cells. (Ford et al 2004b). Misclassification using phenotypic analysis could theoretically occur in patients whose therapy interferes with the in vitro reaction or which induces enzyme activity (Ames et al 1986; Lysaa RA 1996; Szumlanski & Weinshilboum 1995; Woodson et al 1983; Xin et al 2005).

1.5.2 Genotyping

The TPMT cDNA and gene were cloned and characterized by Honchel et al in 1993. The TPMT gene is located on chromosome 6 and is approximately 34 kb in length. It consists of 10 exons, 8 of which encode for a 245 amino acid long protein. The allele for normal TPMT activity (wild-type) has been designated TPMT *1 (Szumlanski et al 1996). At least twenty five genetic polymorphisms have so far been identified (Tamm et al 2008), the majority of which are associated with decreased levels of TPMT activity. Differences in specific mutant allele frequency have been observed between population groups (Otterness et al 1997). The predominant variant alleles are TPMT*3A, TPMT*3C and to a lesser extent TPMT*2. In Caucasians the most common variant is TPMT*3A (~5%) which has two nucleotide polymorphisms (SNPs), on exon 7 and 10 (Yates et al

1997). In African-Americans the most common mutant allele is TPMT *3C, which has the same, exon 10 SNP as for TPMT *3A (Szumlanski et al 1996), this is also the most common mutation in East Asians (Collie-Duguid et al 1998). It has also been observed that the frequency of mutations in the TPMT gene is lower in Chinese populations (Engen et al 2006).

Techniques used for TPMT genotyping include:

- **Restriction Fragment Length Polymorphism (RFLP)** - differentiation by analysis of patterns derived from cleavage of DNA, using restriction endonucleases, which cleave DNA molecules at specific nucleotide sequences.

- **Amplification Refractory Mutation System (ARMS)** - takes advantage of the fact that the 3'-OH end of a primer bound to template DNA is required for the polymerase enzyme to synthesize a new copy of DNA. Any mismatch leads to PCR failure. Consequently, any point mutation can be detected by synthesizing two primers specific to the wild type or mutant DNA. Several mutations can be screened for at once, using multiplex reactions containing more than one set of primers.

- **Single Strand Conformational Polymorphism (SSCP)** - this is a method to screen the exons of the TPMT gene for mutations using

electrophoresis. This method, although relatively simple and low cost, is time consuming and is not suited for routine use in a clinical setting.

- **Denaturing High-Performance Liquid Chromatography (DHPLC)** - identifies mutations by detecting sequence variation in re-annealed DNA strands (hetero-duplexes). This method efficiently detects single nucleotide and insertion/deletion variation in crude PCR products directly without DNA sequencing.

- **DNA microchip technology** - fluorescently labelled amplified DNA is hybridized with oligo-nucleotide DNA probes immobilized in gel pads on a biochip. This specially designed, costly biochip can recognize six point mutations in the TPMT gene and seven corresponding alleles associated with TPMT deficiency (Nasedkina et al 2006).

- **Mass spectrometry** - highly multiplexed genotyping of TPMT variants has been performed using matrix-assisted laser desorption/ionization - time of flight analysis (MALDI-TOF) (Schaeffeler et al 2008). A matrix is used to protect the molecules during ionisation, transforming the energy from the laser into excitation energy. The time-of-flight analyser separates the ions according to their mass (m) to charge (z) (m/z) ratio by measuring the time it takes for them to travel through a field free region.

Most of these techniques require polymerase chain reaction (PCR), which amplifies a specific region of DNA, defined by a set of two ‘primers’. DNA synthesis is initiated by a thermo-stable DNA polymerase. It was developed in 1987 by Kary Mullis and associates. Usually, at least a million-fold increase of a specific section of a DNA molecule can be realised, the amplified products (amplicons) are usually between 150-3,000 base pairs in length and can be separated and detected using gel electrophoresis.

One of the main limitations of PCR based genotyping techniques is that they do not identify on which allele a mutation lies. Consequently, where two different heterozygous mutations are identified, it is not possible to distinguish whether they lie on the same allele or on different alleles. If they lie on the same allele, one allele is wild type. This is a heterozygote genotype which will result in low activity, requiring treatment with a reduced dose of thiopurines. If the mutations lie on different alleles the patient would be classed as a compound heterozygote. They would have deficient TPMT status and thiopurine drug therapy would be contraindicated. It is rarely practical to screen for all known TPMT mutations, so rare mutations are likely to be missed as well as any new deficient TPMT activity causing mutations. Another important disadvantage of TPMT genotyping is that it does not identify the small percentage of high methylators which are at risk of hepatoxicity (Dubinsky & Lamothe 2000). Genotypic analysis has the advantage of not being affected by drug interactions, RBC transfusions or sample age but methods are slower, much more costly than phenotyping, can only detect common mutations and do not give a quantitative result. Consequently,

genotyping is not often the first-line approach to routine TPMT screening. However, it has a clear complementary role, alongside phenotyping in a routine TPMT service, in the following scenarios:

- To confirm the deficient TPMT status of patients identified by phenotyping.
- Phenotypic / genotypic correlation. This will establish a clearer reference interval between patients heterozygous for the deficient TPMT activity allele and those who are homozygous for normal TPMT activity alleles.
- To determine the TPMT status of patients who have recently been transfused, for whom phenotyping is not reliable.

The concordance between phenotyping and genotyping has been reported to be approximately 60-70% for patients with low TPMT activity and >90% overall (Ford et al 2009; Schaeffeler et al 2004).

1.6. City Hospital Birmingham International TPMT Screening Service

At City Hospital a nationwide referral service was established in 2003. This is now used by over 200 external laboratories. This service has made TPMT testing in the UK quicker, cheaper and more accessible. In this time the laboratory has received over 60000 requests for TPMT phenotypic analysis. Approximately 12% of these were reported as a low TPMT activity, while over 200 patients with undetectable TPMT activity have been identified. At present, (Sept 2009) over 350 samples are analysed per week, with the capability for over 160 per day.

The service provided for TPMT phenotyping used the determination of thiopurine S-methyltransferase activity in erythrocytes using 6-thioguanine as substrate and a non-extraction liquid chromatographic technique (Ford & Berg 2003). This was an improvement on older assays, making it more suitable for routine use. This was the first NHS hospital in-house assay to be CE marked. This has required the use of a rigorous vigilance system, described in Chapter 5, which has helped control this inherently complex assay.

A strategy for routine genotyping TPMT alleles is also in place which underpins the phenotyping service. Based on the multiplex amplification refractory mutation system (ARMS) of Roberts et al, modifications make it more suitable for routine use. This offers a screening method for the TPMT gene, without having to resort to expensive molecular biology equipment. In comparison to restriction digest techniques, where incomplete cutting of the target DNA can lead to inaccurate results, it seems to be more reliable and is less time consuming. This can detect the common mutations of the TPMT gene, TPMT*2, TPMT *3A and TPMT *3C.

1.7. Thioguanine Nucleotides

The next step to aid management of patients on thiopurine drugs is the measurement of 6TGN. Monitoring of 6TGN levels can increase drug efficacy and reduce the likelihood of side effects as it guides dosage (Gearry et al 2006). A correlation has been demonstrated between 6TGN concentration and clinical remission rates (Cuffari et al 2001 & 2004; Dubinsky 2005). An inverse correlation has also been observed between the level of RBC 6TGN and

leucocyte counts, which is consistent with the immunosuppressive effects 6TGN (Lennard et al 2004).

6TGN are formed as shown in Figure 1.3. This figure also shows the TPMT catalysed methylation of 6-thioinosine 5'-monophosphate which leads to the production of 6-methylthioinosine 5'-monophosphate. This metabolite is found in relatively high concentrations. It is an inhibitor of de novo purine synthesis and high levels are associated with hepatotoxicity (Gardiner et al 2008).

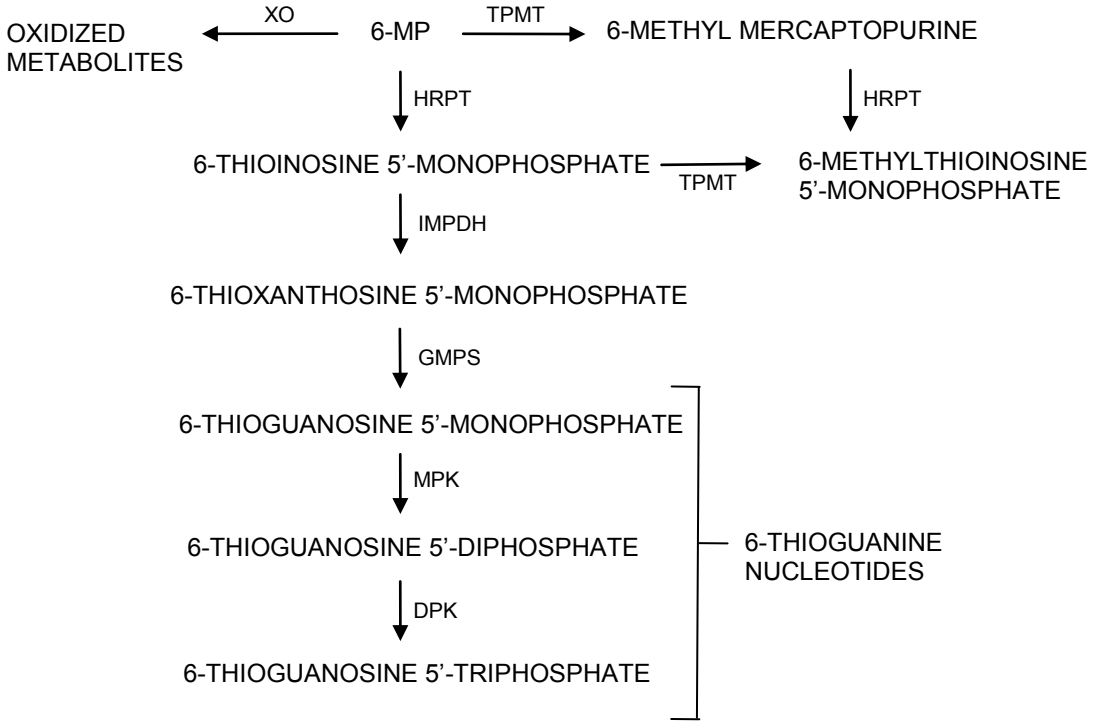


Figure 1.4 ***Thioguanine nucleotide formation***

HPRT=hypoxanthine phosphoribosyltransferase, XO=xanthine oxidase, TPMT=thiopurine methyltransferase, IMPDH=inosine monophosphate dehydrogenase GMPS=guanosine monophosphate synthetase. The diphosphates and triphosphates are formed by their respective monophosphate (MPK) and diphosphate (DPK) kinases

Therapeutic drug monitoring of thiopurine drugs is complex. Plasma 6MP levels and the measurement of plasma and urine thiouric acid, the xanthine oxidase pathway metabolite, have been utilised (Weller et al 1995; Bruunshuus & Schmiegelow 1989). Thiouric acid is only useful in assessing compliance, as it does not give any indication of therapeutic response. Both these assays are limited in their reliability to predict efficacy or toxicity. A greater understanding of thiopurine metabolic pathways has led to the development of methods to estimate 6TGN concentration. These assays use erythrocytes as an indicator of intracellular levels of metabolites in the target cells i.e. peripheral blood leucocytes or bone marrow pre-cursors. The RBC is not an ideal surrogate for these target cells as it is not nucleated. It therefore lacks full purine functionality, as IMPDH, one of the enzymes required for the conversion of 6MP to 6TGN, is not expressed in erythrocytes. The 6TGN produced, as a result of 6MP metabolism, are made indirectly, largely in hepatic tissue. Therefore erythrocyte 6TGN levels are more likely to reflect liver cell, rather than target white blood cell metabolism. (Dulay & Florin 2005). This is different for patients being treated with 6-thioguanine (6TG), a guanine analogue that can be converted to 6TGN via HRPT. This direct incorporation of 6TG into 6TGN, compared with the indirect route followed by 6MP, results in relatively higher RBC 6TGN in patients receiving 6TG than those on 6MP. Patients who prove intolerant to 6MP may benefit from 6TG therapy. (Dulay & Florin 2005). Ideally 6TGN should be measured in non-stimulated target cells. This would be difficult in practice as it would involve the laborious purification of leucocytes. In the measurement of

RBC 6TGN it is difficult to avoid contamination with white blood cells, which could affect results. Further problems arise when considering sample stability between collection and analysis. The degree of degradation of the labile cell nucleotides depends on factors such as storage conditions, time and anti-coagulant used.

Increased RBC 6TGN and improved efficacy of 6MP and azathioprine have been noted when these thiopurines are co-administered with allopurinol (Teml et al 2007). This is a treatment which prevents hyperuraemia, gout and formation of calcium oxalate renal stones. Allopurinol inhibits xanthine oxidase, theoretically increasing the conversion from 6MP to 6TGN. A patient receiving this drug would require a reduction in their thiopurine dose, to a third or less, suggesting that two thirds of a normal dose is catabolized by xanthine oxidase. This contradicts the experience of patients that are deficient for TPMT who are successfully treated with 5% of a normal dose. This would suggest that 95% of 6MP is catabolized by TPMT. It has been found that allopurinol co-therapy with low-dose Azathioprine can alleviate thiopurine hepatotoxicity (Ansari et al 2008).

Studies have demonstrated elevated 6TGN levels and subsequent thiopurine sensitivity in patients treated with methotrexate, an antifolate drug. It has been suggested that a polymorphism of a key folate gene (MTHFR) may be responsible. This gene regulates the supply of the s-adenosyl - methionine (SAM) co-factor.

Complex variations e.g. differing tissue distribution, possible inhibition or induction of enzymes, require research to explain these drug interactions further.

1.8. Research Objectives

1.8.1 Development and implementation of whole blood TPMT assay

Since its introduction, improvement of the TPMT service at City Hospital has been ongoing. Pre-analytical steps of TPMT phenotype analysis, using the method of Ford & Berg 2003, were quite laborious, using a series of centrifugation and washing steps to prepare erythrocytes, from which a lysate was finally produced. This required a relatively large primary sample volume. Also, it had become apparent that the pellet of washed red blood cells, produced as part of this process, was not homogeneous, as repeat aliquots did not give the same result. Further washing and mixing was required to obtain a sample that could be aliquoted repeatedly to give acceptable precision.

Further issues involved higher than expected numbers of patients having results reported in the low activity range. It was suspected that the cut-off between low and normal activity was set too high. Possible explanations for this include:

- Reference ranges were established using results from samples that had been taken in the last 24hrs, which is not typical for the samples routinely received for analysis, although our studies have shown TPMT activity to be stable at 4°C for up to 8 days.

- The patient group from which samples are received is not a 'normal' population; many could be undergoing therapy that could affect observed TPMT activity.

- As the method has become optimised, under the efforts of continuous quality improvement, variables that could impact on results have come under more vigilant control.

A major research objective in this work has been to develop a new method for analysis of TPMT in whole blood. Research would then be carried out to determine if whole blood is better than RBC for monitoring TPMT activity and detection of any induction effects as the new assay entered routine use. A major comparative study between activity in RBC preparations and activity in whole blood samples alongside relevant screening for common genetic mutations is also presented.

1.8.2 Monitoring the new assay

This work assesses the whole blood TPMT phenotyping methodology. This has involved looking at all aspects of the method, identifying actual and potential causes of variation and implementing limiting measures wherever possible. Reagents, enzyme kinetics, operator variability and instrumentation are all evaluated. The effect of sample age and haemoglobin concentration will also be considered. Finally the use of the service will be assessed.

1.8.3 Development and implementation of whole blood 6TGN assay

A method for 6TGN analysis in RBC lysates has already been developed in the City Hospital Laboratory. This technique is rapid and well suited for routine use in a clinical laboratory and provides a service for 6TGN monitoring in the UK. A further aim of this work has been to improve this method and assess the feasibility of measuring 6TGN in whole blood rather than washed erythrocytes.

1.8.4 Induction study

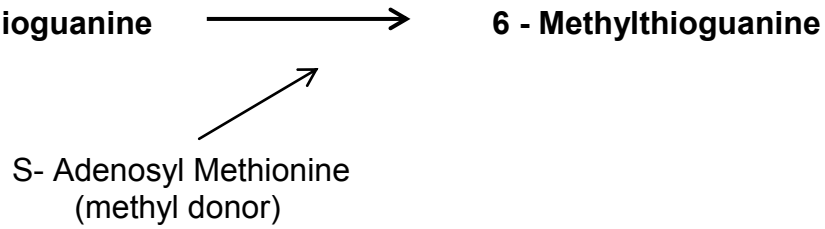
It has been suggested that TPMT activity may be induced by thiopurine drugs (Weyer et al 2001; Thervet et al 2001). If induction occurs this may necessitate dose adjustment during therapy to maintain adequate active metabolite levels and associated therapeutic effects. Induction effects may be more apparent in whole blood samples, due to the presence of nucleated cells which unlike erythrocytes can continually synthesize protein. To test this hypothesis it is intended to monitor TPMT activity and 6TGN levels in patients commencing on azathioprine treatment.

2. MATERIALS AND METHODS

2.1 TPMT Phenotyping

2.1.1 Assay principle

TPMT catalyses the methylation of 6-thioguanine (6TG), in the presence of a methyl donor, to produce highly fluorescent 6-methylthioguanine (6MTG). The amount of 6MTG produced is directly proportional to the amount of enzyme in the sample. Results are expressed in nmol 6MTG/g Hb/hour.



2.1.2 Red blood cell (RBC) method

A lysate of RBC was prepared from a whole blood sample containing the anticoagulant ethylenediaminetetraacetic acid (EDTA). The sample was centrifuged at 2000g for 5 minutes then the plasma and buffy coat removed. An equal volume of sterile physiological saline was added to the cells; the sample was then mixed and re-centrifuged. The supernatant and white blood cells (WBC) were removed and the RBC rewashed in saline, retaining the WBC for genotyping if required. After a third centrifugation the supernatant and any white

cells were discarded, 200ul of the washed, packed RBC were then mixed with 1ml of phosphate buffer (0.02M KH_2PO_4 *(Sigma-Aldrich)* titrated with 0.02M K_2HPO_4 *(Sigma-Aldrich)* to pH 7.4). The hypotonic shock lysed the RBC.

The haemoglobin concentration of the lysate was measured using a colorimetric method. The reagent *(Bayer ADVIA Haemoglobin)* is 20mM potassium cyanide in a 2% dimethyllaurylamine oxide solution. In this method the haem iron in the haemoglobin is oxidised from the ferrous to the ferric state. It is then combined with cyanide to form the reaction product, which is measured at 546nm. *(Instrumentation Laboratories ILab 600 analyser).*

$$\underset{Fe^{++}}{\text{Haemoglobin + Reagent}} \longrightarrow \underset{Fe^{+++}}{\text{Methaemoglobin}} \longrightarrow \underset{Fe^{+++}.CN}{\text{Cyanide haemoglobin}}$$

2.1.3 TPMT incubations

200ul of lysate was added to 500ul of Test Reagent (59mg S-adenosyl methionine *(Sigma-Aldrich)* and 140mg 6TG *(Alpha Aesar)* in 1 litre of 0.1M phosphate buffer 0.01M KH_2PO_4 *(Sigma-Aldrich)* titrated with 0.01M K_2HPO_4 *(Sigma-Aldrich)* to pH 7.4). This mixture was incubated at 37°C, for one hour. Protein was precipitated by heating to 90°C and removed by centrifugation.

2.1.4 HPLC analysis

The 6MTG produced was measured by reverse phase HPLC, using a Kingsorb 3µ C18 90A, 75 x 4.6mm column *(Phenonomex)*. The mobile phase used was 0.05M phosphate buffer (0.05M KH_2PO_4 *(Sigma-Aldrich)* titrated with 0.05M

K_2HPO_4 *(Sigma-Aldrich)* to pH 6.2), acetonitrile *(VWR)* and tetrahydrofuran *(VWR)* in the ratio 89:7:4. Fluorescence detection was performed at an emission wavelength of 390nm, excitation 315nm *(Shimadzu HPLC, LC Solutions software)*. A 0.11nmol/L 6MTG *(Sigma-Aldrich)* one-point calibrator (20ng/L) was used. Normal and low TPMT control samples were prepared from volunteers with a known genotype. The Sandwell and West Birmingham Ethical Committee have given approval for collection of these samples. Calibrators and controls were treated in the same way as patient samples.

This method used is based on that of Ford & Berg, published in 2003. Reference ranges were published based on 1000 results from out-patients in the local population (Ford et al 2004a).

	nmol/6MTG/gHb/h
TPMT Deficient	<5
Low Activity	6-24
Normal Activity	25-55
High activity	>55

2.2 TPMT genotyping extraction

2.2.1 Cell lysis

0.5ml of the white blood cell rich buffy coat was treated, on ice for 20 minutes, with 1ml buffer (0.32M sucrose, 1% Triton-X 100, 0.01M Tris pH 7.5, 0.005M $MgCl_2$ *(Sigma-Aldrich)*), to lyse the cells The samples were then centrifuged, the cell lysis step repeated and centrifuged once more, before removal of the

supernatant. The resulting nuclear material pellet was re-suspended in 100µl buffer (10mM Tris-HCl, 0.4M NaCl, 2mM EDTA titrated to pH 8.2 with NaOH *(Sigma-Aldrich)*) and 5µl 10% sodium dodecysulphate *(Sigma-Aldrich)*. Protein digestion was achieved by incubation with 10µl 10mg/ml DNAse free Proteinase K *(Sigma-Aldrich)* in a water bath, for 2.5 hours at 65°C or 37°C overnight.

2.2.2 Phenol/chloroform extraction

The protein digests were mixed gently with an equal volume of buffered phenol pH 8.0 *(QBiogene),* then centrifuged and the upper aqueous layer removed and retained. Treatment with phenol and centrifugation was repeated until the resultant aqueous layer was clear. 300ul of Chloroform/Butanol 80:20 *(Sigma-Aldrich)* was added to the aqueous phase and mixed gently by inversion, followed by centrifugation. The supernatant was transferred to a sterile micro-centrifuge tube.

2.2.3 DNA precipitation

Sodium acetate (3M) was added to the supernatant in the ratio 1:10. The sodium ions shield the negative charges on the DNA phosphates. This decreases inter-strand repulsion and allows the nucleic acids to aggregate. Four volumes of ice-cold 100% ethanol *(Sigma-Aldrich)* were then added, the precipitated DNA appeared as white strands. After gentle mixing, followed by centrifugation for 10 minutes, the ethanol was removed. 300µl of ice-cold 70% ethanol was added to the pellet of DNA. After gentle mixing, the ethanol was removed and the pellet

left to dry at room temperature for approximately 20 minutes. The dried pellet was re-suspended in 50-200µl (depending on the size of the pellet) buffer (10 mM Tris pH 8.0 and

1 mM EDTA *(Sigma-Aldrich)*). The pellet was left to dissolve at room temperature for a minimum of 30 min then stored at -80°C.

2.2.4 Purity and concentration of DNA

Care must be taken to ensure protein and phenol are absent from the isolated DNA as both can interfere with PCR reactions. The purity of the isolated DNA can be assessed by measuring the absorbance at 260 and 280nm. Pure preparations of DNA have ratios of optical density at 260nm / optical density at 280nm of 1.7-2.0 (a ratio < 1.7 suggests phenol or protein contamination, >2.0 suggests RNA contamination).

The DNA concentration in µg/ml can be estimated using the Beer-Lambert Law, $A = \varepsilon cl$, which relates the amount of light absorbed at a given wavelength to the molar extinction coefficient and concentration of an absorbing molecule. Where A is absorbance, ε is the molar extinction coefficient, c is the concentration of the solute, and l is the length of the light path of the solution measured (cm). Maximal spectral absorption by double stranded DNA occurs at 260 nm and is characterized by a specific absorption coefficient of 0.02(µg/ml) $^{-1}$ cm $^{-1}$. This absorption is a function of the molar extinction coefficients of the substituent nucleotide bases.

If the DNA concentration is very low (<50 μg/ml) the DNA can be concentrated by re-precipitation in ethanol, drying then re-suspension in a smaller volume of buffer. If the DNA concentration is very high (>500 μg/ml), it can be diluted with buffer, until a final concentration of 500 μg/ml is achieved.

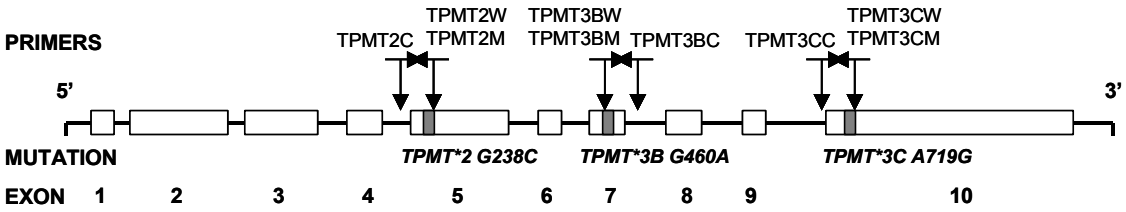
2.3. TPMT genotyping polymerase chain reaction

This simplified version of the ARMS of Roberts et al uses a PCR master-mix which contains pre-optimised concentrations DNA polymerase, $MgCl_2$, deoxynucleotide triphosphate and buffer. Using a master-mix reduces the number of pipetting steps which decreases the time taken to set up a reaction, reducing the likelihood of pipetting errors and increasing reproducibility.

Primers used for ARMS analysis were for the common mutations of the TPMT gene TPMT*2, and TPMT*3C and were custom-synthesised to order. The synthesis scale used was Standard >15 O.D. units.

The specificity of the primers was checked using NCBI BLAST programme (www.ncbi.nih.gov/BLAST/)

2.3.1 Primer sequences



Primer	Type (wildtype/mutation)	Direction	Sequence (5' – 3')
TPMT2C	Common wild type	Sense	ATCTgCTTTCCTgCATgTTCT TTgAAACCC
TPMT2W	Wild type	Antisense	CACACCAACTACACTgTgTCCCCggTCTCC
TPMT2M	TPMT*2 mutation	Antisense	CACACCAACTACACTgTgTCCCCggTCTCg
TPMT3CC	Common wild type	Sense	ATTTTTAgTAgAgACAgAgTTTCACCATCT
NTPMT3CW	Wild type	Antisense	TgTCTCATTTACTTTTCTgTAAgTAgTT
NTPMT3CM	TPMT*3C mutation	Antisense	TgTCTCATTTACTTTTCTgTAAgTAgTC

Table 2.1 ***Primer sequences***

Primer positions indicated are relative to the sequence of human TPMT gene, Genbank Accession number AB045146.

2.3.2 Primer composition

Two different multiplex reactions are required for TPMT genotyping:

TPMT32Wild

TPMT32Mutant

The primer composition of each multiplex reaction is shown in the table below:

Multiplex Reaction	Reaction	Forward Primer	Reverse Primer	Predicted Product Size
TPMT32Wild	B2M (Control)	BMIF	BMIR	574
	WildType TPMT*2	TPMT2C	TPMT2W	194
	WildType TPMT*3	TPMT3CC	NTPMT3CW	325
TPMT32Mut	B2M (Control)	BMIF	BMIR	574
	Mutant TPMT*2	TPMT2C	TPMT2M	194
	Mutant TPMT*3	TPMT3CC	NTPMT3CM	325

Table 2.2 ***Multiplex reaction primer composition***

Method

For each multiplex reaction, 20 μl of the appropriate 100ρmol/μL primer stocks (*Alta Bioscience)* were added and made up to a final volume of 1 ml using sterile water. After mixing by inversion 10 x 100 μL fractions were aliquoted into 1.5 ml labelled sterile micro-centrifuge tubes and store at -20°C. Before use, the multiplex PCR master mix kit, multiplex primer mixes and patient DNA samples were completely thawed at room temperature.

To guard against cross-contamination a blank containing no DNA template was performed for both the TPMT32Wild and TPMT32Mut multiplex reactions

Analysis of TPMT*1/*1, TPMT*1/*2, TPMT*1/*3C and TPMT*3C/*3C control DNA was included each time the PCR analysis was performed

Both TPMT32Wild and TPMT32Mut multiplex reactions were performed for every patient sample. 2.5μl of the appropriate primer master mix and 1ul of DNA was

added to a 0.2ml thermal tube containing 9µl of RNase-free water and 12.5µl multiplex master mix *(Queen)*.

The tubes were processed in the thermal cycle using the following parameters:

Stage	Temp	Time	Cycles	Prig N°	Link
HotStarTaq DNA polymerase activation	95°C	15 min	1	4	5
PCR Amplification:			30	5	7
Denaturing	94°C	30s			
Annealing	62.5°C	90s			
Extension	72°C	30s			
Final Primer Extension	72°C	2 min	1	7	8
Hold at 4°C	4°C	Hold	1	8	E

Table 2.3 ***PCR Parameters***

2.4. TPMT genotyping electrophoresis and visualisation

Agarose (2g) *(Sigma-Aldrich)* was heated until dissolved in 100ml of a 1:5 dilution of Tris borate EDTA (TBE) buffer (54g Tris base *(Sigma-Aldrich)*, 27.5g boric acid *(Sigma-Aldrich)*, 7.4g EDTA *(Sigma-Aldrich)* made up to 1 litre in distilled water). After cooling to approximately 65°C, 5µl ethidium bromide *(Sigma-Aldrich)* was mixed in and the molten gel poured into a welled casting tray. When set the gel was placed in the electrophoresis gel chamber and submerged in 1:5 TBE buffer.

A loading dye was prepared from (25mg Orange-G *(Sigma-Aldrich)*, 1.5g Ficoll *(Sigma-Aldrich)*, 100µl 1M Tris pH 8.0 *(Sigma-Aldrich)*, 20µl 0.5M EDTA *(Sigma-Aldrich)*). 5µl of dye was mixed with each 25ul of ARMS PCR reaction mix, 20µl of this mixture was loaded into the wells. Electrophoresis of the PCR products

was carried out at 100V for approximately 45 minutes, until adequate separation was achieved as indicated by the dye front.

The resulting DNA fragments were visualised under UV light using a Gene Genius Bio-imaging system *(Syngene).*

2.5. Thioguanine nucleotides

2.5.1 Assay principle

6TGN extraction is performed using a single reagent containing perchloric acid, an internal standard (IS) and an anti-oxidant. 6TGN are hydrolysed back to 6TG which is measured using isocratic HPLC with UV detection.

2.5.2 Method

RBC were first isolated from other blood components in a whole blood EDTA sample. This was achieved by centrifugation, removal of plasma and buffy coat and washing RBC in an equal volume of physiological saline. These steps were repeated and the resulting RBC re-suspended in saline, aiming for an approximate red cell count of $8x10^{12}$ cells/L.

A red cell count was performed *(Bayer Advia 120)*. Two 100µl aliquots were pipetted into labelled micro-centrifuge tubes, using a displacement pipette, these were frozen for a minimum of 15 minutes at -80°C before analysis.

A concentrated calibrator stock solution (20mg/100ml) was prepared from 20mg 6TG *(Alpha Aesar)* dissolved in 4ml of 0.1M NaOH *(Sigma-Aldrich)* made up to 100ml with distilled water. This was diluted 1:20 in distilled water to give 1mg/100ml. This was further 1:20 diluted with Paratek *(Alpha Laboratories)* a

haematology blood control (checked to contain no measurable 6TGN/6TG), to give a working concentration of 50ng/ml.

An internal quality control sample was also prepared from pooled patient samples. Samples where clinical details indicated that the patient was on thiopurine therapy were collected and the RBC washed as previously described. These were pooled, mixed and aliquoted into 100µl fractions and stored at -80°C. This was initially analysed 5 times to give a target internal quality control concentration.

Two 100µl aliquots of calibrator, internal quality control and each patient sample were allowed to defrost at room temperature. 500µl of hydrolysing reagent (10ml 70% perchloric acid *(Sigma-Aldrich)*, 400µl 10mM 5-bromouricil *(Sigma-Aldrich)*, 500mg DL-Dithiothreitol (DTT) *(Sigma-Aldrich)* made up to 100ml with distilled water) was added to each aliquot. Each aliquot was capped and vortex mixed immediately after addition of the hydrolysing reagent. The samples were then centrifuged, to separate the precipitated proteins. The supernatant from each micro-centrifuge tube was then transferred, using a micro-pastette, into appropriately labelled HPLC vials. The vials were then capped and heated at 95°C for 1 hour to hydrolyse liberated 6TGN back to their parent 6TG. The addition of DTT prevents oxidative degradation of 6TG, which is promoted by contaminant ions and UV light and prevents the binding of 6TG to acid denatured RBC proteins. The 6TG concentration is measured using HPLC with UV detection at a wavelength of 304nm. Any losses are accounted for by inclusion

of the internal standard, 5-bromouracil, which is detected at a wavelength of 342nm.

The hydrolysing reagent was found to stable for at least 1 week at room temperature. Aliquots of a sample stored at -80°C were analysed over an eight day period with both freshly prepared reagent and reagent prepared on Day 1.

Results are as follows:

Day	6TGN pmol/8x10^8 cells *Fresh Reagent*	6TGN pmol/8x10^8 cells *Pre-prepared Reagent*
1	215	215
4	248	232
6	249	261
7	201	201
8	257	253

Table 2.4 ***Stability of 6TGN hydrolysing reagent***

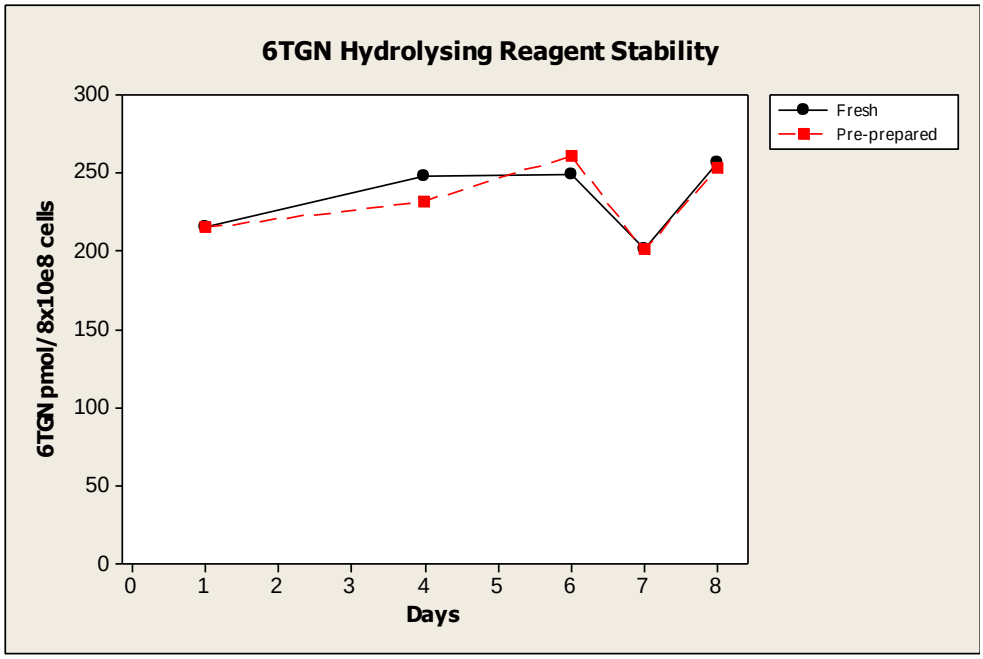


Figure 2.1 ***Stability of 6TGN hydrolysing reagent***

3. WHOLE BLOOD TPMT ACTIVITY

3.1 Development of a new phenotyping assay underpinned by genotyping

This section of work develops a new TPMT phenotyping method, which analyses whole blood rather than washed RBC. This new method uses freezing at -80C as the method of cell rupture, instead of using a lysis buffer.

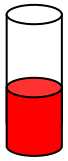
Whole blood samples (n=402) that had been collected into EDTA tubes and received for routine analyses of TPMT activity, were selected randomly over a four-week period. Samples were stored for a maximum of 2 days at 4°C prior to analysis. Samples greater than 8 days old at time of analysis were excluded from the study as earlier work in the department has shown TPMT activity to decline after a week at room temperature.

3.1.1 Phenotype

The whole blood samples were mixed well by inversion and two 200 μl aliquots were accurately pipetted into test tubes and capped. The tubes were frozen for 15 minutes at -80°C to disrupt the cells, percentage cells lysed is detailed in Chapter 5.3. The cells were shock thawed by the addition of 600 μl of whole blood suspension buffer (0.1 M KH_2PO_4 titrated with 0.1 M K_2HPO_4 *(Sigma-Aldrich)* to pH 7.4), then vortex mixed. RBC lysates were also prepared in duplicate, from the same EDTA blood samples. The buffy coats were retained in separate labelled test tubes for DNA extraction and TPMT genotyping.

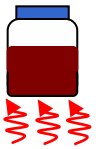
Freeze 200µl whole blood at -80°C for 15 minutes. Add 600µl buffer and mix to produce a whole blood lysate

Determine lysate haemoglobin concentration

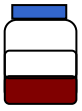


Incubate 200µl of lysate with 500ul of test reagent for exactly 1 hour at 37°C

Stop the reaction by heat inactivation at 95°C for 10 minutes



Centrifuge vials to produce a clear supernatant



Measure 6MTG by HPLC fluorimetry

Figure 3.1 ***Whole blood TPMT analysis***

The TPMT enzyme reaction, determination of haemoglobin concentration and HPLC analysis were performed on all whole blood and RBC lysates, as described in the methods chapter.

For both the standard RBC and whole blood methods, TPMT activities were calculated from 6-methyl-thioguanine (6MTG) peak height, and expressed in relation to haemoglobin concentration (nmol 6MTG/gHb/h).

3.1.2 Genotyping

DNA was extracted from buffy coats using phenol-chloroform extraction and our multiplex amplification refractory mutation system (ARMS) strategy was used to screen for the common TPMT mutations, TPMT*2 and TPMT*3 (TPMT*3A, TPMT*3C and TPMT*3D). The gel provides the support medium for the separation of the DNA fragments which were visualised using ethidium bromide, under UV light as shown in Figure 3.1.

For each PCR run it was checked that there were no products in the blank reactions. If there were, due to cross-contamination, the run was rejected and repeated.

For each DNA sample it was checked that the control B2M product was present in both the wild and mutant reactions, if it was not the ARMS reaction was rejected and repeated.

As part of the DNA method, quality control checks were made to ensure that the TPMT*1/*1, TPMT*1/*3C, TPMT*1/*2 and TPMT*3C/*3C control DNA reactions had all worked. If these QC checks failed the run was rejected and repeated.

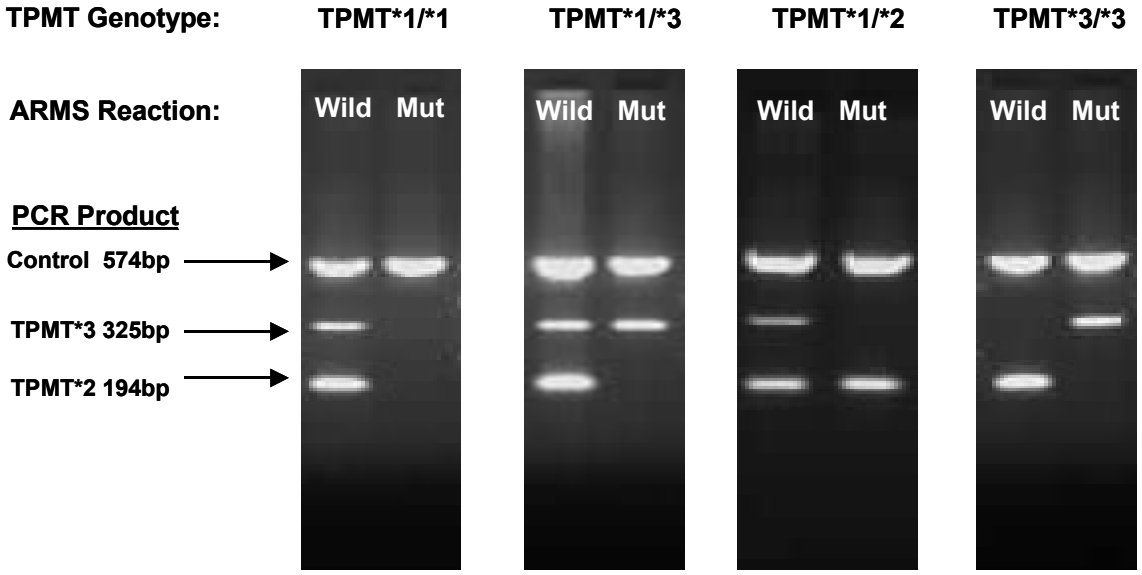


Figure 3.2 ***Examples of DNA fragments visualized for wild type, heterozygous and homozygous deficient genotypes***

To evaluate this new technique the whole blood results were compared to both the established RBC lysate method results and the TPMT genotype. Agreement between duplicates for each assay was compared and the percentage difference between methods was calculated. Reference intervals were determined for the new whole blood assay, in-line with the RBC method, by using the already established percentages for deficient, low and normal TPMT status individuals, previously obtained from the RBC method phenotyping-genotyping data (Ford et al 2004a).

3.1.3 Results

The data for this study can be found in Appendix 2

Method	Mean TPMT	Median	Std Dev	SE Mean	Min	Max
RBC	37.0	36.0	11.3	0.56	0.0	82.0
Whole Blood	51.0	50.0	13.6	0.68	0.0	102.0

Table 3.1 ***Summary of TPMT activities in nmol 6MTG/gHb/h for RBC / whole blood comparison study. 402 samples analysed in duplicate by both methods***

The intra and inter batch variation for the new method was determined using EDTA whole blood collected from a healthy volunteer.

Method	Imprecision	Mean TPMT activity (nmol 6MTG/gHb/h)	CV (%)	n
Conventional RBC	Within batch	39	3.6	10
	Between batch	53	8.0	20
New Whole Blood	Within batch	45	2.7	10
	Between batch	53	7.6	20

Table 3.2 ***Within batch and between batch imprecision data for RBC and whole blood phenotyping methods***

3.2 Correlation of enzyme activities

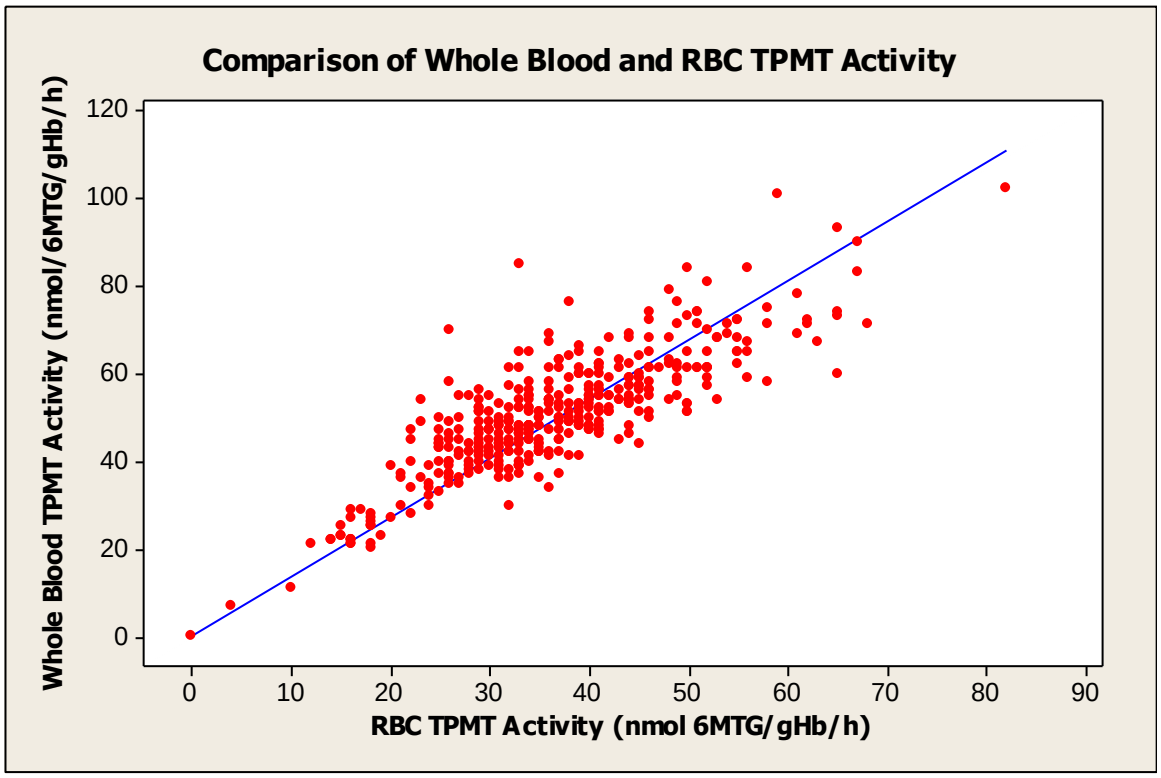


Figure 3.3 ***Relationship between mean whole blood and mean RBC TPMT activity r = 0.834***

3.3 Comparison with genotyping

TPMT activities were higher, per g of Hb, in whole blood than in RBC, in 98.5% of samples. The mean percentage increase observed in whole blood was calculated to be 42%, with a range of -8.5% to 172.5%. The mean TPMT activity for whole blood was 51 nmol 6MTG/gHb/h compared to the RBC lysate mean of 37.

The normal, low and deficient TPMT groups give rise to tri-modal distributions of activity. These three groups can be seen for both methods in Figure 3.4.

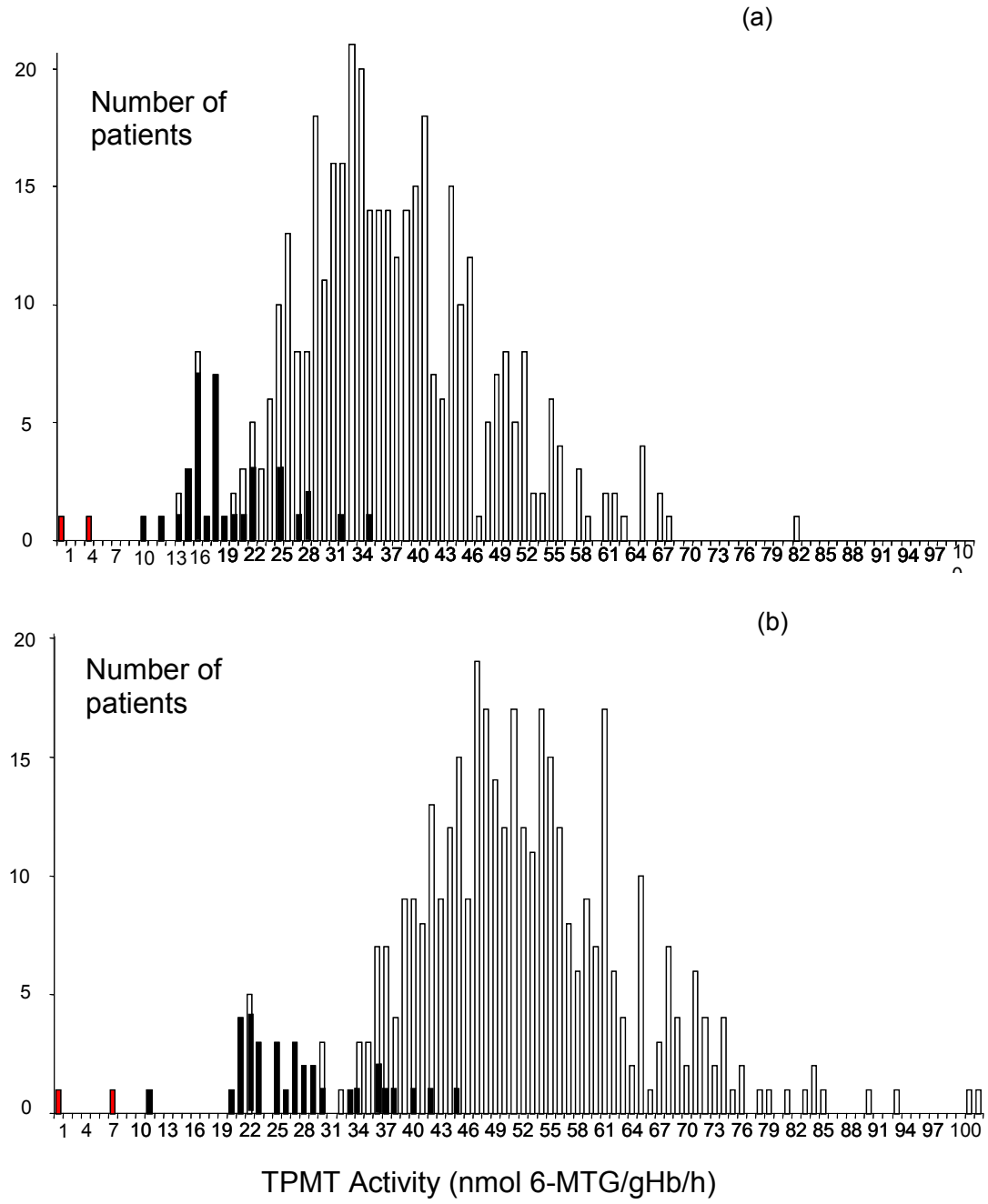


Figure 3.4 *Distribution of TPMT activity and genotype in 402 individuals*
*(a) TPMT activity measured using red blood cell lysate. (b) TPMT activity measured using Whole blood lysate. Individuals with a TPMT*3/*3 genotype are shaded red, a heterozygous genotype, either TPMT*1/*2 or TPMT*1/*3 are shaded black and TPMT*1/*1 genotype white. TPMT, thiopurine S-methyltransferase; 6-MTG, methylthioguanine*

Genotype	Number of Patients	% of Patient Total
TPMT *3/*3	2	0.5
TPMT *1/*3	31	7.7
TPMT *1/*2	3	0.8
TPMT *1/*1	366	91

Table 3.2 ***Summary of TPMT genotypes for RBC / whole blood comparison study***

Concordance between low TPMT activity and heterozygotes was 79% for both the new whole blood and standard RBC lysate methods, while overall concordance with genotypic analysis was 97% for both phenotypic methods. This is as expected as the RBC assay has been used to determine the whole blood assay reference intervals.

3.4 Advantages of new assay

Offering whole blood TPMT phenotyping has a number of benefits:

- **Easier and quicker** - Washed RBC do not need to be prepared. Elimination of washing the cells twice in physiological saline saves around two hours of biomedical scientist time on an average working day. This has obvious cost and turnaround time implications.
- **Safer** - Sampling is directly from the primary tubes which are used to take blood from the patient and are fully labelled. This minimises any sample handling errors.
- **More reproducible** - The original whole blood sample is more homogeneous than a pellet of washed cells. The mean percentage difference between duplicates was 9.4% for RBC lysates and 5.1% for

whole blood. This shows that the new whole blood method can inherently give more reproducible results

- **Increased accuracy** - Whole blood may give a better indication of what is happening in vivo rather than RBC only, being representative of the reactions taking place in drug metabolizing tissues.

Multiplex ARMS testing for TPMT*2 and 3*C mutations has been used to define the reference ranges for low and normal TPMT activity. This has been used to demonstrate that the new whole blood TPMT phenotyping method performs as well as the conventional RBC lysate assay. This new method overcomes the need to prepare washed RBC, a process that is time-consuming, and increases analytical variation. The resulting assay is better suited to a regional or national TPMT phenotyping service.

When whole blood samples were analysed, using the RBC method, higher activity was virtually always observed. It was postulated that this increase was due to the contribution to TPMT activity by leucocytes. In some samples, the whole blood activity was markedly higher than in RBC. This led to the hypothesis that TPMT activity maybe induced in some patients, which would be seen primarily in the nucleated white blood cells.

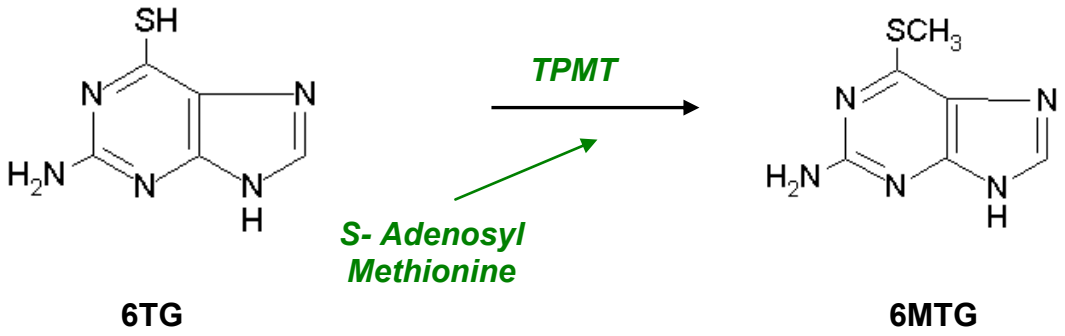
The development of this assay has been published by us; Ford L, Graham V, Berg J. (2006) Whole-blood thiopurine S-methyltransferase activity with genotype concordance: a new, simplified phenotyping assay. Ann Clin Biochem. Sep;43(Pt 5):354-60

4. EVALUATION OF PHENOTYPING

4.1 Enzyme Assay

4.1.1 Introduction

Our TPMT phenotyping method depends on the measurement of the product formed in the reaction:



6TG **6MTG**

Figure 4.1 ***TPMT phenotyping reaction***

A constant rate of reaction, where velocity throughout the incubation time is directly proportional to enzyme concentration, is required in order to calculate enzyme activity.

In reactions of this type reaction rate can decline due to:

- **Substrate depletion** - velocity decreases as the enzyme becomes less saturated
- **Product inhibition**
- **Instability of components**

- **Assay method artefact** - if the specific detection procedure ceases to respond linearly to increasing product concentration, this can lead to a decline in the measured rate of reaction with time
- **Change in assay conditions** - for example if a reaction causes a pH change, this will affect the rate if adequate buffering systems are not in place.

These factors are controlled in the assay and the reaction is stopped after a fixed incubation time, within the linear period.

4.1.2 Establishing substrate concentrations

Enzyme kinetics are examined periodically, usually when a new lot of substrate is received, or when problems are encountered, to ensure measurement is being performed in the linear range.

In our assay, where the amount of enzyme present is being determined, the amount of product needs to be influenced by the level of enzyme activity only. Therefore zero order kinetics are established, with regard to our two substrates. The concentration of 6TG and SAM is determined using a Michaelis Menten approach to ensure there is sufficient of each substrate in the reaction mixture to achieve such zero order kinetics. This is carried out by analysing a high activity sample using the routine phenotyping method, but varying the substrate concentrations independently as shown in Figures 4.2 and 4.3.

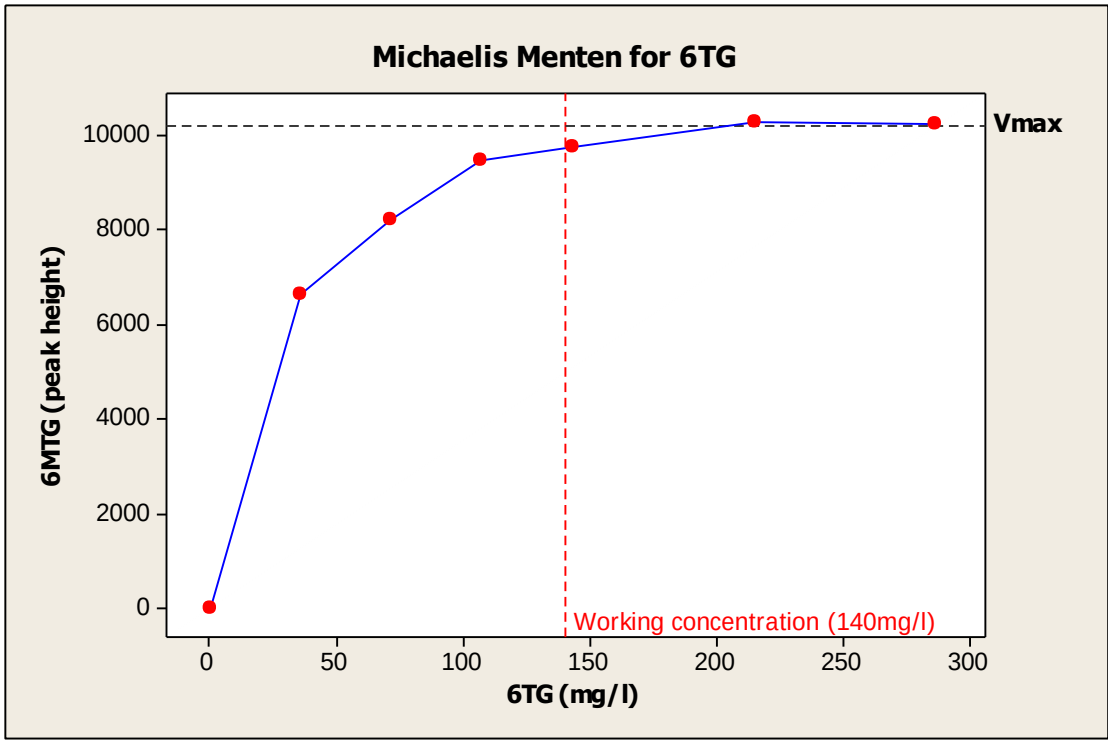


Figure 4.2 ***Michaelis Menten plot for 6TG showing the final choice of substrate concentration used in the assay***

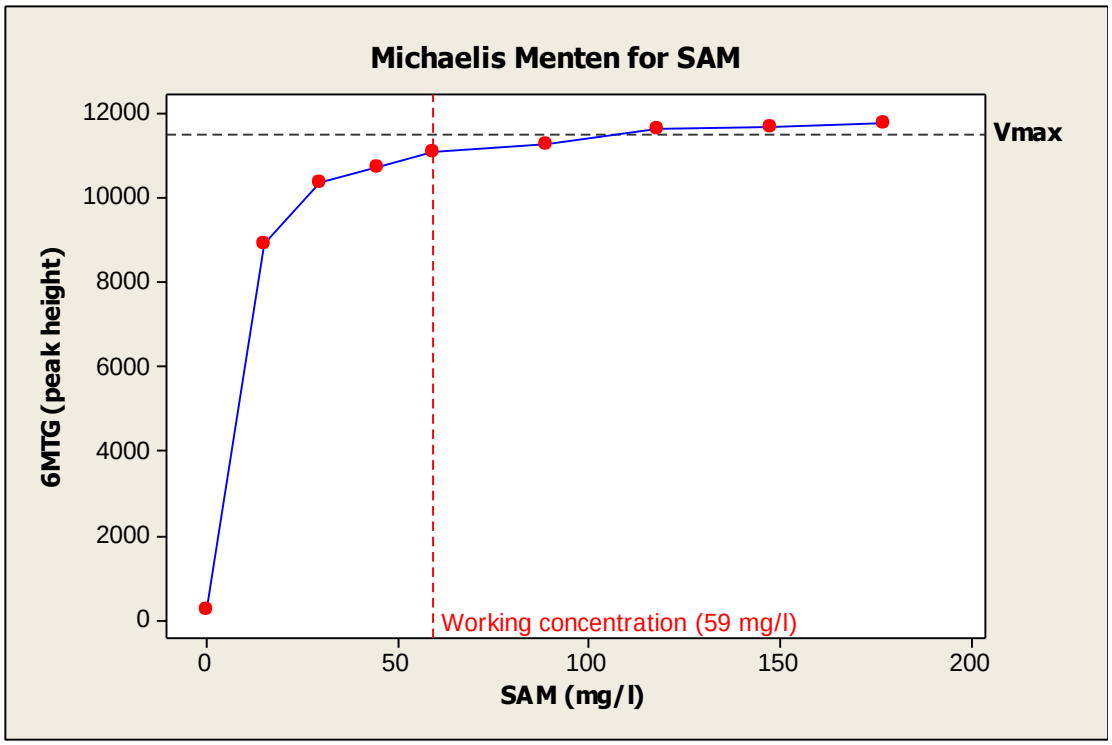


Figure 4.3 ***Michaelis Menten plot for S-adenosyl methionine showing the final choice of substrate concentration used in the assay***

Working concentrations of the substrates have been selected so that the reaction is at, or close to, its maximum rate (Vmax). Concentrations above this are avoided, as this excess substrate is seen to contribute to increased blank readings.

4.1.3 Linearity of the reaction

It is also required that the kinetics of the reaction are first order in relation to the enzyme. This is demonstrated in Figure 4.4, where the enzyme concentration is directly proportional to the product formed.

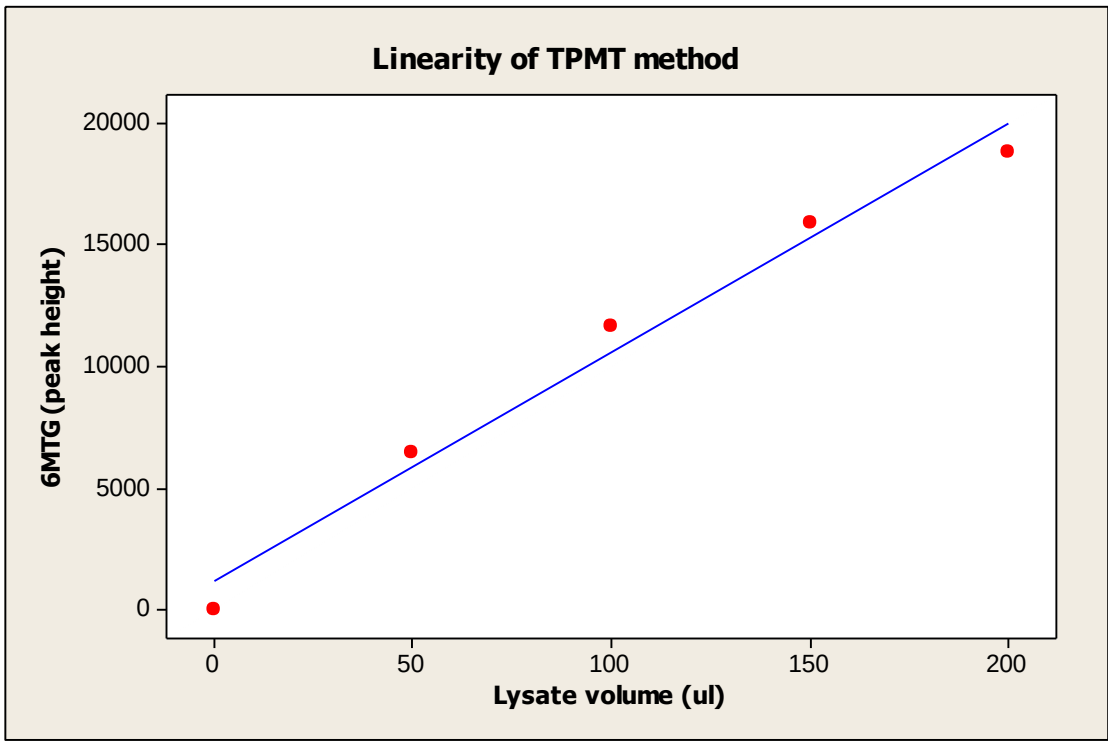


Figure 4.4 ***Linearity of the TPMT assay***

Another experiment was carried out to check the linearity of the TPMT phenotyping method. Four samples covering a range of TPMT activity were carried through the method using a series of dilutions of the whole blood lysate. Activity per gram of Hb was calculated for each reaction. Results are presented in Figure 4.5.

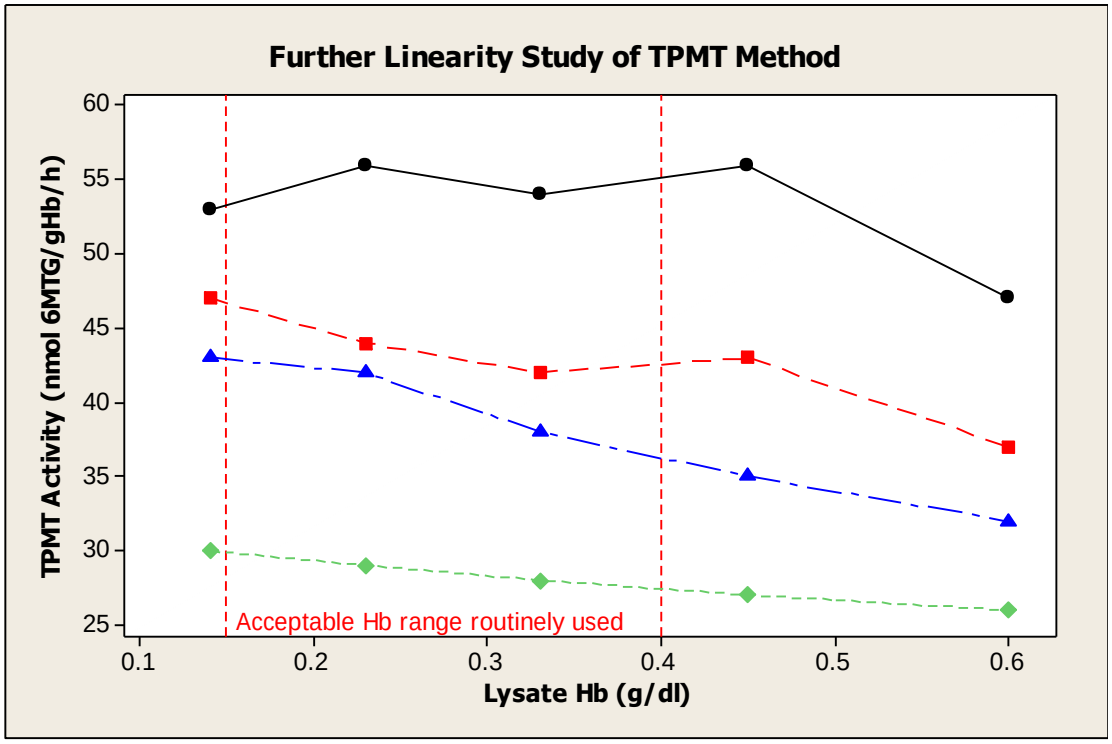


Figure 4.5 ***Study of linearity using a range of lysate dilutions***

It can be seen in Figure 4.5, that as the amount of sample in the reaction mixture is increased, activity tends to reduce slightly, this may reflect substrate depletion. For this reason, only lysates with a Hb concentration within the range indicated are acceptable.

4.1.3 Controlling reaction conditions

Accurate reaction start times may be difficult to determine and bursts or lag phase in product formation can occur, which could distort the reflection of the linear reaction. Effects of these are minimised in our assay by using sand in the hot block wells to dissipate heat, thus avoiding spikes in temperature and by pre-warming all components of the reaction mixture prior to their combination.

Additional important practical considerations include purity and stability of all reagents and samples. This necessitates appropriate supply, storage and handling. The chemicals for our assay are purchased in bulk from consistent sources. Reagents are prepared in lots then stored appropriately. Preparation, freezing and thawing is carried out as quickly as possible, to avoid any reagent deterioration. Samples are stored at 4°C and analysed on the next working day, to minimise further loss of enzyme activity.

4.1.4 Importance of blank

Background rates of reaction can be observed in the absence of TPMT. Causes for these readings include contamination of one of the components or non-enzymatic reactions. It is important to compensate for these ’blank’ readings, or falsely high readings could be recorded. This is achieved for our assay by preparing a vial containing all components except the sample lyste which is replaced with saline. This vial is carried through the method and the resulting chromatography is automatically subtracted from that of the unknown samples.

4.1.5 Checking for artefactual product peaks

It is routine practice to check for any underlying peaks for samples with very high TPMT activity. Any substance present in the sample, which elutes at the same retention time as 6MTG and exhibits similar fluorescent properties, could lead to falsely elevated results. This could cause major consequences if such a peak 'masked' a deficient result. The original method for checking for any such interfering substances was to immediately move the reaction vial to the 90°C precipitation block as soon as the test reagent had been added to the sample. In theory this was a zero incubation time. On several occasions a 6MTG peak was observed on these samples. This seemed to be the result of some reaction actually taking place, as these were very high activity samples and a finite period of time was being taken to reach a sufficient temperature to stop the reaction. An attempt has been made to overcome this problem by a change of practice. Reaction vials containing the test reagent are preheated to 90°C, before addition of the sample lyste. At this temperature no reaction can occur, as all protein is denatured immediately. Even though this is a true zero time incubation some 6MTG is still observed. This seems to be due some methylation of the 6TG in the preheating stage. For this reason a blank zero minute incubation reagent vial is prepared and the blank reading subtracted from the tests. To date well over 1000 such checks on high TPMT activity samples have not identified any directly interfering substances.

4.1.6 HPLC

After the reaction, the incubate is treated so that it is suitable for analysis by HPLC with fluorescence detection. The metabolites of the TPMT reaction are injected into a stream of high pressure liquid (mobile phase) which carries the sample into a column and then to the detector. Separation takes place in the column as different components have a different affinity for the column packing. Those with least affinity will exit first. Each component elutes from the column as a narrow band or peak. The retention time on the column is specific to the compound and the peak height is proportional to its concentration.

The retention time is influenced by:

- Characteristics of the stationary phase (pore diameter, particle shape, surface area and pH)
- Composition of the mobile phase - the elution power increases as the polarity of the solvent decreases
- pH and salts
- Temperature

Fluorescence is the detection method of choice for our assay as it has greater specificity and sensitivity than UV detection. Fluorescence-based photometric detection methods are capable of much greater sensitivity than absorbance assays as comparisons are being made between a reading and zero, in contrast to the comparison between two relatively high readings.

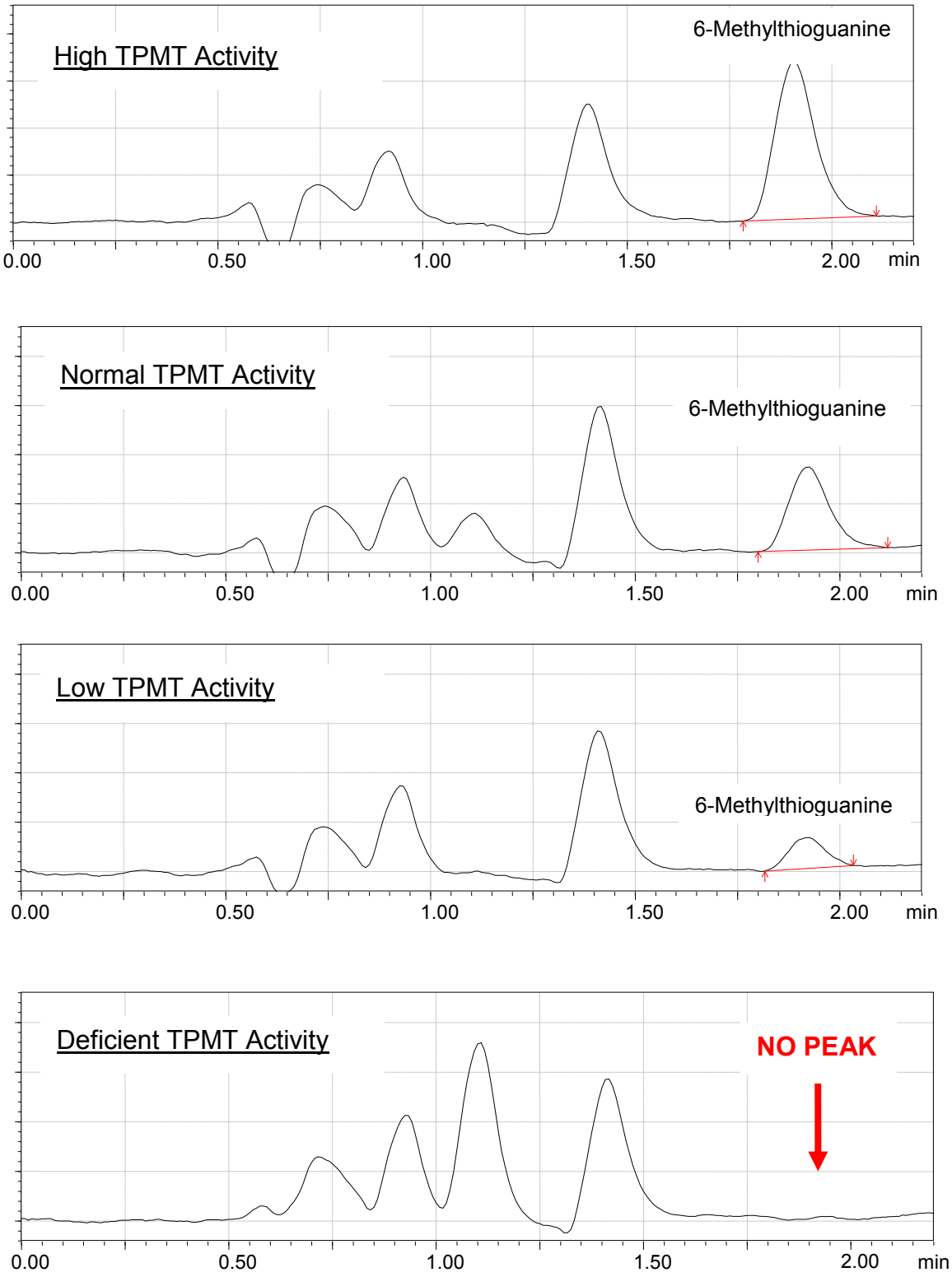


Figure 4.6 ***Chromatography typical of high, normal, low and deficient TPMT***

Originally, a calibration curve was constructed to cover the range expected from the assay. This exhibited a linear relationship; therefore calculations are now based upon the measured fluorescence of a single point calibrator, whereby a 6MTG standard of known concentration is on each run.

4.2 Effect of sample age

The samples received for TPMT phenotyping come from all over the world and can take several days to reach the laboratory. The storage and transport conditions prior to receipt are unknown, therefore it is important to have some understanding of the stability of TPMT at various temperatures.

An experiment was carried out looking at the change in TPMT activity for 5 donor samples. These samples were aliquoted immediately after taking and stored at three different storage temperatures.

The results of this study are presented graphically in Figures 4.6 - 4.8.

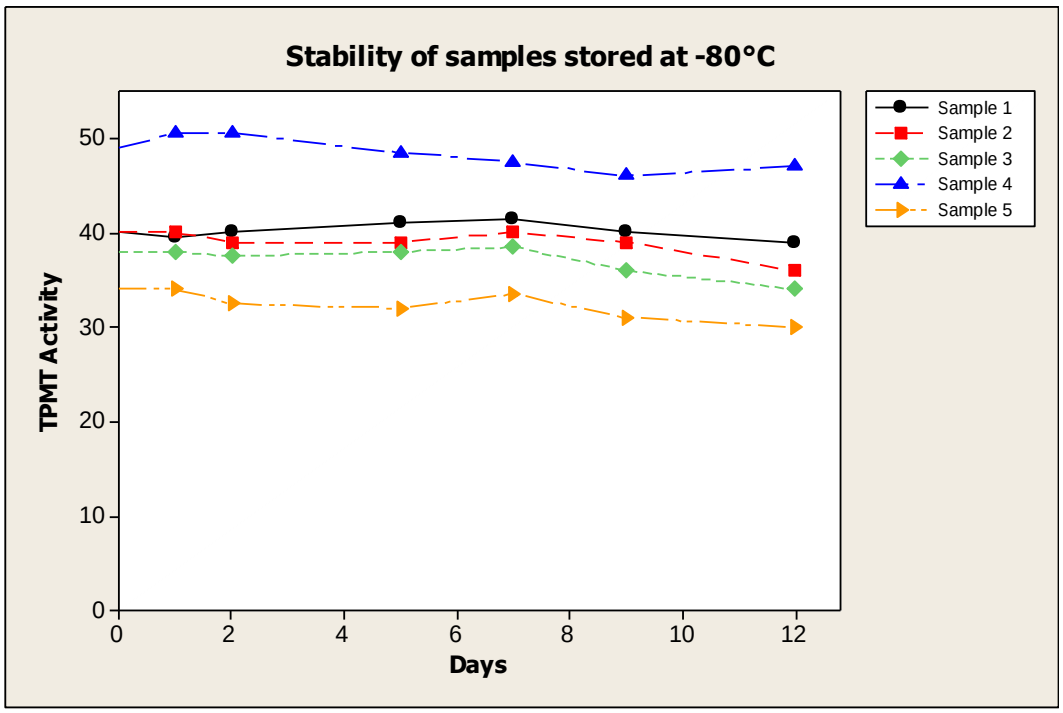


Figure 4.7 ***TPMT activity in samples stored at 80°C***

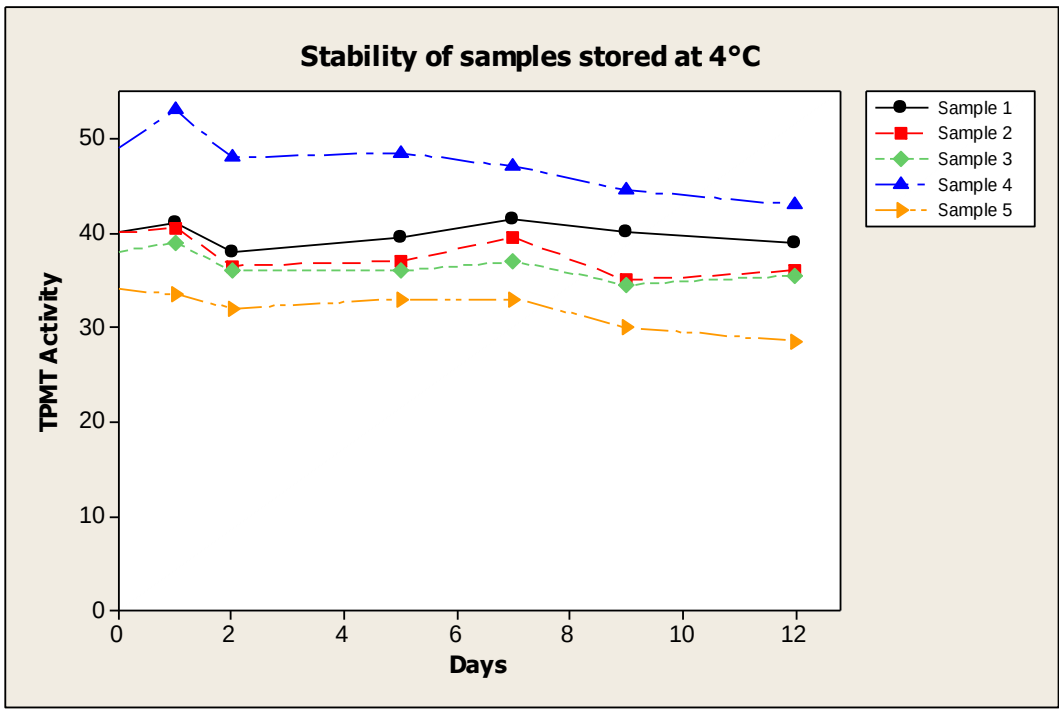


Figure 4.8 ***TPMT activity in samples stored at 4°C***

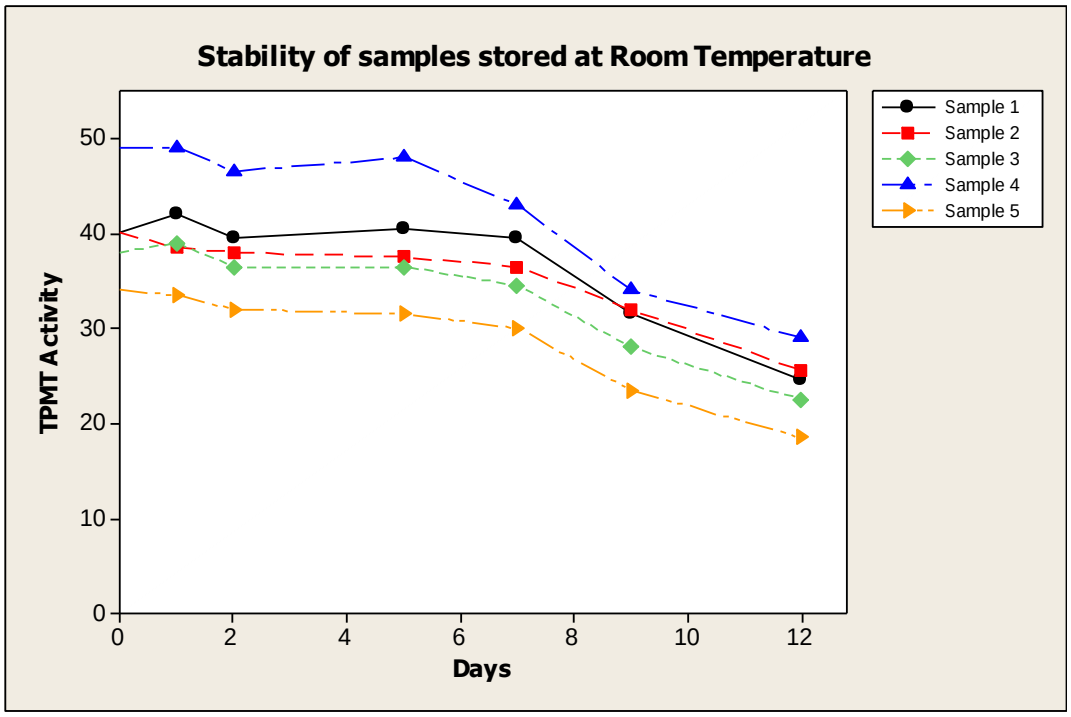


Figure 4.9 ***TPMT activity in samples stored at room temperature***

These results show that TPMT is stable for over 10 days at -80°C and 4°C, but activity is seen to fall after about 6 days at room temperature.

A second experiment was carried out where blood from a suitable donor was collected into 3 specimen tubes. These were stored as follows:

1. Aliquoted into 200µl fractions and stored at -80°C
2. 4°C
3. Room Temperature (ranged from 18.5 - 24°C)

On each working day a frozen aliquot and 200µl of whole blood from each of the other storage conditions were analysed using the whole blood TPMT method.

4.2.1 Results

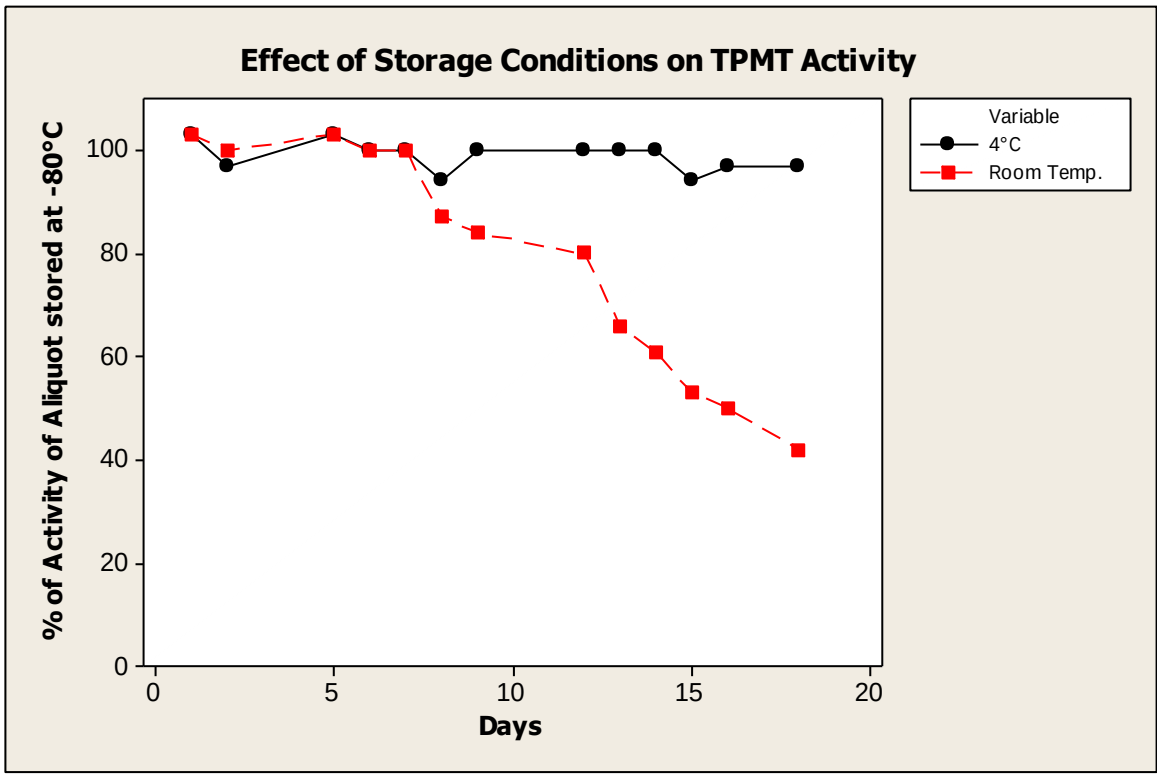


Figure 4.10 ***TPMT activity of samples stored at 4°C and room temperature compared to activity when stored at -80°C***

Activity of 4°C and room temperature samples were calculated as a percentage of the 80°sample activity, on each day.

The results from both these experiments suggest that TPMT activity is stable for over 10 days at 4°C but begins to fall after 6 days at room temperature.

4.3 Haematology effects

4.3.1 Low patient haemoglobin

TPMT activity is expressed per gram of haemoglobin. The RBC act as a surrogate marker for cells, such as the liver, where TPMT activity takes place. (Szumlanski et al 1996). The inter-relationship between Hb and calculated TPMT activity has been studied in several ways:

- From 14/08/07 to 28/08/07 527 samples were phenotyped for TPMT. In this period there were 13 high TPMT activity results, 54% of these had a low lysate haemoglobin concentration (defined as <2.6g/dL). If the applied haemoglobin correction for these samples is removed, all but one of these samples would be classified as normal.
- Nineteen EDTA samples from patients with low Hb (<7.6g/dL) were collected from the haematology department. The whole blood and RBC TPMT activity of these patients was determined. This was found to be high (>80 nmol 6MTG/gHb/hr) in 42% of the specimens, as shown in Table 4.1. This is a significant proportion as routinely only 2 to 3% of our results fall into this high category.
- In 2005 a questionnaire was sent out with all high TPMT activity reports. Of the 42 responses received 45% of patients were classed as anaemic at the time of testing.

RBC TPMT (nmol 6MTG/gHb/h) Normal (25-56)	**Whole blood TPMT** (nmol 6MTG/gHb/h) Normal (35-79)	**Sample Hb** (g/dL) Normal Female 11.5-16.0 Male 13.2-17.5
78	103	6.9
113	117	11.8
48	61	6.5
127	134	6.6
45	65	6.9
71	88	6.9
22	27	4.1
24	32	6.0
119	160	6.4
39	45	7.3
74	96	6.3
32	52	7.3
28	50	7.1
31	45	7.3
75	94	7.3
33	71	7.5
119	129	7.5
26	28	7.5
36	68	7.5

Table 4.1 ***RBC and whole blood TPMT for patients with low Hb***

This combined evidence shows that a relatively high proportion of samples that have a low haemoglobin concentration have abnormally high calculated TPMT. This suggests that when the Hb content of RBC is low, it may actually be a poor marker of cellular contents. This raises the question, as to whether elevated

results on patients with low Hb are misleading, sometimes resulting in misclassification of the TPMT status.

Previous experimental work (4.1.3) has shown that differing concentrations of lysates prepared from the same sample give similar results, once corrected for Hb. This demonstrates that the increased substrate/enzyme ratio that exists, in lower concentration lysates, should not significantly affect the reaction rate.

4.3.2 TPMT activity uncorrected for Hb

For the reasons highlighted in 4.3.1 and 4.3.2, it was studied whether it would it be more straightforward to express TPMT activity in units per litre of whole blood (U/L) instead of using haemoglobin correction. This could have advantages over the existing method, as it would be simpler, faster and cheaper and may avoid misclassification of patients with low Hb.

The whole blood phenotype/genotype concordance study data was re-analysed. Comparisons were made between genotype and TPMT activity corrected and not corrected for Hb concentration.

Looking at the whole blood results of samples from the whole blood study (Chapter 3), phenotyping and genotyping for 400 wild type or heterozygous samples were available.

This data was examined more closely to look for differences in phenotype/genotype concordance between activity expressed per gram of Hb and TPMT activity not corrected for Hb.

Results corrected for Hb **Total Concordance = 96.5%**

Low Phenotype (<35 nmol 6MTG/g/Hb/h)		Normal/High Phenotype (>34 nmol 6MTG/g/Hb/h)	
33 (8.3%)		367 (91.7%)	
Wild Genotype	Heterozygous Genotype	Wild Genotype	Heterozygous Genotype
6	27	359	8

Table 4.2 ***Phenotype / Genotype concordance when Hb correction applied***

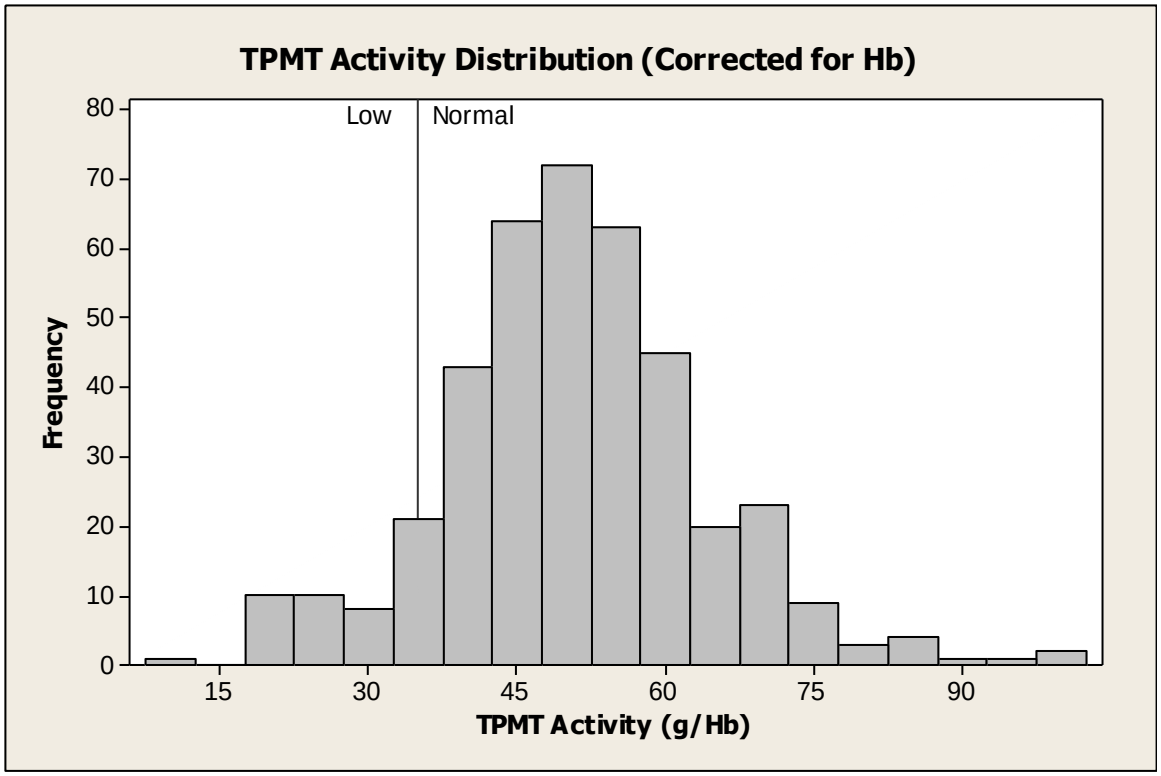


Figure 4.11 ***TPMT activity distribution for 400 study samples, corrected for Hb***

Results not corrected for Hb **Total Concordance = 95.6%**

Low Phenotype (Peak Height 6MTG <7.5)		Normal/High Phenotype (Peak Height 6MTG >7.4)	
36 (9.0%)		364 (91%)	
Wild Genotype	Heterozygous Genotype	Wild Genotype	Heterozygous Genotype
9	27	356	8

Table 4.3 ***Phenotype / Genotype concordance without Hb correction***

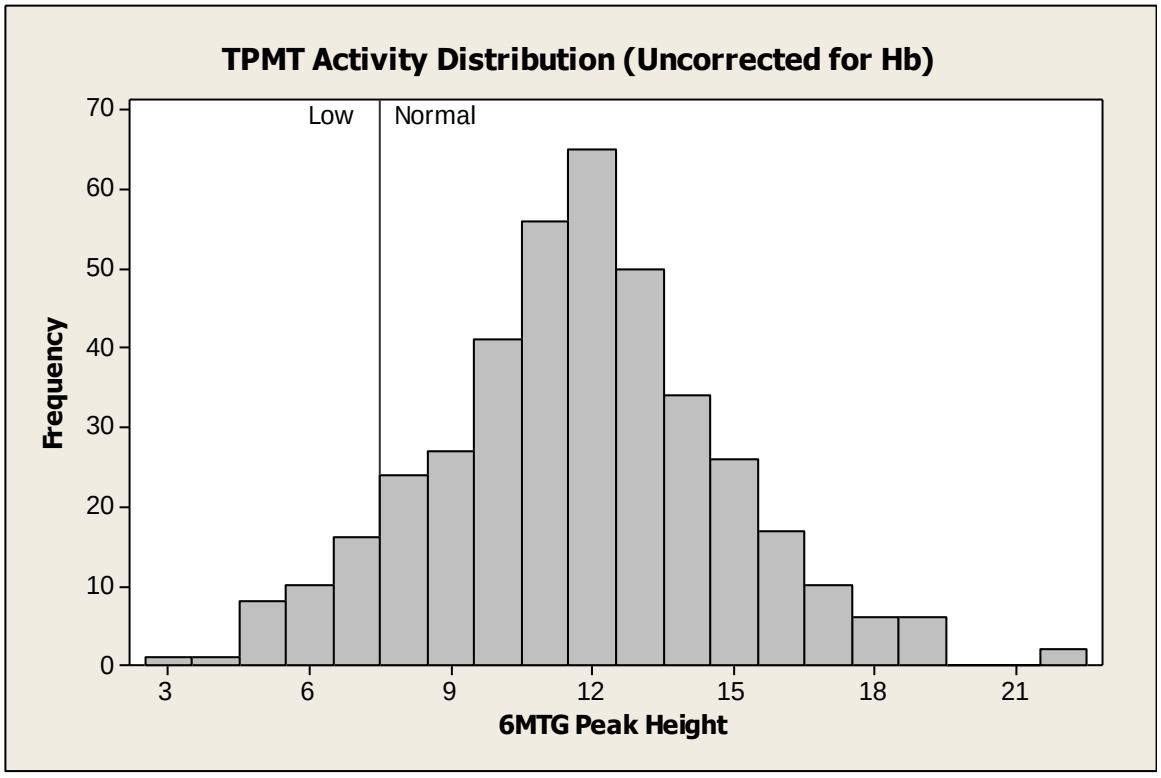


Figure 4.12 ***TPMT activity distribution for 400 study samples, uncorrected for Hb***

A cut-off of 6MTG peak height of 7.5 as the bottom of the 'normal' range for results uncorrected for Hb was set. This gave very similar genotype concordance data to that obtained for results corrected for Hb.

Discordance where the phenotype is low but a wild type genotype is observed is likely to be due to sample deterioration or the presence of a mutation that is not being screened for. Discordance due to the samples that have a normal activity but exhibit a TPMT mutation can be due to one allele coding for relatively high enzyme production or misclassification.

Looking at the results corrected for Hb, there were 8 samples where a 1/*3 genotype was determined but the calculated TPMT activity was in the normal range.

Calculated TPMT activity (nmol/6MTG/gHb/h)	6MTG (Peak Height)	Genotype
36	6.8	1/*3
36	8.7	1/*3
37	7.3	1/*3
38	8.8	1/*3
40	7.3	1/*3
42	7.4	1/*3
45	7.8	1/*3
60	12.4	1/*3

Table 4.4 ***Data for samples with phenotype / genotype discordance***

Looking at 6MTG peak heights, half of these patients would not have been misclassified if interpretation had been based on activity uncorrected for Hb concentration. These results raise the question whether there is any advantage and indeed possibly a disadvantage in correcting for Hb.

4.3.3 Haemoglobin concentration and white blood cell counts

Full blood counts were carried out on 55 samples that had a high calculated TPMT activity. These results suggest a strong correlation between low Hb and high calculated TPMT activity, with approximately 80% of these samples having a Hb concentration below the quoted reference intervals. No obvious correlation was observed between level of TPMT and WBC count.

RBC TPMT (nmol 6MTG/gHb/h) *Normal (25-56)*	**Hb** (g/dL) *Normal Female 11.5-16.0 Male 13.2-17.5*	**WBC count** ($x10^9$/L) *Normal (4.0-11.0)*
79	7.1	4.03
80	8.5	6.61
80	9.6	2.93
80	9.5	7.67
80	12.4	12.38
81	13.7	14.71
81	13.8	12.41
82	11.3	14.20
82	9.9	5.37
82	9.8	13.04
83	13.7	5.74
84	8.8	1.29
84	9.6	5.35
84	11.7	5.36
84	11.2	4.55

RBC TPMT (nmol 6MTG/gHb/h) *Normal (25-56)*	**Hb** (g/dL) *Normal Female 11.5-16.0 Male 13.2-17.5*	**WBC count** ($x10^9$/L) *Normal (4.0-11.0)*
85	8.1	5.97
85	10.3	3.33
85	16.2	6.73
86	11.7	3.79
86	9.0	7.47
86	9.4	4.75
86	13.2	6.00
87	9.5	10.31
87	13.9	8.35
87	10.5	4.68
88	7.3	5.39
88	12.0	8.62
88	11.8	9.52
88	10.7	12.74
89	9.7	6.57
89	9.6	12.99
89	12.9	4.39
87	10.9	7.06
90	10.3	8.42
91	10.7	12.13
92	10.3	7.19
92	11.3	12.48
92	9.5	11.73
92	9.5	5.08
92	9.5	6.23
94	10.3	7.06
95	10.4	5.03
96	9.5	11.93
96	11.0	4.90
97	8.5	7.17
97	12.6	8.20
97	10.2	6.63
100	13.4	3.58

RBC TPMT (nmol 6MTG/gHb/h) *Normal (25-56)*	**Hb** (g/dL) *Normal Female 11.5-16.0 Male 13.2-17.5*	**WBC count** ($x10^9$/L) *Normal (4.0-11.0)*
102	6.6	11.32
102	6.2	5.92
112	7.9	12.59
113	7.6	5.91
115	9.8	6.09
135	7.7	13.34
163	9.8	9.10

Table 4.5 ***Haematology of high TPMT activity samples***

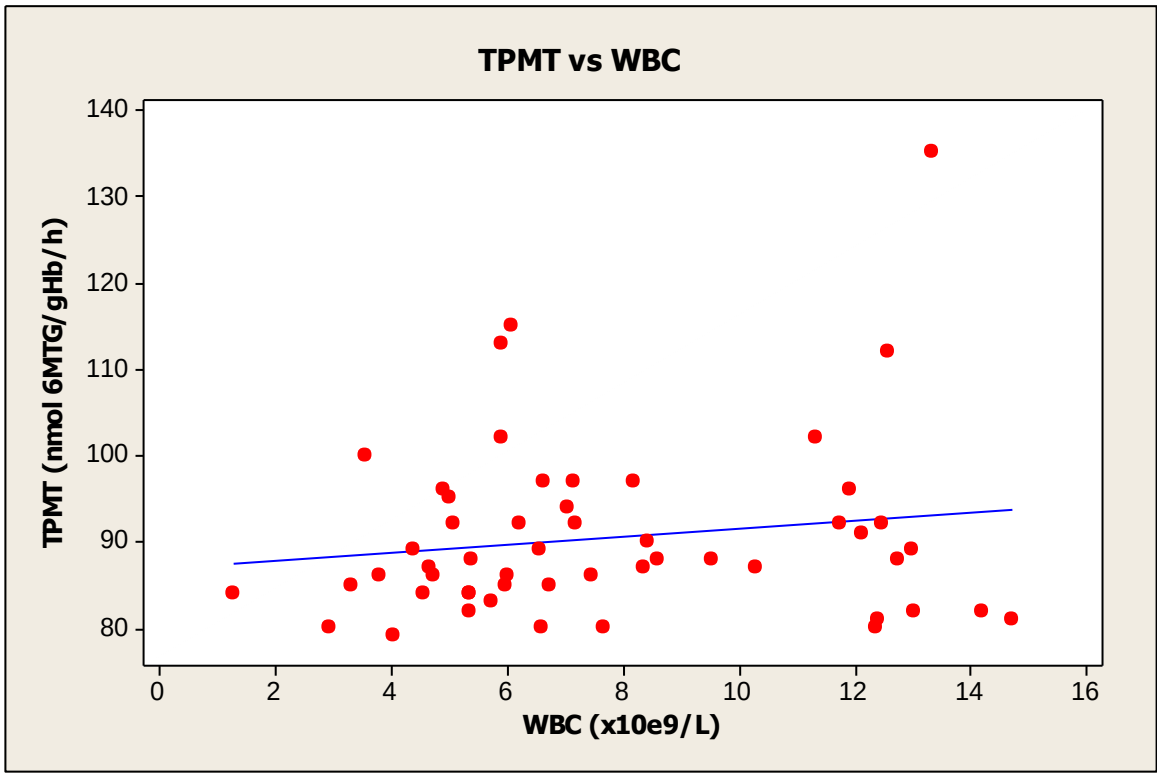


Figure 4.13 ***Correlation of WBC count and TPMT activity*** ***r=0.156***

4.4 Phenotype / genotype comparison

4.4.1 Audit data

	Requests	Deficient <6		Low 6-34		Normal 35-79		High >79	
		No.	%	No.	%	No.	%	No.	%
Jun-07	682	1	0.1	98	14.3	577	84.8	6	0.8
Jul-07	1078	1	0.1	160	14.8	910	84.4	7	0.7
Aug-07	1100	5	0.5	137	12.5	942	85.5	16	1.5
Sep-07	1136	1	0.1	109	9.6	990	87.1	36	3.2
Oct-07	1336	1	0.1	134	10.0	1148	85.9	53	4.0
Nov-07	1305	6	0.5	118	9.0	1146	87.8	35	2.7
Dec-07	1103	1	0.1	106	9.6	964	87.4	32	2.9
Jan-08	1315	4	0.3	129	9.8	1146	87.1	36	2.7
Feb-08	1248	5	0.4	103	8.3	1112	89.1	28	2.2
Mar-08	1113	5	0.5	116	10.4	974	87.5	18	1.6
Apr-08	1372	3	0.2	121	9.0	1216	88.5	32	2.3
May-08	1353	8	0.6	121	8.9	1203	88.9	21	1.6
Jun-08	1360	7	0.5	144	10.6	1180	86.8	29	2.1
Jul-08	1496	10	0.7	125	8.4	1322	88.3	39	2.6
Aug-08	1326	3	0.2	104	7.8	1180	89.1	39	2.9
Sep-08	1464	10	0.7	148	10.1	1278	87.3	28	1.9
Mean	**1237**	**4.4**	**0.3**	**123**	**10.2**	**1081**	**87.2**	**28**	**2.2**

Table 4.6 ***Breakdown of phenotyping results***

	Deficient <6	Low 6-23	Low 24-29	Low 30-34	Normal >34	Heterozygote Concordance
Jun-07	100.0	100.0	69.3	8.0	92.4	63.2
Jul-07	100.0	100.0	84.0	79.6	100.0	67.3
Aug-07	100.0	84.6	91.8	53.6	80.9	78.1
Sep-07	100.0	89.5	94.4	82.9	68.8	89.8
Oct-07	100.0	76.5	90.6	78.0	75.0	83.7
Nov-07	83.3	89.5	64.1	82.1	42.8	82.9
Dec-07	100.0	95.8	75.0	79.3	33.3	73.9
Jan-08	75.0	86.7	95.6	90.0	100.0	86.0
Feb-08	80.0	100.0	94.1	83.9	50.0	85.1
Mar-08	100.0	91.3	93.9	86.2	33.0	88.1
Apr-08	100.0	95.2	90.9	76.5	91.0	87.5
May-08	100.0	100.0	87.0	58.0	94.0	81.7
Jun-08	100.0	87.1	94.8	73.3	94.3	85.1
Jul-08	100.0	100.0	94.5	90.0	76.6	94.8
Aug-08	100.0	100.0	97.5	78.6	60.5	92.0
Sep-08	100.0	97.0	91.5	81.2	82.4	89.9
Mean	**96.1**	**93.3**	**88.1**	**73.8**	**73.4**	**83.1**

Table 4.7 ***Phenotype/Genotype Concordance***

The overall concordance between TPMT phenotype and genotype reported in this audit was 83.1%. This is lower than our previous concordance rate of 96.8% in the whole blood study. However, this audit concentrated on TPMT activity results either side of the cut-off between low and normal TPMT activity rather

than all results, which is the likely explanation for the lower phenotype-genotype concordance found.

The mean concordance between deficient TPMT activity and the 3*/3* genotype is 90%, with the concordance being 100% in thirteen out of the sixteen months the audit was performed. It is likely, where the concordance for deficient TPMT samples is less than 100%, that these samples have TPMT mutations not screened for using our current genotyping strategy. Where no or only one mutant allele was identified these individuals are either compound heterozygous or homozygous for rare mutations, which are not screened for. These findings are highly significant as it demonstrates the importance of our testing strategy, using phenotyping to screen all samples and genotyping for selective testing. Using genotyping alone these patients would have been misclassified and could have been treated with thiopurine drugs with serious consequences. In the period April 2007 to April 2009, 10 patients were identified possessing both*2 and *3 mutations, two of these had deficient activity and 8 had low activity.

Sample instability may have impacted on concordance in some instances where delays in transit meant many samples were older than 8 days when analysed. This could result in a normal genotype being classified as 'low' using phenotyping only. All such 'old' samples are flagged as such on patient reports.

This vigilance system requires constant communication and interaction between the DNA and TPMT laboratories in our department. This produces clinically valid and reproducible results that aid the appropriate treatment of patients screened

by our test. This type of audit is invaluable in monitoring the performance of the phenotyping assay and will remain a part of the stringent quality system.

4.4.2 Mutation type

There have been over 25 mutations in the TPMT gene identified. The location of the mutation and its effect on the protein, for the most common mutations are detailed in Table 4.6.

Designation	Mutation	Exon	Effect on TPMT protein
TPMT*2	G238C	5	80 (Ala) A → (Prol) P
TPMT*3A	G460A	7	I54 (Ala) A → (Thr) T
Double mutant allel	A719G	10	240 (Tyr) Y → (Cys) C
TPMT*3B	G460A	7	I54 (Ala) A → (Thr) T
TPMT*3C	A719G	10	240 (Tyr) Y → (Cis) C
TPMT*3D	T146C	5	98 (Glu) E → (Stop)X
Triple mutant allele	G460A	7	I54 (Ala) A → (Thr) T
	A719G	10	240 (Tyr) Y → (Cys) C

Table 4.8 Common TPMT mutations

The presence of TPMT*2 or TPMT*3C results in a significant decrease in TPMT activity and protein expression, while TPMT*3A results in a more dramatic reduction (Tai et al 1996). As each type of mutation has a different effect on the protein coding for the enzyme, type of mutation may influence the degree of activity reduction. To test this hypothesis, audit data for one year was examined to look at the correlation between mutation type and TPMT activity. This did not show any significant difference in activity between samples that were genotyped as 1/*2 and those identified as 1/*3.

An explanation for this could lie in the screening strategy. One of the mutations screened for is A719G, which is present in both *3A and *3C genotypes, as can be seen from Table 4.8. If this mutation is detected, it is not possible to tell whether patient is a *3A or a *3C genotype.

Several allelic variants are silent, producing no detectable effect, the most common being TPMT *1s (Alves et al 2000).

4.5 Use of the service

4.5.2 Requestors

The demand for the service has increased steadily over the last three years. This is demonstrated graphically in Figure 4.14.

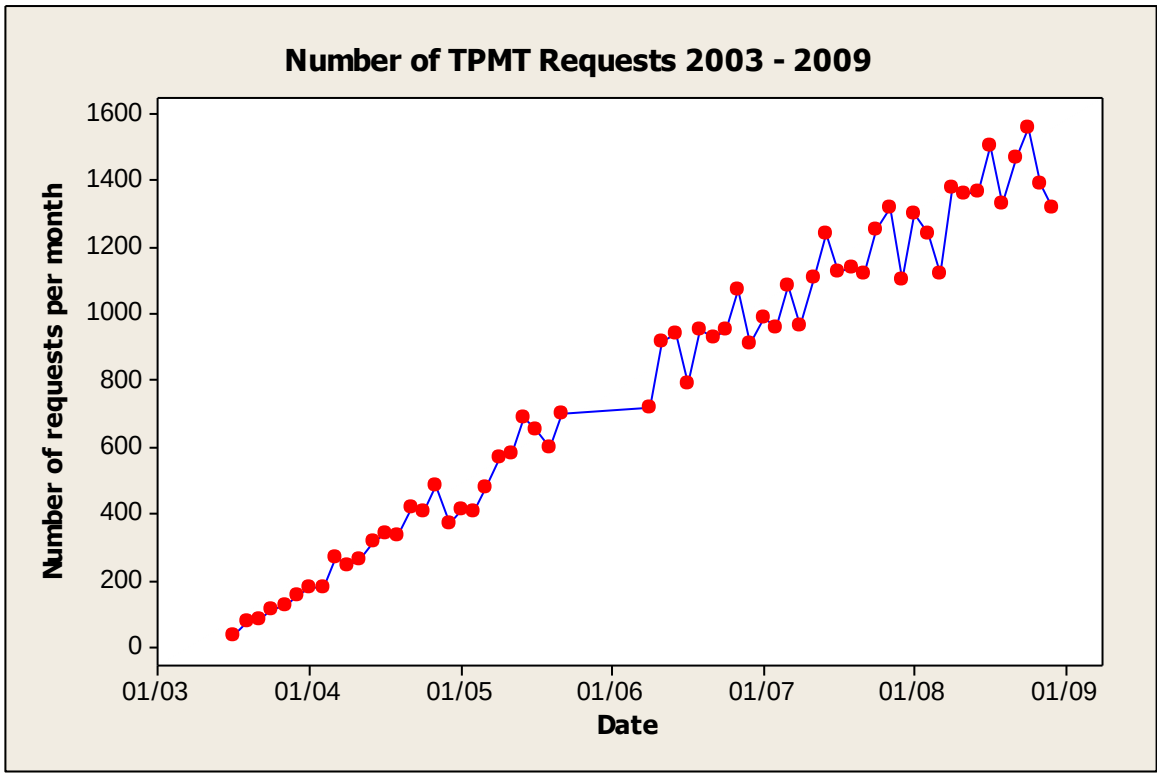


Figure 4.14 ***Increase in TPMT requests from July 2003 to January 2009***

The clinical disciplines requesting TPMT phenotyping are represented diagrammatically in Figure 4.15. These data were obtained by looking at the location and clinical details of requests over a one month period.

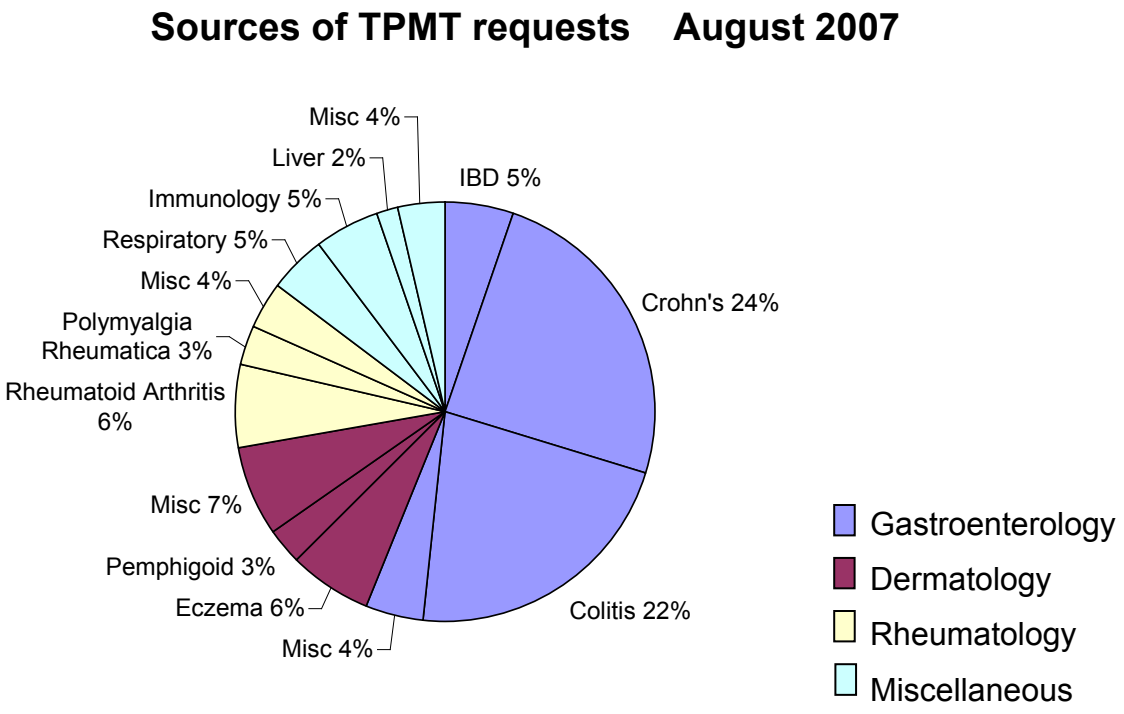


Figure 4.15 ***Breakdown of TPMT requests by clinical discipline and clinical condition. Data for August 2007***

4.6 Individual variability in TPMT activity

4.6.1 Repeat testing

To look at individual TPMT variability, results for samples received for repeat testing were analysed. Change in activity for patients where two samples had been assayed at a time interval greater than one month but no more than six

months apart were recorded. The correlation between the two results for each patient is shown in Figure 4.16.

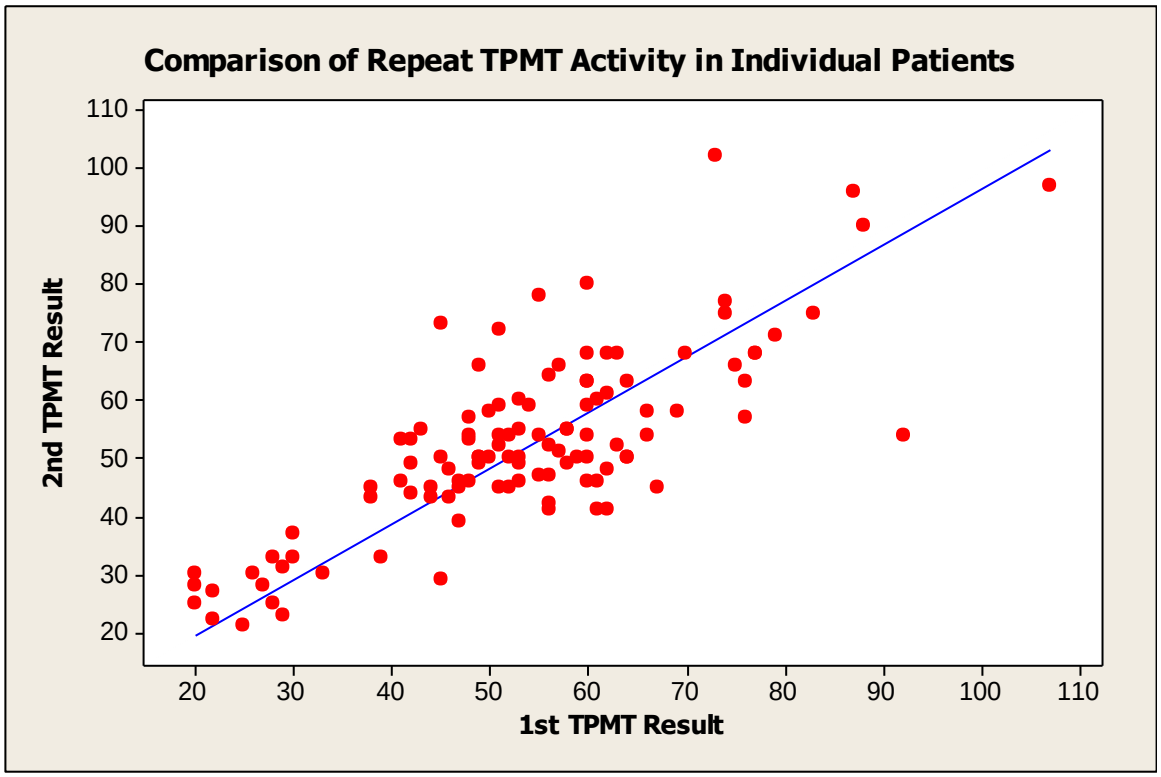


Figure 4.16 ***Correlation between activities for patients repeat tested for TPMT***

The r value is 0.791, this indicates a fairly strong positive correlation between the first TPMT result and the second TPMT result.

Variables beyond our control in this study included:

- Age and storage of samples - after approximately 6 days at room temperature TPMT activity begins to fall, as shown in Chapter 4.2. It is not known what conditions the samples were subject to before receipt, which could account for differences in activity.

- Change in patient haemoglobin - as results are expressed per gramme of Hb, fluctuations in a patient's Hb status could influence the calculated TPMT activity. High TPMT activity per gramme of Hb is often observed in patients with very low Hb concentration, as shown in Chapter 4.3.
- Drug treatment, which may affect activity - for example possible inhibition of activity by non-steroidal anti-inflammatory drugs (Oselin & Anier 2007).

A paired T-test was also performed. The results are shown in Table 4.7.

	n	Mean	Standard Deviation	SE Mean
1st TPMT result	111	53.4	16.1	1.5
2nd TPMT result	111	52.4	15.6	1.5
Difference	111	1.0	10.3	1.0

Table 4.9 ***Paired T for C1 - C2***

With a null hypothesis that the mean difference is equal to zero and an alternative hypothesis that it does not equal zero gives 95% confidence intervals for mean difference (-0.90484, 2.95890)

T-Test of mean difference = 0 (vs not = 0): T-Value = 1.05 P-Value = 0.294

With a level of significance of 0.05 the T value does not exceed 2.96, implying there is no evidence to reject the null hypothesis. The data strongly suggest that the mean for the first TPMT test is the same as the mean for the second TPMT test.

4.6.2 Gender / age differences in TPMT activity

All 15580 TPMT results from May 2008 through to May 2009 were analysed with respect to gender and age. (Results <10 or >100 were excluded from the calculated mean).

Female	Number of samples	Mean	SD	High Activity (>100)
<20	694	52.3	15.6	11
21-40	2374	50.4	12.9	6
41-60	2453	50.8	12.9	8
61+	2688	51.4	13.1	8
All females	8209	51.0	13.2	
Male (Years)	**Number of samples**	**Mean**	**SD**	**High Activity (>100)**
Age <20	760	52.8	14.0	4
21-40	1900	51.8	14.0	10
41-60	2079	51.6	13.6	8
61+	2632	52.4	13.8	10
All males	7371	52.1	13.8	
	Total	**Overall Mean**		**Proportion of total results**
	15580	51.7	13.5	0.42%

Table 4.10 ***Gender / age differences in TPMT activity***

This data shows no variation in TPMT activity with respect to gender or age. This is based on a t-test at 99.9% level of significance which suggests values outside the range 38.8 to 64.6 for differences to be significant.

5. INTEGRITY OF RESULTS

5.1 Quality system

An incorrect TPMT result has the potential to cause serious harm; therefore a Quality Control System is in place to minimise errors. This vigilance system has CE marking of this assay as an integral component and it covers the following elements:

5.1.1 Failure modes and effect analysis (FMEA)

This is a bottom up approach, where every element of the system is examined with regard to the potential for failure. Following FMEA, mechanisms are put in place to eliminate or minimise the risks identified.

An example of how analysis of the method in this way can prevent erroneous results has been demonstrated by identifying the effects of column age on peak resolution. It has been found that salicylate elutes and is detected slightly after 6MTG. As column use increases the resolution between these two peaks diminishes, to the extent that a deficient activity could be missed as the saliyclate peak could be mistaken for a 6MTG peak. For this reason columns are changed after approximately 1000 injections. The degree of separation between 6MTG and salicylate is monitored by including salicylate in our quality control material. The column is changed when resolution of the 6MTG and salicylate peak back to baseline is lost. Reduction of peak height and resolution is shown in Figure 5.1 and 5.2.

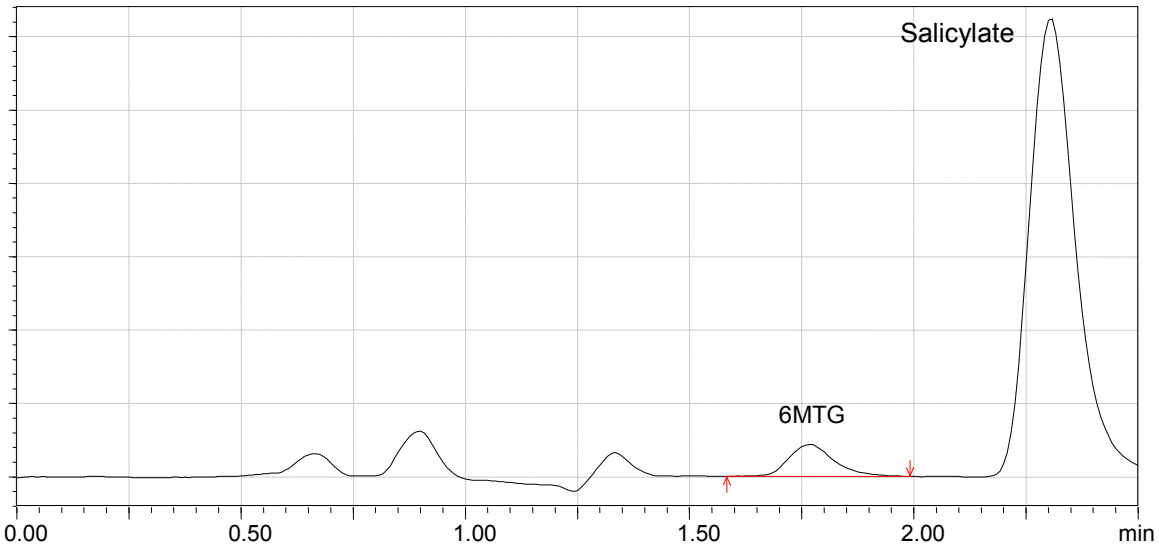


Figure 5.1 ***Separation between 6MTG and salicylate using a new column***

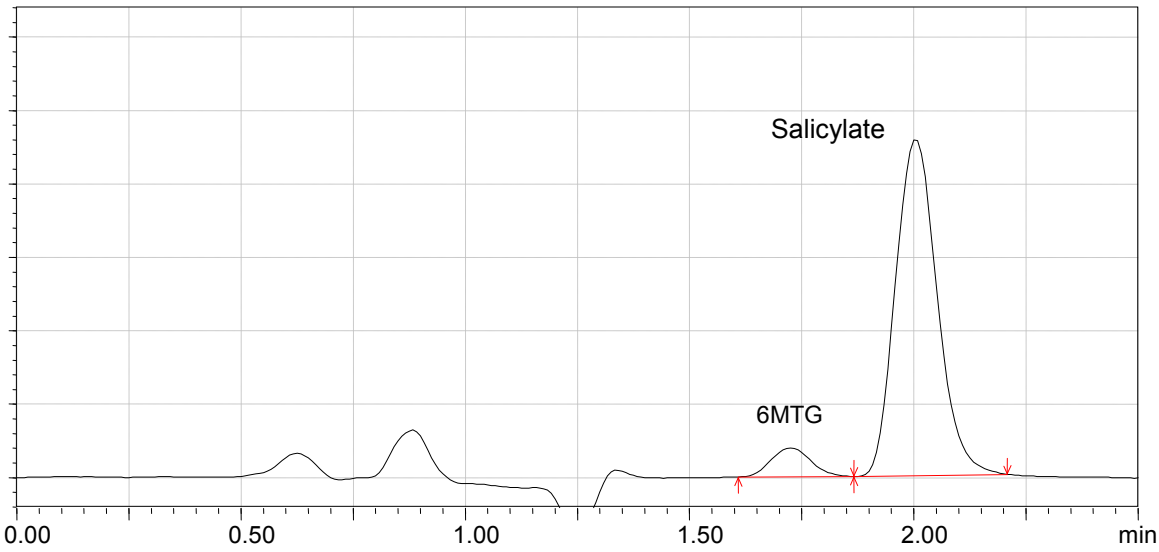


Figure 5.2 ***Separation between 6MTG and salicylate using a column after approximately 1000 injections***

5.1.2 Acceptance criteria

Once a run is complete the quality of the chromatography is examined to ensure 6MTG peaks are fully resolved and correctly integrated. The 6MTG peak in a blank sample is automatically subtracted from all subsequent chromatography. Quality control samples are included in every run, results of which must be within two standard deviations of the target, for both lysate haemoglobin concentration and TPMT activity.

Recovery of a 6MTG calibrator placed at the end of the run must be in the range 85 – 115%.The calculated mean TPMT activity for all patient samples on the run must be within the range 40-55 nmol 6MTG/gHb/hr, excluding a very high or very low TPMT result.

TPMT results are automatically calculated from the 6MTG concentration and the lysate haemoglobin concentration, by the laboratory data management system. At least three patient results for each run must be manually calculated to check this is functioning correctly.

5.1.3 Introduction of changes

Before being put into routine use, any changes to the method must be fully validated to ensure there is no effect on the quality of the result. All relevant experimental data is held on record and a FMEA performed to identify any new areas of risk.

5.1.4 Reagent manufacture

A log sheet is used to record the manufacture of all reagents used to obtain a TPMT result. This includes the name of the operator, lot number of chemicals used and the preparation and expiry dates of the manufactured reagents. Each reagent is given a unique batch no, so that for any result the reagents used can be identified.

5.1.5 Competency checks

After training, new operators must satisfactorily complete a competency check, before being allowed to run the assay unsupervised. For this, they must perform a run in which all acceptance criteria are met and a sample analysed five times within that run gives an intra-run CV of <5%.

5.1.6 Daily maintenance logs

Each time an assay is performed the details are recorded e.g. operator, reagents / controls used, incubation temperature, pump pressures. Any calibration details, operator comments and whether a run has been accepted or rejected are also recorded.

5.1.7 Introducing a new QC

Control samples are prepared from screened volunteers. These are analysed alongside an established QC for a minimum of ten runs. A mean target concentration is then calculated and a Levy Jennings chart prepared.

5.1.8 Repeat samples

All patient samples found to have deficient activity are confirmed by repeat analysis. A second patient's sample is also requested.

Samples with confirmed lysate haemoglobin concentrations outside the range 1.5 - 4.5 g/dL are put for repeat analysis using a fresh, more concentrated or dilute lysate as appropriate.

Patient samples with TPMT activity >89 are checked by repeat analysis. These samples are also checked for interfering substances as outlined in 4.1.5.

5.1.9 Genotyping confirmation

TPMT genotyping is used to confirm the status of patients in the following situations:

- Samples with deficient TPMT activity
- Recently transfused patients
- Samples with low TPMT activity
- Where there is a change of status between present and previous results
- Patients known to have had an adverse reaction to thiopurine drugs

5.1.10 Reporting

All new users of the service are sent communication introducing the service.

Any relevant comments e.g. sample age are added to reports before issue. Deficient TPMT activity results are telephoned to the requesting clinician as soon as possible, requesting an additional sample for confirmation.

5.1.11 Logs

A user feedback log is in place which provides a summary of all contacts with end users. All communications are reviewed at the end of each month. If this identifies an area in which the service can be improved a record of action taken should be made.

A TPMT incident log also exists which serves as an important troubleshooting guide for operators. All major equipment failures and reasons for rejecting runs must be recorded in detail in this log. This is also reviewed every month.

5.2 QC data

The graphs in Figure 5.3 represent the data from over 800 separate TPMT analytical runs.

The JB and VG controls are blood samples that are collected from two laboratory donors, who have been screened negative for hepatitis and HIV. Blood is donated approximately every 3 months, aliquoted, frozen, then thawed and analysed on every run. Levy-Jennings charts are constructed for each new batch of control.

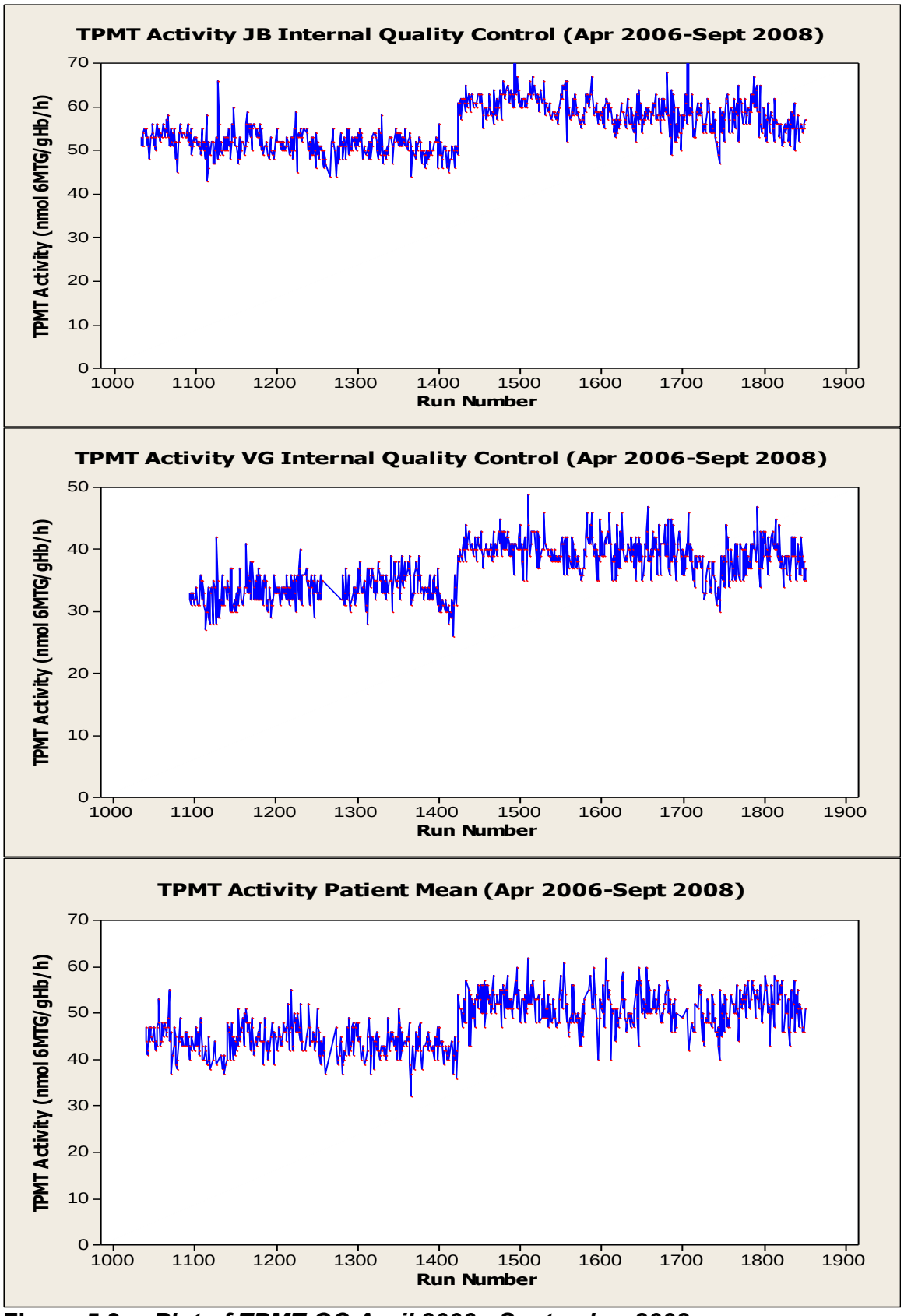


Figure 5.3 ***Plot of TPMT QC April 2006 - September 2008***

The Levy-Jennings assessment is one of the main criteria when determining whether a run is acceptable to report.

Due to the relatively high numbers of samples analysed, looking at the patient mean for each run also gives an indication of the acceptability of the run. Any deficient or results over 90 nmol 6MTG/gHb/h are excluded when calculating the patient mean.

It can be clearly seen from these graphs that the QC and patient mean results have moved in parallel over the time period studied. Shifts in the graph have reflected issues with the assay such as reagent lot changes and problems with incubation temperature control. For example, around run 1420 a new lot of one of the substrates (SAM) was introduced. This resulted in a considerable shift upwards in results. This gave better genotype/phenotype concordance and the percentage of low results became more in line with published data. This overcame concerns, within the laboratory, that our results had been slightly low at that time.

The JB and VG internal QC results demonstrate the consistency in activity for each individual. The data has been looked at in a different way by calculating the mean and confidence limits for each batch of QC. This is represented in Figure 5.4.

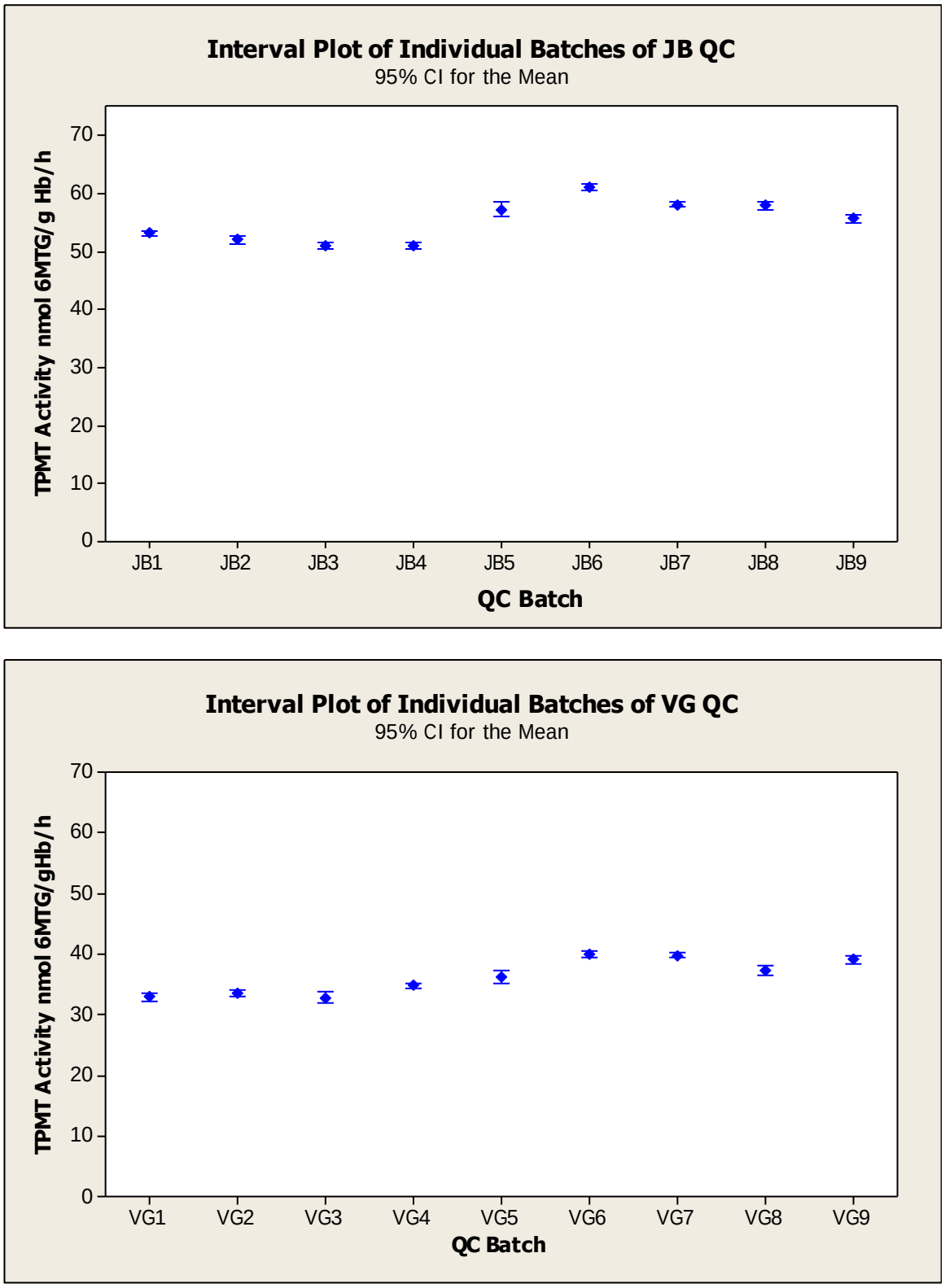


Figure 5.4 ***Longitudinal study of TPMT activity in two individuals used as Quality Control April 2006 - October 2008***

5.3 Reducing variability

Sample handling has been standardised as much as possible to minimise operator variance. The whole blood is thoroughly mixed, to give a homogeneous sample, before aliquoting. The aliquots are then frozen for a minimum of 15 mins to ensure maximum lysis. An experiment was performed to determine the extent of blood cell lysis in this time. Cell counts were performed pre and post freeze using a Bayer Advia 120.

Sample	Pre freeze Red blood cell count	Post freeze Red blood cell count	% RBC lysis
1	4.65	0.16	96.6
2	4.57	0.29	93.7
3	5.09	0.34	93.3
4	4.84	0.40	91.7
5	3.95	0.22	94.4
6	4.21	0.13	96.9
7	4.90	0.48	90.2
8	4.43	0.27	93.9
9	4.77	0.26	94.5
10	4.56	0.31	93.2
Mean			**93.8**

Table 5.1 ***% RBC lysis achieved after 15 minutes at -80°C***

Sample	Pre freeze White blood cell count	Post freeze White blood cell count	% WBC lysis
1	5.92	4.58	22.6
2	5.92	5.07	14.4
3	4.88	4.22	13.5
4	7.02	5.85	16.7
5	11.09	8.72	21.4
6	5.36	3.48	35.1
7	3.54	2.70	23.7
8	5.41	4.90	9.4
9	18.29	14.14	22.7
10	10.69	8.26	22.7
Mean			**20.2**

Table 5.2 ***% white blood cell lysis achieved after 15 minutes at -80°***

Repeated freeze thaw cycles did not show any further cell lysis. RBC counts are very low after freezing and no longer in the usual measuring range. The accuracy of these results is questionable as levels are outside the confidence limits of the analyser.

5.3.1 Freeze thawing techniques

Incomplete lysis has been observed in samples that have been covered or stood on boxes in the freezer, therefore this practice is avoided.

Once removed from the freezer buffer must be added to the aliquots immediately to prevent sample degradation. The samples are then allowed to thaw for approximately 5 minutes, before mixing thoroughly. Deviations from these practices have resulted in unacceptable quality control.

5.3.2 Incubation temperature control

It has been found that the rate of methylation of 6TG is extremely temperature dependent. This has necessitated strict vigilance systems to keep the assay in control. The 1 hour 37ºC incubation was initially performed in a water bath; this was substituted by a hot block, for convenience, as sample numbers increased. The temperature of the blocks is monitored closely and adjusted when necessary to maintain the required temperature. Problems with quality control seem to be more frequent when the temperature in the laboratory is low. The installation of air-conditioning has helped minimise any changes in ambient temperature.

Ideally, the reaction rate will be linear over the total incubation time. To allow this, the test reagent is brought up to 37ºC prior to addition to the sample lysate. This reagent preparation is crucial as if the starting temperature is too low an initial lag in the reaction will occur. Conversely, if the reagent is over heated, non-enzymatic methylation of 6TG to 6MTG can occur. This could contribute to a high 6MTG peak in blank samples. If the pre heating process is unduly long the reagent can begin to degrade, with substrates, being seen by visual inspection, precipitating out of solution.

5.3.3 Sampling errors

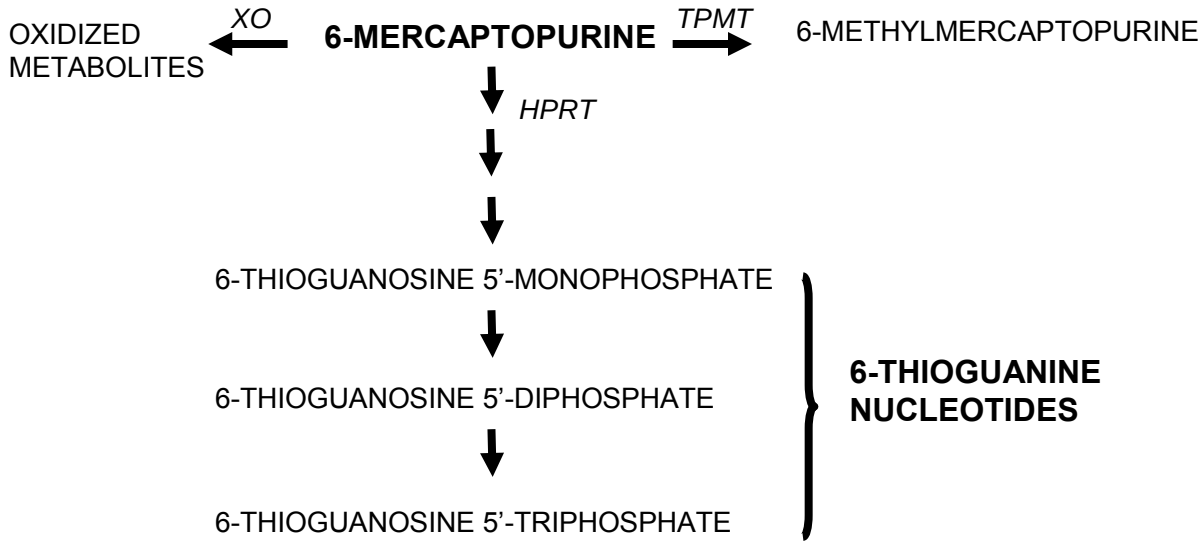
Precise injection volumes of only 2µl are required to be dispensed by the HPLC autosampler, therefore a system has been put in place that would detect a 'short' sample. This risk has been overcome by the addition of L-tryptophan to the whole blood suspension buffer. This acts as an internal standard and the L-tryptophan peak is monitored by the UV detector. Monitoring these peak heights will highlight any imprecision in injection volume.

6. THIOGUANINE NUCLEOTIDES

6.1 Thiopurine drug monitoring

Plasma 6MP levels have been used in an attempt to aid dosing strategies for thiopurine drugs, this assay has been limited in its reliability to predict efficacy or toxicity. The measurement of urine thiouric acid, the xanthine oxidase pathway metabolite has also been tried. This is only useful in assessing compliance, as it does not give any indication of therapeutic response.

More effective monitoring has been achieved, through greater understanding of thiopurine metabolic pathways. The 6TGN are the active metabolites of thiopurine treatment. They achieve their immunosuppressive effect by virtue of the fact that they are incorporated as false bases into DNA, preventing cell proliferation.



XO= xanthine oxidase, *TPMT=thiopurine methyltransferase, HPRT=hypoxanthine phosphoribosyltransferase.*

Figure 6.1 ***Formation of 6-thioguanine nucleotides***

Another explanation of the immunosuppressive mechanism of thiopurine metabolites is induced T cell apoptosis by modulation of Rac1 activation (Tiede et al 2003).

At the moment most 6TGN quantification methods measure nucleotides indirectly. Direct measurement is possible using mass spectrometry and ion-exchange HPLC. These methods can differentiate 6TGN from 6TG and can also measure thio-ITP from 6MP which may be useful in studying ITPA deficient patients. Other direct assays include enzymatic conversion of 6TGN to nucleosides with reverse-phase HPLC measurement.

Many of the assays developed use erythrocytes as a surrogate pharmacokinetic marker, to indicate the intracellular levels of metabolites in the target cells i.e. peripheral blood leucocytes or bone marrow pre-cursors. In the measurement of RBC 6TGN it is difficult to avoid contamination with white blood cells, which may affect results. Further problems arise when considering the degree of possible degradation of the labile cell nucleotides between collection and analysis.

A 6TGN concentration of >235 pmol/10^8 RBC is associated with therapeutic efficacy, while concentrations of >450 pmol/10^8 are linked with myelotoxicity (Dubinsky et al 2000).

It is important that measurement is performed when a steady state has been achieved, after approximately four weeks of treatment, in order to give a reliable prediction of response to therapy.

6.2 Reasons for testing

It is argued that TPMT phenotyping, genotyping and measurement of 6TGN all have value in the clinical evaluation of patients receiving thiopurine therapy.

Thiopurine metabolism is complex, with three competing enzyme pathways. Also, polymorphisms exist in the TPMT gene and may also exist for genes encoding for other metabolic enzymes. These factors result in a wide range of metabolite concentrations, for a given dose, in each individual patient. The poor correlation between dose and 6TGN concentration supports measurement of 6TGN, to aid rational dose adjustment as increased 6TGN has been correlated to reduction in disease activity (Hindorf et al 2004).

The usefulness of 6TGN measurement is to monitor the patient, in response to treatment, to help achieve a dosing regime with maximum therapeutic effects and minimal toxicity. This quantification allows confident escalation of dose in non-responders and dose reduction for those with unexpectedly high, potentially harmful, 6TGN concentrations. It is particularly useful in assessing:

- patients heterozygous for TPMT activity who may not be receiving standard dosage
- patients who fail to respond to therapy
- cases where compliance is questionable.
- patients receiving the XO inhibitor allopurinol
- hypermethylators, that have higher than normal TPMT activity, who could require treatment with a higher dose to achieve adequate response

A study of samples received in our laboratory from patients being treated with Azathioprine indicated that a large proportion of patients were being treated with a sub-optimal dose. These findings were summarized and presented as a poster at the EuroMedLab conference in Amsterdam 2007; a copy is included as Appendix 4.

A simplified assay for determination of red blood cell 6-thioguanine nucleotides; early evidence suggestions UK patients are receiving sub-optimal doses of thiopurine drugs Graham V, Ford L T, Berg J

Similar findings by Morales et al in 2007 highlighted a high proportion of low 6TGN level in patients using the standard mg/kg strategy.

Higher TGN levels have been observed in Crohns disease (CD) patients than in those with Ulcerative Colitis (UC), on similar dosing regimes (Hande et al 2006). This is unexpected as reduced absorption would be expected in those with Crohns disease. This could be indicative of an independent association between disease type and nucleotide levels.

If 6TGN concentrations are low this could indicate abnormal absorption or metabolism, this may be warrant measurement of 6MMP concentration. If this is also low it would suggest under dosing or non-adherence. While elevated 6MMP coupled with low 6TGN indicates preferential metabolism of 6MP to 6MMP by TPMT.

6.3 Results of provisional study and comparison with New Zealand laboratory

In 2006, a 6TGN assay was put into use at City Hospital. This assay was adapted from the methods of (Dervieux & Boulieu 1998; Lennard 1987) and was performed using washed RBC. Initial comparisons were carried out using RBC samples from a laboratory with an established service in New Zealand.

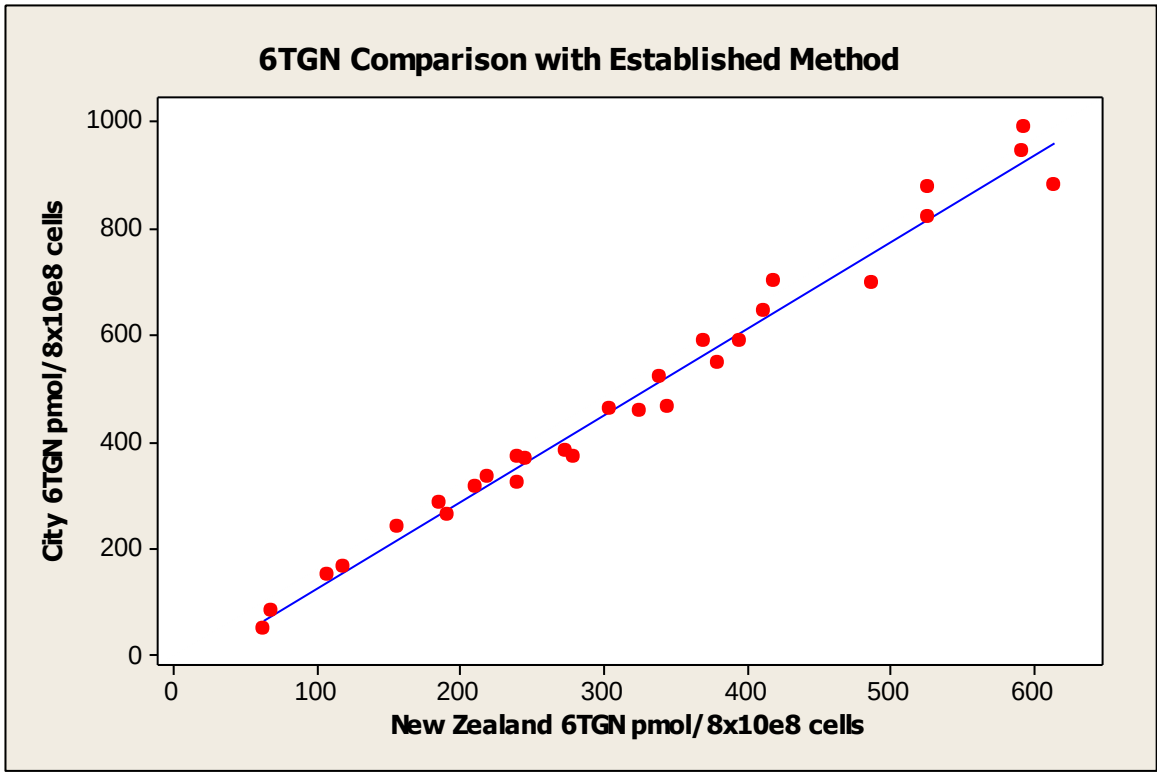


Figure 6.2 ***Initial 6TGN comparison with New Zealand laboratory***
y = 1.6165x - 35.395 ***r = 0.99***

The City laboratory assay results initially showed a positive bias of 25-30%. It was discovered that if the prepared 6TG calibrator was heated alongside the samples the 6TG peak height increased. This heating of the calibrator was

introduced into the method; this change resulted in a lowering of results, and brought the comparison results much more in line with the New Zealand laboratory.

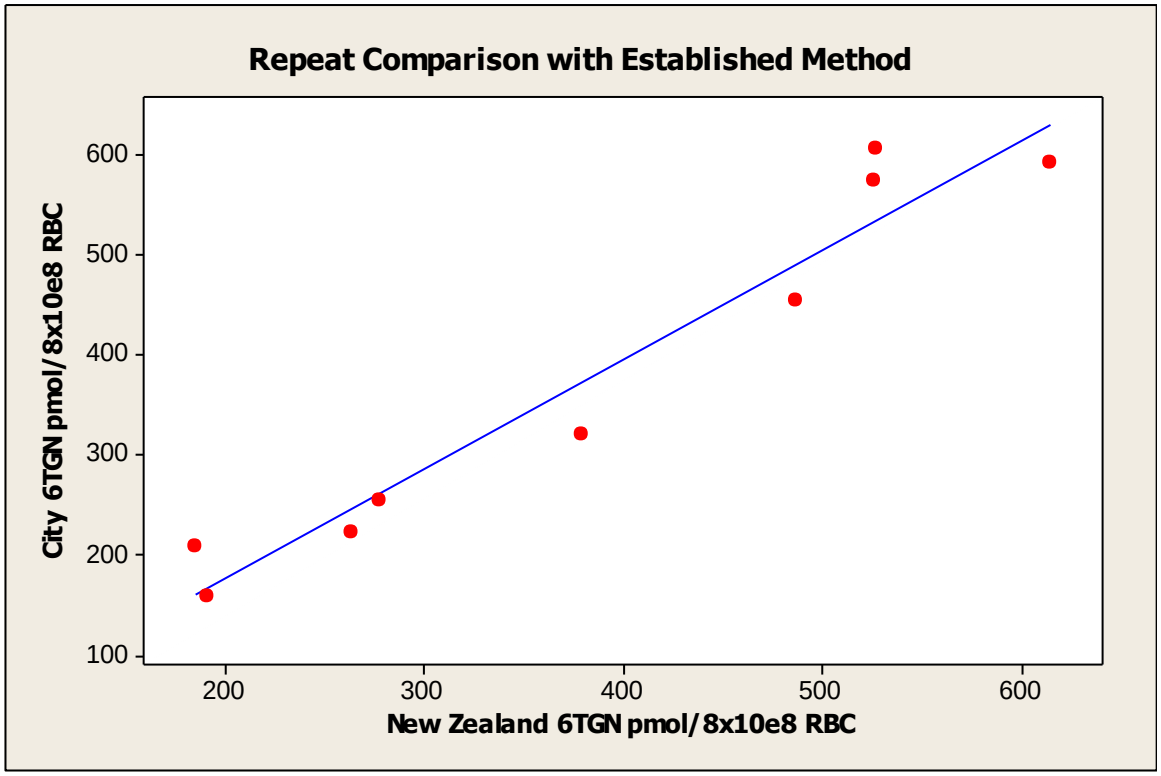


Figure 6.3 ***Repeat 6TGN comparison with New Zealand***
y = 0.8635x + 58.674 ***r = 0.97***

6.4 Method development

The original method developed liberates 6TGN from RBC, using perchloric acid at 95ºC, hydrolysing them back to their parent thiopurine, 6-thioguanine (6TG). The 6TG concentration is measured by HPLC, any losses are accounted for by inclusion of an internal standard.

This method requires a relatively large sample volume, as RBC are washed and counted prior to analysis. This preparation procedure is laborious and can introduce errors in the cell counting step. A radical approach was therefore

adopted, similar to that taken with TPMT, to assess the feasibility of measuring 6TGN using whole blood.

Whole blood aliquots and red cell counts were taken from 70 patient samples before they were prepared for the existing washed red blood cell method. These whole blood samples were analysed alongside the routine samples and results compared.

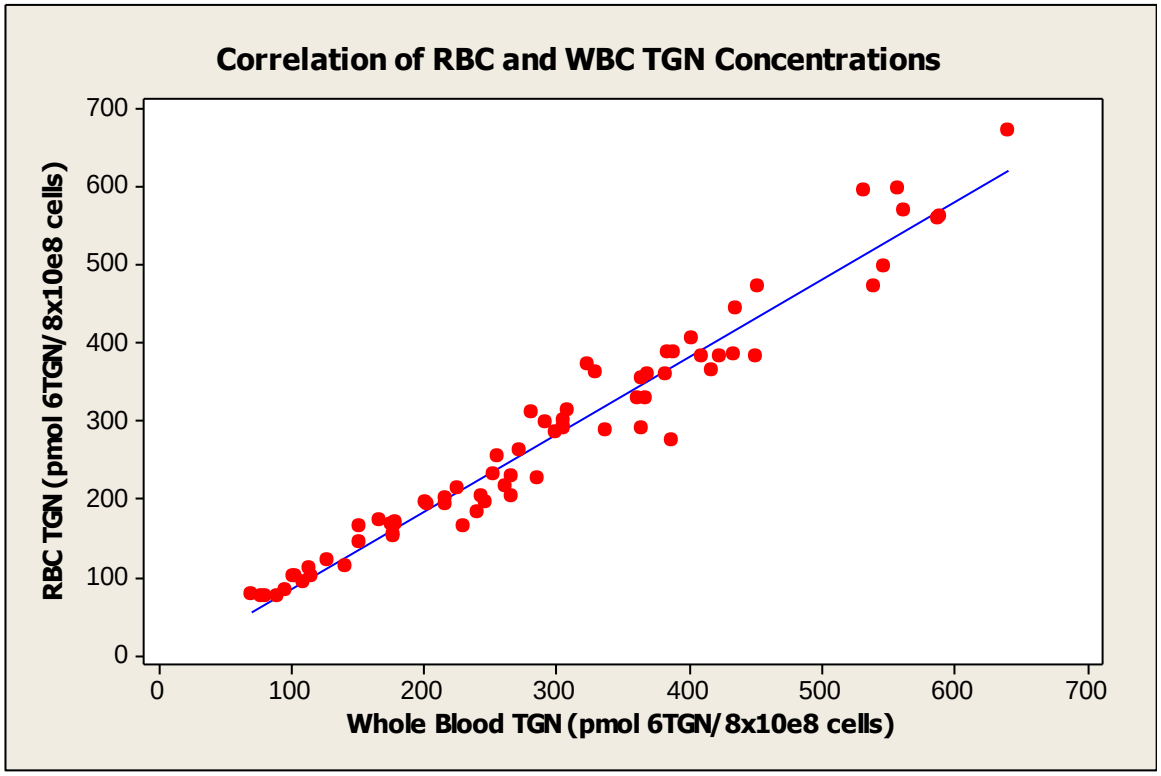


Figure 6.4 ***Comparison between 6TGN concentrations measured in washed RBC and whole blood r = 0.978***

The correlation between washed red cell and whole blood results was excellent which is in concordance with a study carried out at the Mayo Foundation in the USA (Pike et al 2001).

The quality of the chromatography for this method was poor, due to several factors including:

- the low concentrations of solvent in the circulating mobile phase meant the system was prone to microbial growth. Resulting in frequent pump problems and blocked filters
- using an isocratic system, the analysis time allotted to each sample, was too short to allow complete elution of some unknown compounds. This resulted in huge, very late peaks appearing in subsequent chromatography, as shown in Figure 6.2

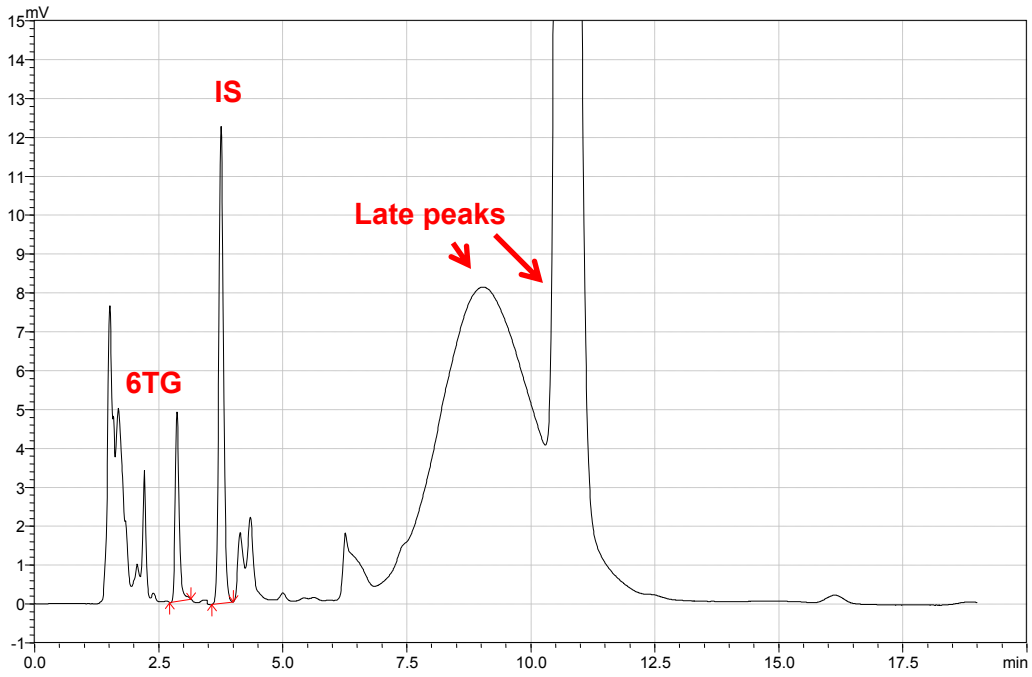


Figure 6.5 ***Late peaks on 6TGN chromatography***

These problems were solved by the introduction of a gradient elution method. The theory behind this change was to bring in a more hydrophobic mobile phase after elution of the peaks of interest. This was designed to achieve elution of all

residual compounds from the column in a relatively short time frame. Then the system would return to the original mobile phase in preparation for the next sample injection. Several compositions of mobile phase and pump settings were trialled to achieve optimum settings, as show in Figures 6.6 - 6.8. The rate of introduction and duration of the more hydrophobic mobile phase is as shown.

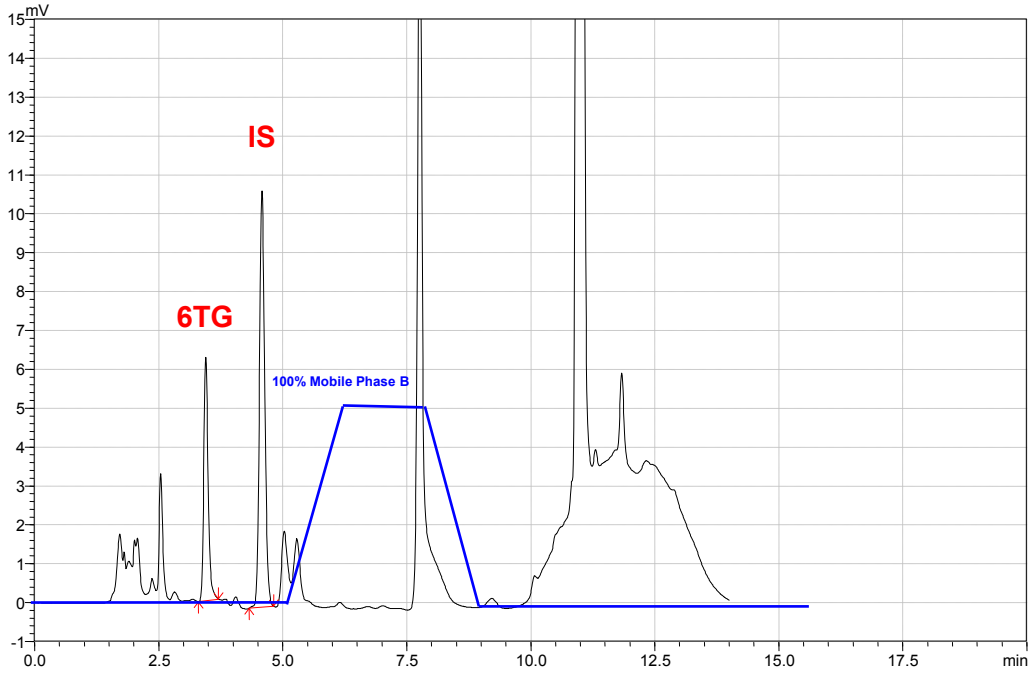


Figure 6.6 ***6TGN chromatography using mobile phase B composition: 15% Acetonitrile / 5% Methanol / 80%Buffer***

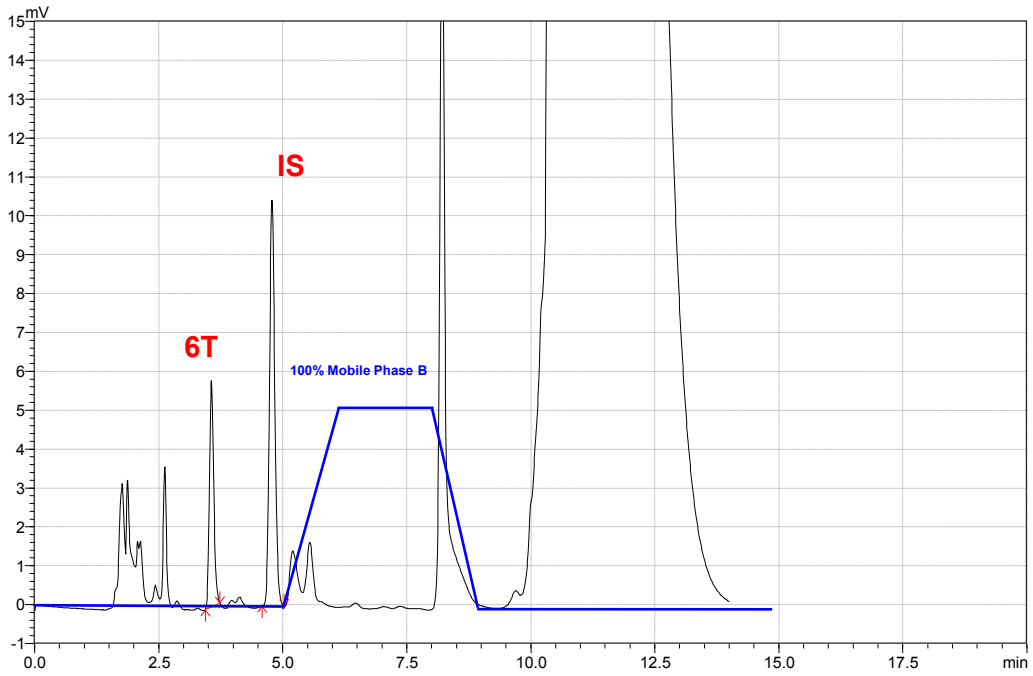


Figure 6.7 ***6TGN chromatography using mobile phase B composition: 30% Acetonitrile / 10% Methanol / 60%Buffer***

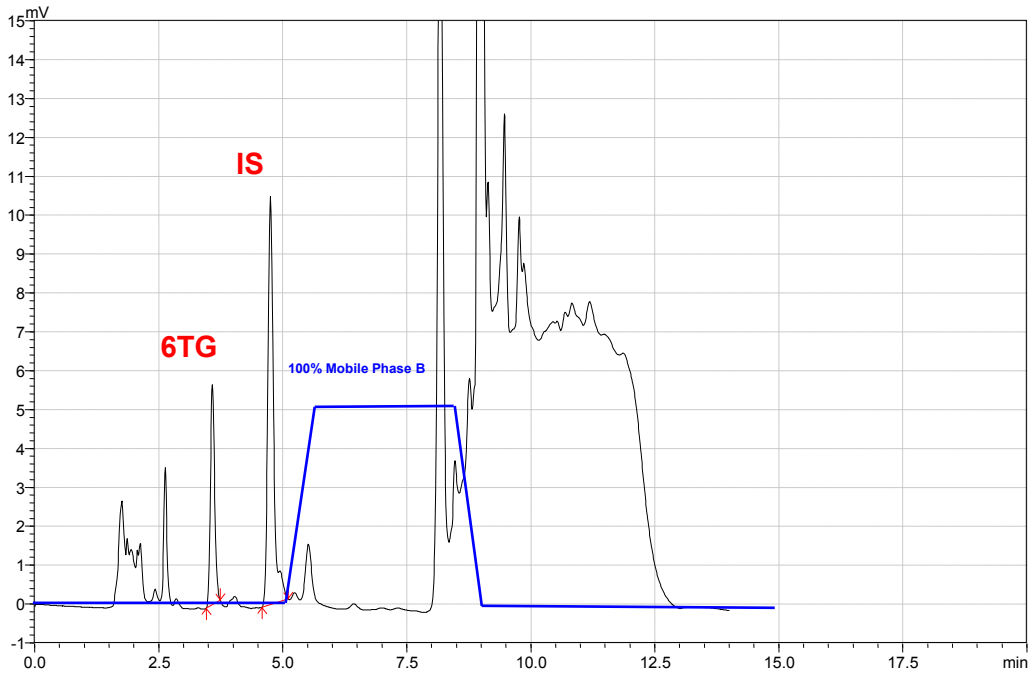


Figure 6.8 ***6TGN chromatography using mobile phase B composition: 15% Acetonitrile / 5% Methanol / 80%Buffer. Increased ramp and duration.***

The intra batch variation for this new method is 8.3% (n=10) and inter batch variation 10.2% (n=18).

A study of 6TGN stability in whole blood has shown levels to fall rapidly after around 5 days at room temperature (Figure 6.9). For this reason, results for samples that are over 5 days old on receipt are flagged to be interpreted with caution.

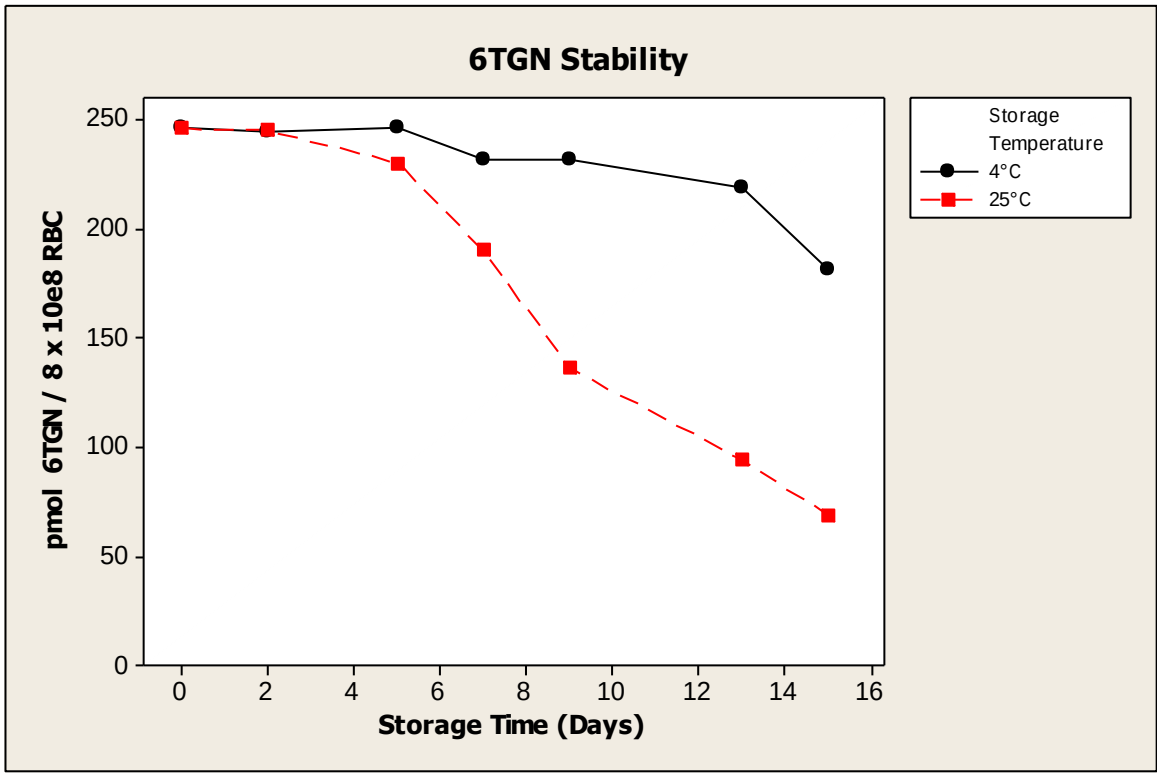


Figure 6.9 ***6TGN sample stability***

The new whole blood assay, developed as part of this research, has taken this test forward in several ways:

- sample preparation is less labour intensive
- cell counting and pipetting errors are minimised
- the sample volume required is reduced

- the quality of the chromatography is much improved and less failed runs are encountered since the change to the gradient method
- the assay is quicker and more cost effective

A poster summarising some of this work is included as Appendix 5.

Improving a test that has a growing clinical demand; blood 6TGN and its role in pharmacogenomics. (2008) Graham V. Berg J.

Now in routine use, this simplified assay will allow more confident escalation of dose in non-responders and dose reduction for those with high, potentially harmful, 6TGN concentrations. It will be particularly useful in assessing patients heterozygous for TPMT activity, who receive a reduced dose and for which achieving therapeutic levels can be problematic.

6.5 Use of the 6TGN service

The number of users of this service is still relatively small, at around 15 laboratories. Those who do request 6TGN often send a number of samples on the same patient. The number of requests received per month has risen by approximately 100% in the last year as shown in Figure 6.10.

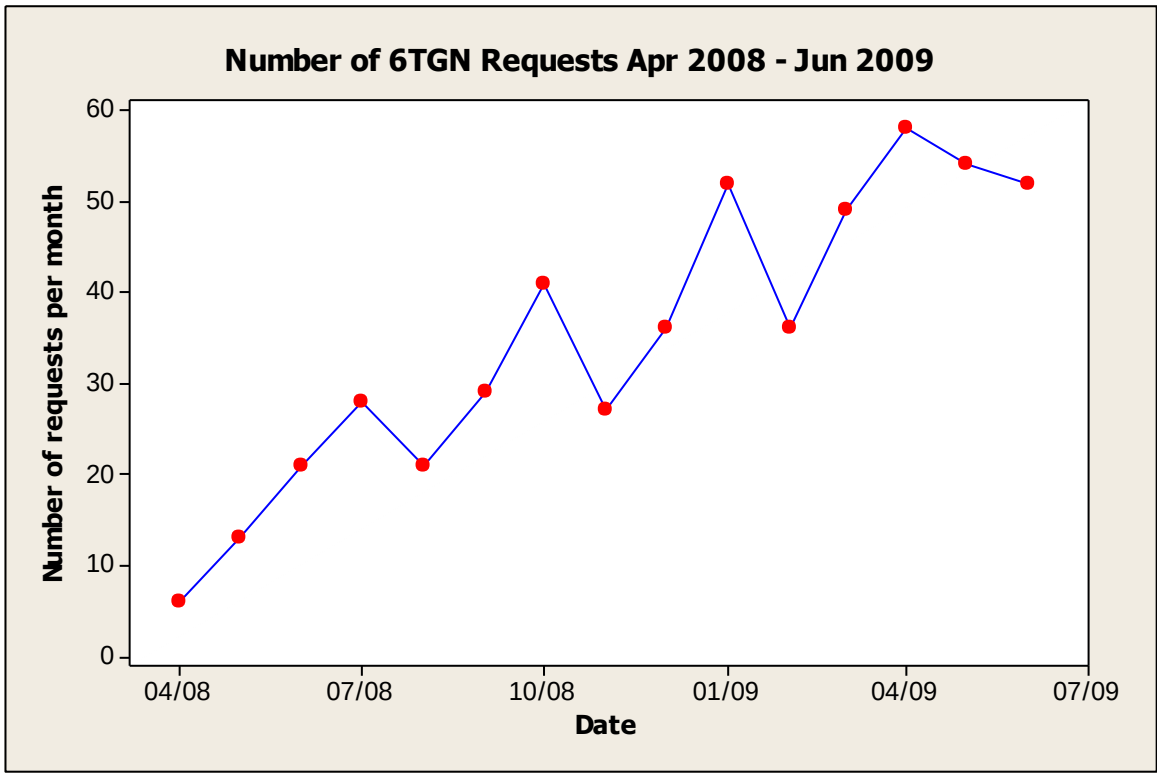


Figure 6.10 ***Increase in demand for 6TGN analysis***

If it becomes common practice to monitor 6TGN levels huge escalation in demand for this test could be seen. As unlike TPMT testing, which usually only needs to be done once per patient, this is a therapeutic drug monitoring assay, where a number of determinations on one patient are more likely.

TPMT activity was been performed on 166 samples requesting 6TGN and the correlation between TPMT activity and 6TGN concentration examined. For samples with a high 6TGN concentration (>450 pmol/8x10^8 RBC), which is associated with myelotoxicity, 24% exhibited a low TPMT activity compared to only 11% low activity overall.

The correlation between 6TGN concentration and mean cell volume was also examined for patients samples received for routine analysis.

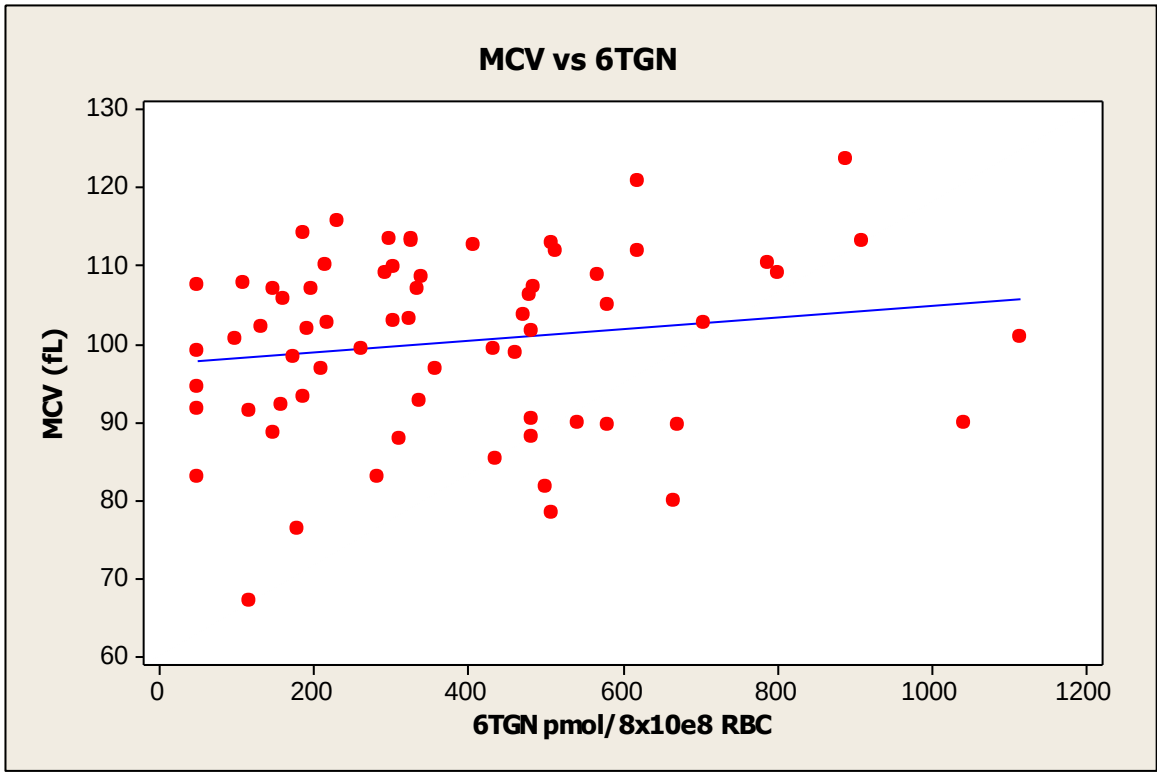


Figure 6.11 ***Mean cell volume / 6TGN level correlation***

No significant correlation was observed but this data is flawed as most of the samples analysed were several days old, which can result in falsely high MCVs.

7. INDUCTION STUDY

7.1 Aims

Many of the enzymes involved in drug metabolism may be up-regulated by exposure to drugs and environmental chemicals leading to increased rates of metabolism. This phenomenon is known as enzyme induction. Studies on renal transplant patients in 2001 showed increases in patient TPMT activity, possibly due to induction by Azathioprine (Weyer et al 2001; Thervet et al 2001). More recent studies in Sweden do not support this hypothesis (Lindqvist et al 2006) but these researchers used RBC TPMT. The research in this chapter looks at possible TPMT induction in whole blood.

Eight patients from within our hospital, which were about to commence on Azathioprine were selected at random. Patient details, other than hospital number, were not recorded. As haematology samples were received for routine monitoring on these patients they were tested for TPMT activity and 6TGN.

The levels of 6TGN achieved and variation in TPMT activity observed over a 5-6 month period is represented in Table 7.1, with a graphical representation of variation of TPMT activity in Figure 7.1.

Week	TPMT Activity (nmol 6MTG/gHb/h)							
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
1	38	*	*	*	42	*	*	*
2	*	39	*	*	*	*	*	*
3	*	*	52	52	*	35	*	*
4	*	*	*	*	*	*	*	*
5	*	*	*	*	*	*	*	*
6	37	43	58	45	39	*	35	*
7	*	*	*	48	42	30	*	37
8	*	*	*	48	40	37	31	35
9	35	*	51	*	41	33	*	37
10	*	*	*	*	51	38	35	44
11	36	43	*	*	48	39	*	*
12	*	*	*	*	49	41	33	42
13	*	*	*	*	45	42	*	*
14	36	*	*	63	*	*	37	44
15	*	42	*	*	*	*	*	*
16	*	*	*	*	*	43	39	*
17	*	*	*	*	*	*	*	41
18	38	*	*	*	*	*	40	*
19	*	41	*	*	*	42	*	43
20	*	*	50	68	*	*	34	*
21	*	*	54	*	*	*	*	44
22	*	*	*	*	*	*	35	*
23	*	46	*	68	42	40	*	41
24	*	*	*	*	*	*	36	*
25	36	*	55	*	*	*	*	41

6TGN level sub-therapeutic

Therapeutic 6TGN level

6TGN level above therapeutic range

Table 7.1 ***TPMT and 6TGN levels of patients starting azathioprine treatment***

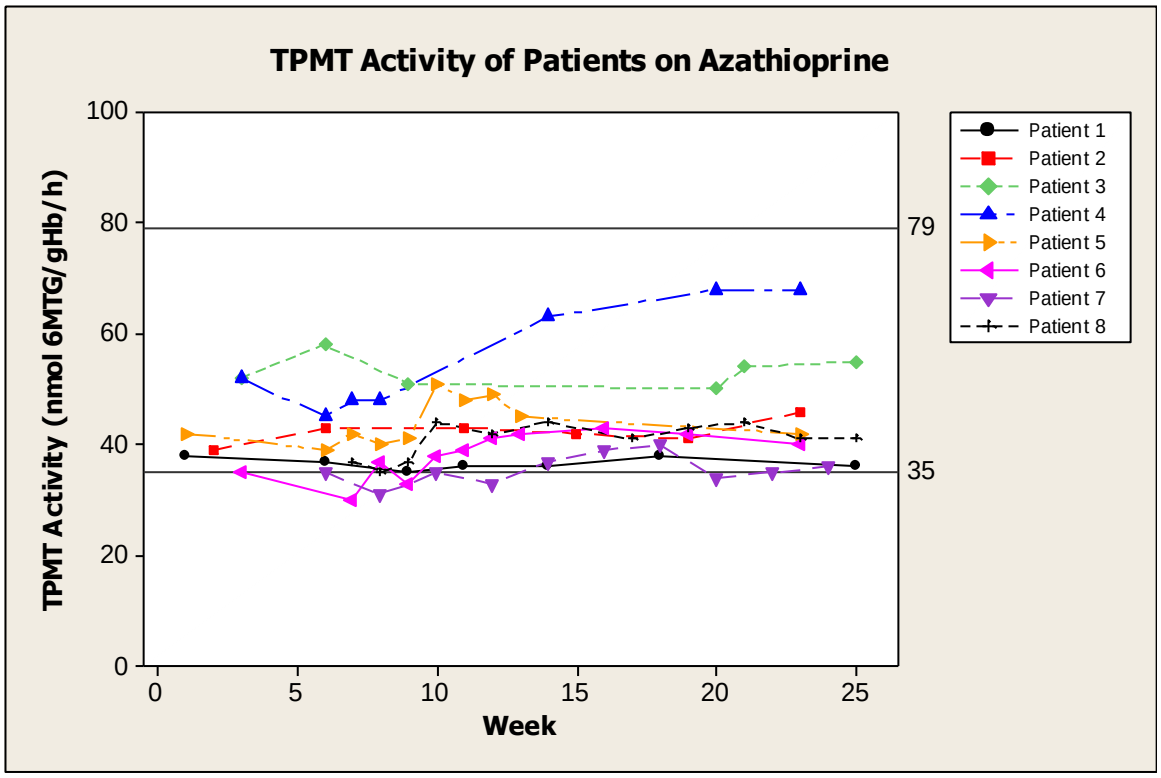


Figure 7.1 ***TPMT induction in patients on Azathioprine***

It can be seen from the data in Table 7.1 that 7 of the 8 patients in this study achieved at least therapeutic levels of 6TGN. But it can be seen from Figure 7.1 that, with the exception of one patient, no increase in TPMT activity was observed. The apparent increase in this one patient may have been due to factors other than induction e.g. possible fall in patient Hb levels, which could give rise to high calculated activity as explained in Chapter 4.3.

8. DISCUSSION

8.1 Introduction

There is an increasing interest and availability of pharmacogenetic products and services, many of which, including TPMT testing, detect polymorphisms associated with severe adverse drug reactions. These tests have obvious theoretical clinical advantages, some have relatively strong evidence bases and there can be a commercial interest in their development. Yet, validation and approval can take a very long time. Expense and poorly defined legal frameworks have contributed to this 'logistical challenge' and this is delaying widespread clinical use (Hopkins et al 2006). The thiopurine drugs which are the basis of TPMT testing are 'off patent', making them open to generic competition. Therefore, there is little incentive for manufacturers to invest in pharmacogenetic services. Despite these facts, TPMT testing is one service which has become well established in the UK and is currently the best example of applied clinical phamacogenetics. The work of this thesis has researched and developed the pharmacogentic services of City Hospital Birmingham, part of the Sandwell and West Birmingham NHS Trust, which is currently a major provider of TPMT testing in the UK.

In a 2007 study, it was found that over two thirds of UK National Health Service consultants were requesting determination of TPMT before initiation of Azathioprine treatment (Fargher et al 2007). This is compared with virtually zero requests in 1997 (Tan BB et al 1997). This increase in testing, which has been

demonstrated in this thesis, reflects to a certain extent, changes in national guidelines. However, the guidelines are not strictly adhered to and advice relating to TPMT testing can be discipline specific, for example resulting in a higher uptake from dermatologists, compared with gastroenterologists and rheumatologists (Fargher et al 2007b). Pre-treatment and regular measurements of full blood count, liver function tests, urea and electrolytes are still strongly recommended in all clinical situations.

Training and education of healthcare professionals in the field of pharmacogenetics is taking place (Higgs et al 2008) but this requires escalation to meet patients' expectations (Fargher et al 2007). Emerging tests need to be explained and interpreted. It has become necessary for clinical pharmacists to be able to understand both enzyme level and DNA based TPMT tests in order to guide optimal prescribing rather than solely relying on the British National Formulary recommended dosing based on mg/kg body weight (between 1 and 3 mg/kg). The national survey by Fargher et al 2007 shows how well Azathioprine is tolerated, with around 67% of patients having good, 17% having reasonable and 15% having poor tolerance. With more informed dosing this should continue to improve.

8.2 Research outcomes

8.2.1 Whole Blood Assay

The work in this thesis has helped develop a new TPMT assay that has improved clinical utility. This whole blood assay has been introduced after comparing RBC TPMT with whole blood TPMT. This work involved comparison of the phenotyping for both methods, with genotyping, for 406 patient samples. The new whole blood assay is simpler and requires less sample volume. The fundamental changes to the method are that whole blood is used rather than washed RBC and cell lysis is achieved using freezing rather than using a lysis buffer. A strong correlation (r=0.834) was observed between the RBC and whole blood phenotyping methods. Both techniques demonstrated 97% genotype concordance and 79% concordance between low TPMT results and heterozygous genotypes.

8.2.2 Whole blood assay precision profile

One of the main reasons for the development of a whole blood TPMT method was to improve the relatively high imprecision of the conventional RBC assay. This imprecision was shown to be due to the washed cell pellet not being homogeneous. This results in varying mixtures of cell type being aliquoted on repeat analysis. Variation in activity on repeat analysis may also have been due to differences in the amount of old blood cells or white blood cells present in the aliquot. Studies by Lennard L et al in 2001 showed that older RBC, which could be partially separated during centrifugation, have a lower than average TPMT

activity. While in 2008, de Boer et al found extremely high TPMT levels in patients that had a high percentage of young RBC. This supports the hypothesis that all RBC in a sample do not have similar activity. The activity in the aliquot could also be affected by the amount of WBC present. The data from the whole blood study shows that whole blood activities are always higher but the degree of increase can vary enormously. The higher TPMT activities seen in whole blood compared to washed red cell preparations are likely to be due to white cell activity. Preliminary studies by ourselves have shown the activity per white cell to be very high but considering the haematology of high activity samples in Table 4.5, there is no obvious correlation between WBC and TPMT activity. WBC TPMT activities would be an interesting area of research, but is beyond the scope of this thesis.

8.2.3 Whole blood assay utility

Another main driver for the whole blood method development was to try to eliminate the RBC washing steps. The new method has achieved this, saving several hours biomedical scientist time every day, making the assay more cost effective. These time and cost savings have impacted positively on the turnaround time of results, which has typically been kept to less than two working days. This short time frame enables rapid, informed dosing of thiopurines for patients that may be extremely ill. The importance of emphasis on turnaround is demonstrated by this service now being used by over 200 external laboratories. The income the test has generated has allowed development in

other areas of the department, including work presented here on the 6TGN assay.

8.2.4 Phenotype / genotype concordance

Now the whole blood method is in use, all samples with a TPMT of less than 41 (normal range 35-79) are routinely genotyped (Ford et al 2009). This selective genotyping underpins the whole blood phenotyping service providing a valuable quality control function. This gives confidence in an assay which can be difficult to control, due to in-house reagent production and a number of variables, including operator technique.

Additional samples selected for genotyping include those where the clinical details suggest that the patient has been transfused. Genotyping these samples overcomes one of the disadvantages of phenotyping where the measurement of activity from the donor blood masks the true TPMT status. In a worst case scenario, a deficient patient could be missed, as TPMT in a donor's cells can produce a significant amount of reaction product. The transfused blood usually contains only a very small percentage of nucleated cells; therefore the genotyping should still give a true indication of the patient's TPMT status. In transfusion cases it is suggested that TPMT phenotyping is repeated at least 120 days after the last transfusion, so that no donor cells remain in circulation. A case study where a transfusion masked a deficient patient, tested at City Hospital, is described by Ford et al 2004b. After eight weeks treatment with Azathioprine this patient developed neutropenic sepsis and staphylococcal skin impetigo. They

had become pancytopenic and a bone marrow examination revealed severe aplastic anaemia. The azathioprine was stopped after nine weeks and the following week blood was analysed for TPMT. The RBC activity was found to be low, 16 nmol 6MTG/gHb/h (normal 24-55). At a review eight weeks later the patient's TPMT activity was found to be undetectable at <2 nmol6MTG/gHb/h. The sample was genotyped and found to be homozygous for *3A, the most common TPMT mutation in Caucasians, demonstrating a clear phenotype/genotype concordance. Due to the apparent change in TPMT status, from low to deficient, the clinical history for the patient was re-examined. It was discovered that the patient had received a blood transfusion of one unit of blood and two units of platelets six days before the first sample for TPMT measurement was taken. In cases like this, the activity detected can be that of the donor cells. This is the likely cause of the discrepancy in this case and the cause of the initial misleading result. This highlights the importance of interpreting the TPMT results with caution in patients who have been transfused. It also highlights one of the reasons why our service genotypes all low activity samples and all samples where the clinical details indicate a recent transfusion.

The testing strategy of phenotyping to screen all samples and genotyping for selective testing should minimize the risk of missing ‘deficient’ patients which may have gone undetected using a single technique.

As well as genotyping assurance, all aspects of the method have been examined and adjusted if necessary to reduce variability and ensure the integrity of the results reported. A comprehensive vigilance system has been developed as part

of the overall Quality System. This has been necessary to maintain CE marking for this assay.

8.2.5 Variability of TPMT activity

A pre-analytical issue, which may give low results and possible misclassification of status, is sample deterioration due to age or storage conditions. Results in Chapter 4 show that TPMT is stable for over 10 days at -80°C and 4°C, but activity is seen to fall after about 6 days at room temperature. Users of the TPMT service are advised to store samples at 4°C prior to posting at ambient temperature. It is not known what temperatures the samples are subjected to in transit. Therefore, all samples >8 days old are flagged with the comment that the result must be interpreted with caution due to enzyme instability.

The issue of individual variability in TPMT activity was examined in detail. The analysis of two donors over period of several years has shown no significant fluctuation in their respective activities. Repeat samples from patients also suggest that an individual's TPMT activity remains relatively constant.

The data presented in Table 4.10 gives no indication of gender or age differences in TPMT activity. This supports the much smaller study of Keizer-Garritsen et al (2003) but is contrary to the findings of Klemetsdal et al (1993) and Cooper et al (2008), who observed slightly lower activities in females. Slightly lower results for children compared with adults were found by Ganiere-Monteil et al in 2004.

The demand for the City Hospital TPMT service has continued to increase, with an observed 100% increase over a three year period. This has occurred as it is becoming established practice in many NHS Trusts to screen all patients before initiating thiopurine therapy. Users switching their business to us, to take advantage of the rapid service, have also contributed to the increase in requests. The main users of the service continue to be gastroenterology, dermatology and rheumatology (Barlow et al 2009).

8.2.6 6TGN assay

The usefulness of the 6TGN test is still open to debate as it has been suggested that erythrocyte 6TGN levels are highly variable, even in the same patient and that levels do not reliably provide sufficient information to predict efficacy or toxicity (Sandborn 2004; Lennard & Lilleyman 1989; Lowry et al 2001; Mardini et al 2003). There does not seem to be a weight/dose correlation with metabolite levels (Tani et al 2009). Similarly, Morales in 2007 found the level of 6MP dose to be only weakly associated with 6TGN levels, due to individual variability, and for this reason supports the use of 6TGN testing. Work by Hindorf et al 2006 has shown metabolite monitoring to be cost effective and that of Tani et al 2009 suggests it is necessary to adequately personalise dosing. Some health insurance policies in the USA cover the monitoring of thiopurine metabolite levels, as it is considered medically necessary for individuals with inflammatory bowel disease (IBD) to guide dose changes in those who have not responded to

azathioprine (AZA) and / or 6-mercaptopurine (6-MP) therapy or for those suspected of having toxic responses to these medications.

The level of 6TGN required to achieve therapeutic effect may have an element of disease specificity. Lower mean levels have been observed in patients in remission for dermatological conditions compared with those having a positive response to treatment for inflammatory bowel disease (el-Azhary et al 2009).

Most 6TGN methods, including that used at City Hospital, are not ideal as they do not measure nucleotides directly. Some use enzymatic conversion of TGN to nucleosides with reverse-phase HPLC measurement; others depend on the hydrolysis back to the thiopurine bases. In the latter, the efficiency of hydrolysis is difficult to ascertain due to the unavailability of TGN standards. Another major issue concerns patients being treated with 6TG, where it is impossible to distinguish between the hydrolysed derivatives of TGN and the non-metabolised drug present in the blood cells. Another issue with the majority of 6TGN testing approaches is that RBC are used as a surrogate marker for target cells. This is not an ideal, as RBC are not nucleated and therefore lack full purine functionality. The enzyme IMP dehydrogenase is required for the conversion of 6MP, a hypoxanthine analogue, to thio-GM and TGN, this enzyme is not expressed in erythrocytes. Therefore the TGN produced as a result of 6MP metabolism are made indirectly, largely in hepatic tissue. For this reason it is suggested that erythrocyte TGN levels are more likely to reflect liver cell, rather than target white blood cell metabolism (Dulay & Florin 2005). 6TG is a guanine analogue, which can be converted to thio-GMP and TGN via HRPT. This direct incorporation of

6TG into TGN, compared with the indirect route followed by 6MP, results in relatively higher RBC TGN in patients receiving 6TG than those on 6MP. It has been recommended that patients who prove intolerant to 6MP may benefit from 6TG therapy. (Cheung et al 2003, Lennard et al 1993 & 1996). Ideally TGN should be measured in non-stimulated target cells. This would be difficult in practice as it would involve the laborious purification of leucocytes.

This work has included comparisons between whole blood 6TGN levels and levels in a preparation of washed RBC. Furthermore, research undertaken here has enabled a whole blood 6TGN assay to be introduced which is quicker, minimizes errors and is more suitable for routine clinical use than previously published methods. The actual HPLC analysis has also been improved with the introduction of a gradient elution method, which has greatly improved the quality of the chromatography, making the method much more suitable for routine use.

Work by Decaux et al (2000) and Thomas et al (2003) found a correlation between high levels of 6TGN and increased mean cell volume (MCV). Thomas et al suggest that MCV measurement is a simple and inexpensive alternative to measurement of 6TGN concentrations. No such correlation was found in this research although this data is of limited value. Many of the measured MCVs were above the reference range but most of the samples analysed were several days old, which can result in falsely high MCVs.

In Chapter 7, eight patients commencing on azathioprine were monitored for any apparent TPMT enzyme induction in whole blood. With the exception of one patient no significant increase in TPMT activity was observed. This fails to

support the hypothesis that induction effects may be more apparent in whole blood samples than in washed RBC. The work of Arenas et al in 2004 and Hindorf et al, in 2006, saw no increase in TPMT activity during treatment, in contrast to that of el-Azhary et al (2009), of the Mayo Clinic, where apparent induction was observed.

8.3 Future directions

Due to the high number of samples analysed by this service, a huge amount of valuable data has been available in this study. This has made it relatively easy to look at patterns in results. One of the most interesting trends observed has been the apparent correlation between low sample haemoglobin concentration and high calculated TPMT activity; this is detailed in Chapter 4. Evidence presented in this thesis suggests that the convention for reporting TPMT with respect to Hb concentration may give a misleading, high result for some patients with abnormal haematology, where the Hb measurement is a false indicator of cellular contents. These observations are to be followed up with further studies, including performing genomic analysis on low Hb samples that have borderline low/normal TPMT activity, to compare degree of concordance with routine samples. There is the possibility of taking the radical step of reporting TPMT activity in 'units per litre'. Recent literature shows a range of units are being used to express TPMT activity, many not adjusting for Hb concentration.

To give clinicians a better picture of what is happening 'in vivo' a service for 6MMP is being developed. This assay will be performed alongside 6TGN after slight method modifications. It is important to be able to offer quantification of this metabolite as increased levels can lead to hepatotoxicity. If a low 6TGN result is reported without a 6MMP, this does not give enough information to ascertain why. The thiopurine dose maybe too low, the patient may be non-compliant or 6MP could be being preferentially shunted towards 6MMP, due to high TPMT activity or other contributory factors. Such a 6TGN result could lead to inappropriate dose escalation; this risk is highlighted in the work of Gardiner et al (2008).

As seen in section 4.4, genotyping underpins this TPMT phenotyping service. In the absence of an organized external quality assurance (EQA) scheme. Regular audit of phenotype/genotype concordance ensures the validity of the phenotyping results.

City Hospital previously set up a worldwide EQA scheme for TPMT in conjunction with UK National External Quality Assessment Service (NEQAS). This was difficult to keep running due to difficulty in obtaining suitable material to distribute and also due to staffing issues both at City Hospital and NEQAS. A priority area for development of the service is to initiate a form of external quality control for both TPMT and 6TGN, possibly sample sharing with other laboratories.

Thiopurines are widely used as they are an attractive alternative to steroids, both in terms of cost and side effects. In 2004 nearly 0.6 million prescriptions for Azathioprine were dispensed (The Information Centre NHS 2005). Evidence suggests that treating a patient with these drugs, without firstly checking their TPMT status is ethically questionable. Such treatment has a 1% risk of death from associated bone marrow suppression and there is a four fold increased risk of suppression in heterozygotes (Gisbert & Gomollón 2008). Economic evidence in New Zealand has highlighted the cost savings of thiopurine treatment versus non-treatment of IBD patients (Priest el al 2006). Also, TPMT screening, prior to treatment, has been shown to be cost effective, phenotyping more so than genotyping (van den Akker-van Marle et al 2006) (Payne et al 2009). But robust evidence and accurate costs are difficult to obtain, this is due to reasons such as, under reporting of ADR and differences of opinion of what level constitutes neutropenia (Compagni et al 2008). In addition, when considering economics, phenotyping and metabolite monitoring can facilitate 'tailored' dosing. This is much more likely to have a favourable outcome, resulting in direct cost savings as well as improved quality of life.

Conclusions

This work has studied the development of a whole blood assay for TPMT and monitored it as it took over as the routine test, replacing an earlier more labour intensive and less precise assay. This project has confirmed that the new assay not only produces more precise and reliable results but that the concordance with

genotype is essentially unchanged from the previous test based on a RBC lysate preparation. One feature of this assay, and most others offered by other UK providers of TPMT and centres around the world, is expression of the TPMT activity in relation to haemoglobin or RBC content of the blood cell lysate. We have shown that there is little, if any, advantage to this and that expressing patient whole blood activity as U/L will give a result which classifies patients equally well against their underlying genotype. Furthermore, in some patients with low haemoglobin levels, evidence has been presented that misleading high results can be obtained and that this is not seen when results are expressed as U/L TPMT. Bringing the use of U/L expression into routine use would require further presentation of our evidence which would ideally encourage other providers to move to this reporting format. Such a move is to some extent facilitated by a recently launched commercial assay in kit form (BIOLOGIX Research Corporation) which disregards the link to haemoglobin and expresses a semi-quantitative result in U/L.

Our concept of a whole blood assay rather than using a sample based on washed RBC has also been applied to develop an assay for thioguanine nucleotides, another important outcome of this project has been bringing this into routine use. Again this has produced an assay which is much more suited to routine analysis as it reduces the analytical steps considerably. As the interest in therapeutic drug monitoring using 6TGN grows, this is a very useful development.

Alongside the timeline of this research project interest in TPMT analysis prior to starting treatment with thiopurines has grown considerably. Indeed in January 2009 the Drugs and Therapeutics Bulletin recommend that patients should be pre-screened with TPMT before commencing therapy. The provision of a service at City Hospital, which has always been able to maintain a 1-2 working day turnaround, is to a great part related to the process of continuous development and quality improvement that has been the foundation of the work in this thesis. This work has aided UK clinicians prescribing thiopurines, by offering a timely service for determination of TPMT status in patients commencing therapy, which can only impact positively on patient care.

9. APPENDICES

Appendix 1 Consumable and Equipment Suppliers

General

Camlab	Gilson pipettes
VWR	ART 10µl sterile pipette tips with filter
	ART 20µl sterile pipette gel tips with filter
	ART 200µ sterile pipette tips with filter
	ART 1000 sterile pipette tips with filter
Alpha Labs	1.5 ml sterile micro-centrifuge tubes
	Pastettes
	Micro-pastettes

TPMT Phenotyping

Hettich	Rotanda 460 Centrifuge
Grant	UBD Dri Incubation Blocks
Shimadzu	SCL-10AVP System Controller
	SIL-10ADVP Autosampler
	LC-10ADVP LC Pump
	DGU-14A Degasser
	CTO-10ASVP Column Oven with Phenomenex Kingsorb column
	SPD-10 AVP UV Detector
	RF-10AXL Fluorescence Detector

TPMT Genotyping

Grant	Water Bath
LEC	Automatic Ice Maker
Unicam	UV/visible spectrophotometer quartz micro-cuvettes (10mm)
Abgene	0.2 ml Thermo-tube, thin walled tubes with flat caps
Techne	96 well plate Genius thermal cycler
Eppendorf	5415D Micro-centrifuge
Thermo	EC330 Midicell Primo Submarine Gel System
	EC135-90 Power Pack
Syngene	GeneGenius Gel Documentation System
Consisting of:	Gel Documentation Analysis System (IBM Thinkpad Computer)
	Bio Imaging System
	GelVue UV Transilluminator
	Slim Light Panel
Sony	Digital Graphic Printer UDP-D895-MD
Sony	Printing Paper Type IV (Enhanced) UPP-110HA

6TGN

Shimadzu	CTO -20A Column Oven Phenomenex Prodigy 5uODS(2) column
	LC20 -AD HPLC Pump
	SIL -20A Autosampler
	SPD -20A UV/Vis Absorbance Detector
Hettich	MIKRO 200 Eppendorf Microcentrifuge
Stuart Scientific	SHT 100D Dri Block Unit

Appendix 2 Whole blood TPMT study raw data

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
219424	9.2	33	6.6	35	7.9	34	11.1	47	11.3	48	11.2	48	TPMT*1/1
219425	2.5	9	2.8	11	2.7	10	2.4	9	3.3	12	2.9	11	TPMT*1/3
219426	4.8	23	2.0	21	3.4	22	11.6	46	11.4	43	11.5	45	TPMT*1/1
219427	6.4	26	7.9	32	7.2	29	11.0	58	9.8	49	10.4	54	TPMT*1/1
219428	7.8	24	7.3	22	7.6	23	7.5	36	8.3	35	7.9	36	TPMT*1/1
219414	8.0	29	9.8	28	8.9	29	9.5	45	9.7	40	9.6	43	TPMT*1/1
219415	9.5	28	9.8	43	9.7	36	7.4	35	7.6	33	7.5	34	TPMT*1/1
219416	8.7	32	10.5	33	9.6	33	7.5	37	7.8	36	7.7	37	TPMT*1/1
219417	9.7	25	12.6	36	11.2	31	8.3	37	8.6	35	8.5	36	TPMT*1/1
219418	11.0	34	8.9	28	10.0	31	9.6	44	9.9	42	9.8	43	TPMT*1/1
219419	16.3	44	16.2	45	16.3	45	10.5	44	11.3	44	10.9	44	TPMT*1/1
219420	10.1	38	*	*	10.1	38	12.4	41	*	*	12.4	41	TPMT*1/1
219421	9.1	32	10.4	34	9.8	33	8.2	35	10.4	42	9.3	39	TPMT*1/1
219422	15.2	56	*	*	15.2	56	14.5	59	*	*	14.5	59	TPMT*1/1
219423	11.9	40	12.5	37	12.2	39	8.4	40	9.1	42	8.8	41	TPMT*1/1
219429	8.1	33	10.5	34	9.3	34	12.0	46	12.0	47	12.0	47	TPMT*1/1
219431	13.6	35	12.3	31	13.0	33	12.4	43	11.7	40	12.1	42	TPMT*1/1
219432	16.0	37	8.8	37	12.4	37	11.9	55	11.0	55	11.5	55	TPMT*1/1
219433	13.7	47	16.0	57	14.9	52	15.2	60	14.1	57	14.7	59	TPMT*1/1
219434	14.0	50	16.2	59	15.1	55	14.5	66	14.0	64	14.3	65	TPMT*1/1
219435	10.2	31	12.6	36	11.4	34	11.7	46	10.8	46	11.3	46	TPMT*1/1
219436	6.9	28	9.4	33	8.2	31	12.1	43	12.3	44	12.2	44	TPMT*1/1
219437	8.1	31	12.6	36	10.4	34	9.8	45	9.4	45	9.6	45	TPMT*1/1
219438	6.9	20	4.0	23	5.5	22	11.8	46	11.7	48	11.8	47	TPMT*1/1
219440	7.2	26	3.0	22	5.1	24	7.8	34	7.1	35	7.5	35	TPMT*1/1
219442	7.2	25	9.8	27	8.5	26	8.5	36	7.8	34	8.2	35	TPMT*1/1
219443	9.8	35	*	*	9.8	35	11.1	41	11.0	42	11.1	42	TPMT*1/1
219444	9.4	33	9.5	34	9.5	34	10.0	38	10.2	42	10.1	40	TPMT*1/1
219445	7.4	20	10.9	32	9.2	26	9.2	37	9.0	40	9.1	39	TPMT*1/1
219446	9.2	37	13.8	41	11.5	39	12.7	48	12.7	48	12.7	48	TPMT*1/1
219447	5.0	15	5.4	16	5.2	16	5.9	21	5.7	22	5.8	22	TPMT*1/3

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
219448	9.0	30	13.4	35	11.2	33	9.7	44	9.4	45	9.6	45	TPMT*1/1
219449	11.5	57	5.8	49	8.7	53	11.2	56	10.2	51	10.7	54	TPMT*1/1
219450	12.7	58	18.5	64	15.6	61	12.3	80	17.9	76	15.1	78	TPMT*1/1
219451	5.4	17	6.2	18	5.8	18	5.9	26	6.0	28	6.0	27	TPMT*1/3
219452	9.4	29	11.7	32	10.6	31	11.3	48	12.2	48	11.8	48	TPMT*1/1
219453	5.3	23	7.6	26	6.5	25	8.4	40	8.4	39	8.4	40	TPMT*1/1
219454	4.5	15	5.7	17	5.1	16	5.6	21	5.9	22	5.8	22	TPMT*1/3
219455	5.0	39	9.5	42	7.3	41	14.1	50	13.8	52	14.0	51	TPMT*1/1
219456	11.1	34	12.2	37	11.7	36	3.1	53	14.2	54	8.7	54	TPMT*1/1
219457	12.9	35	8.7	26	10.8	31	10.9	52	10.1	53	10.5	53	TPMT*1/1
219459	13.6	36	12.7	37	13.2	37	13.6	50	13.3	54	13.5	52	TPMT*1/1
219460	10.9	39	10.4	39	10.7	39	12.2	48	12.1	49	12.2	49	TPMT*1/1
219461	5.4	33	7.8	37	6.6	35	10.2	42	9.7	41	10.0	42	TPMT*1/1
219462	15.3	43	13.6	44	14.5	44	13.6	55	13.3	59	13.5	57	TPMT*1/1
219463	16.0	44	14.2	43	15.1	44	11.5	55	10.9	52	11.2	54	TPMT*1/1
219464	6.6	32	9.9	31	8.3	32	9.8	36	9.8	39	9.8	38	TPMT*1/1
219465	11.0	39	16.2	48	13.6	44	8.9	51	11.2	54	10.1	53	TPMT*1/1
219466	15.3	54	14.8	58	15.1	56	14.7	67	13.5	62	14.1	65	TPMT*1/1
219468	9.0	30	15.0	37	12.0	34	13.4	51	12.1	44	12.8	48	TPMT*1/1
219469	10.0	39	12.6	40	11.3	40	12.6	58	11.3	52	12.0	55	TPMT*1/1
219476	11.0	36	16.3	44	13.7	40	13.1	53	12.5	55	12.8	54	TPMT*1/1
219477	10.6	26	13.3	32	12.0	29	11.1	49	10.4	48	10.8	49	TPMT*1/1
219478	18.0	44	*	*	18.0	44	11.5	51	10.3	45	10.9	48	TPMT*1/1
219479	7.5	29	9.3	34	8.4	32	11.4	48	12.0	49	11.7	49	TPMT*1/1
219480	11.8	35	15.4	39	13.6	37	14.3	49	13.8	51	14.1	50	TPMT*1/1
219481	6.5	17	8.7	24	7.6	21	6.3	36	6.3	36	6.3	36	TPMT*1/1
219482	5.6	17	6.9	19	6.3	18	4.4	25	4.5	25	4.5	25	TPMT*1/3
219483	11.1	38	15.5	34	13.3	36	11.8	46	12.7	48	12.3	47	TPMT*1/1
219484	13.7	40	17.8	42	15.8	41	13.0	53	12.2	50	12.6	52	TPMT*1/1
219485	8.5	33	13.3	39	10.9	36	18.1	77	14.5	61	16.3	69	TPMT*1/1
219486	13.9	44	18.7	53	16.3	49	14.6	59	13.9	64	14.3	62	TPMT*1/1

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
219487	16.0	57	10.7	44	13.4	51	*	*	12.2	71	12.2	71	TPMT*1/1
219488	13.9	44	15.5	47	14.7	46	20.0	69	18.0	66	19.0	68	TPMT*1/1
219489	6.0	44	5.0	46	5.5	45	11.4	55	10.6	58	11.0	57	TPMT*1/1
219490	3.4	29	2.9	29	3.2	29	12.5	51	12.0	53	12.3	52	TPMT*1/1
219491	6.9	42	4.6	40	5.8	41	7.8	48	7.3	45	7.6	47	TPMT*1/1
219492	5.2	30	4.6	39	4.9	35	10.7	44	10.2	43	10.5	44	TPMT*1/1
219493	4.7	34	4.1	32	4.4	33	17.1	82	17.4	87	17.3	85	TPMT*1/1
219494	5.2	36	2.8	34	4.0	35	10.2	36	9.9	36	10.1	36	TPMT*1/1
219495	6.5	55	4.4	60	5.5	58	16.0	73	15.3	76	15.7	75	TPMT*1/1
219496	7.0	45	5.4	42	6.2	44	10.2	45	10.2	47	10.2	46	TPMT*1/1
219497	3.3	28	4.1	30	3.7	29	12.2	45	12.3	48	12.3	47	TPMT*1/1
219498	3.6	30	2.5	31	3.1	31	12.0	44	11.4	43	11.7	44	TPMT*1/1
219499	4.7	32	3.2	32	4.0	32	9.6	48	9.3	46	9.5	47	TPMT*1/1
219500	4.4	32	3.4	34	3.9	33	15.9	53	15.8	51	15.9	52	TPMT*1/1
219501	2.3	42	2.9	35	2.6	39	14.5	57	14.9	63	14.7	60	TPMT*1/1
219502	5.1	51	3.5	48	4.3	50	12.4	51	11.7	51	12.1	51	TPMT*1/1
219503	4.0	29	2.9	32	3.5	31	9.9	50	9.4	49	9.7	50	TPMT*1/1
219504	2.1	23	1.6	20	1.9	22	6.6	27	6.8	29	6.7	28	TPMT*1/3
219505	7.0	45	3.4	27	5.2	36	7.8	43	7.2	38	7.5	41	TPMT*1/1
219470	9.6	29	15.6	40	12.6	35	11.1	47	11.8	48	11.5	48	TPMT*1/1
219471	7.7	26	8.0	31	7.9	29	10.3	40	11.0	42	10.7	41	TPMT*1/1
219472	5.1	19	9.0	24	7.1	22	6.2	31	7.5	36	6.9	34	TPMT*1/3
219473	12.9	38	15.8	49	14.4	44	13.6	53	14.3	54	14.0	54	TPMT*1/1
219474	5.9	17	9.9	25	7.9	21	6.9	30	6.9	30	6.9	30	TPMT*1/3
219475	10.7	32	20.8	67	15.8	50	12.8	61	13.2	61	13.0	61	TPMT*1/1
219506	5.6	19	9.0	32	7.3	26	7.7	40	8.0	40	7.9	40	TPMT*1/1
219507	6.1	20	10.2	33	8.2	27	9.5	55	9.4	54	9.5	55	TPMT*1/1
219508	6.4	23	10.0	35	8.2	29	8.6	45	8.4	44	8.5	45	TPMT*1/1
219509	8.1	32	12.3	50	10.2	41	11.2	56	10.1	53	10.7	55	TPMT*1/1
219510	5.7	18	9.2	32	7.5	25	9.7	44	9.2	44	9.5	44	TPMT*1/1
219511	9.1	29	12.3	41	10.7	35	10.4	46	9.8	43	10.1	45	TPMT*1/1

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
219512	7.6	33	11.1	37	9.4	35	11.6	51	11.1	49	11.4	50	TPMT*1/1
219513	9.0	34	10.8	40	9.9	37	14.2	52	14.4	53	14.3	53	TPMT*1/1
219518	6.1	23	8.3	28	7.2	26	8.3	35	8.1	36	8.2	36	TPMT*1/1
219519	17.5	69	19.7	66	18.6	68	13.4	74	12.3	68	12.9	71	TPMT*1/1
219520	9.4	30	9.1	30	9.3	30	10.2	47	9.3	46	9.8	47	TPMT*1/1
219521	7.5	28	10.9	33	9.2	31	9.6	44	9.2	42	9.4	43	TPMT*1/1
219522	12.2	41	13.6	43	12.9	42	13.8	56	14.7	54	14.3	55	TPMT*1/1
219523	7.9	33	14.7	34	11.3	34	13.2	48	13.2	47	13.2	48	TPMT*1/1
219524	18.4	70	18.3	59	18.4	65	18.0	91	20.4	94	19.2	93	TPMT*1/1
219515	10.4	24	11.7	27	11.1	26	5.7	37	7.2	36	6.5	37	TPMT*1/1
219516	16.1	52	*	*	16.1	52	16.0	61	*	*	16.0	61	TPMT*1/1
219517	10.4	26	8.9	38	9.7	32	8.9	38	10.6	45	9.8	42	TPMT*1/1
219525	5.8	16	8.3	19	7.1	18	5.5	23	6.4	26	6.0	25	TPMT*1/3
219527	13.0	31	13.6	34	13.3	33	9.4	40	9.0	37	9.2	39	TPMT*1/1
219528	14.2	65	21.2	65	17.7	65	16.0	73	17.0	72	16.5	73	TPMT*1/1
219529	6.6	21	9.4	25	8.0	23	11.9	55	12.3	52	12.1	54	TPMT*1/1
219530	8.7	27	11.0	33	9.9	30	9.3	46	10.2	47	9.8	47	TPMT*1/1
219531	4.6	13	5.1	15	4.9	14	5.3	22	5.8	22	5.6	22	TPMT*1/1
219532	13.4	43	15.1	46	14.3	45	10.9	60	11.6	58	11.3	59	TPMT*1/1
219533	8.4	26	10.1	30	9.3	28	8.8	39	9.3	39	9.1	39	TPMT*1/1
219534	9.8	31	15.5	42	12.7	37	11.0	58	12.6	63	11.8	61	TPMT*1/1
219535	11.8	31	12.4	35	12.1	33	14.0	51	15.1	52	14.6	52	TPMT*1/1
219536	5.3	25	8.0	30	6.7	28	12.4	51	14.2	58	13.3	55	TPMT*1/1
219537	9.7	31	12.5	40	11.1	36	10.4	67	12.0	66	11.2	67	TPMT*1/1
219538	12.1	43	17.8	53	15.0	48	12.4	68	12.9	68	12.7	68	TPMT*1/1
219539	15.8	45	19.1	58	17.5	52	14.7	65	15.7	64	15.2	65	TPMT*1/1
219540	17.4	46	*	*	17.4	46	11.7	56	*	*	11.7	56	TPMT*1/1
219541	12.4	40	16.9	50	14.7	45	13.7	66	13.0	62	13.4	64	TPMT*1/1
219542	15.4	56	14.0	48	14.7	52	14.1	55	15.1	66	14.6	61	TPMT*1/1
219543	16.8	58	18.1	51	17.5	55	12.3	61	11.9	62	12.1	62	TPMT*1/1
219544	7.7	24	9.6	28	8.7	26	13.9	44	13.5	45	13.7	45	TPMT*1/1

Sample	RBC Lysate 1 Peak Ht.	Activity	RBC Lysate 2 Peak Ht.	Activity	Mean RBC Lysate Peak Ht.	Activity	Whole Blood 1 Peak Ht.	Activity	Whole Blood 2 Peak Ht.	Activity	Mean WB Peak Ht.	Activity	Genotype
219545	7.9	24	8.0	30	8.0	27	8.8	36	8.5	36	8.7	36	TPMT*1/3
219546	10.8	52	8.0	33	9.4	43	10.8	42	10.8	48	10.8	45	TPMT*1/1
219547	9.1	29	13.1	38	11.1	34	12.4	52	11.8	52	12.1	52	TPMT*1/1
219548	7.6	30	11.3	30	9.5	30	11.4	42	11.3	44	11.4	43	TPMT*1/1
219549	12.8	38	14.2	42	13.5	40	14.1	55	14.2	56	14.2	56	TPMT*1/1
219550	11.4	34	11.9	34	11.7	34	12.3	45	11.7	44	12.0	45	TPMT*1/1
219551	7.0	28	7.5	32	7.3	30	10.8	40	10.7	42	10.8	41	TPMT*1/1
219552	10.6	28	11.6	36	11.1	32	11.8	52	11.5	51	11.7	52	TPMT*1/1
219553	5.8	16	7.0	20	6.4	18	7.5	27	8.0	29	7.8	28	TPMT*1/3
219554	10.7	29	9.9	38	10.3	34	15.1	69	16.9	60	16.0	65	TPMT*1/1
219555	6.6	21	7.8	28	7.2	25	10.6	47	10.3	47	10.5	47	TPMT*1/1
219556	15.9	50	22.2	53	19.1	52	17.0	69	16.8	71	16.9	70	TPMT*1/1
219557	5.2	36	14.5	39	9.9	38	10.3	54	10.0	52	10.2	53	TPMT*1/1
219558	9.5	33	7.5	27	8.5	30	10.6	45	10.3	45	10.5	45	TPMT*1/1
219559	15.9	42	11.2	33	13.6	38	15.3	56	16.1	55	15.7	56	TPMT*1/1
219560	13.4	38	15.6	42	14.5	40	14.9	59	16.0	61	15.5	60	TPMT*1/1
219561	11.6	35	10.1	32	10.9	34	8.8	57	9.2	53	9.0	55	TPMT*1/1
219562	8.1	28	8.9	30	8.5	29	9.8	47	10.3	54	10.1	51	TPMT*1/1
219563	11.9	31	14.0	28	13.0	30	14.9	55	15.8	54	15.4	55	TPMT*1/1
219565	10.4	34	13.4	33	11.9	34	10.6	42	9.1	39	9.9	41	TPMT*1/1
219567	13.7	38	15.6	40	14.7	39	13.6	53	14.0	53	13.8	53	TPMT*1/1
219568	19.3	66	14.2	68	16.8	67	15.5	81	15.3	99	15.4	90	TPMT*1/1
219569	15.5	41	15.6	39	15.6	40	13.2	52	14.1	53	13.7	53	TPMT*1/1
219570	12.8	45	5.7	57	9.3	51	8.7	60	10.0	61	9.4	61	TPMT*1/1
219571	8.9	33	*	*	8.9	33	8.9	39	9.3	39	9.1	39	TPMT*1/1
219572	8.8	25	7.5	23	8.2	24	7.7	37	5.6	26	6.7	32	TPMT*1/1
219573	5.6	15	6.2	15	5.9	15	6.6	24	6.8	25	6.7	25	TPMT*1/2
219577	4.8	14	*	*	4.8	14	6.3	22	6.7	22	6.5	22	TPMT*1/3
219578	15.0	50	14.4	50	14.7	50	13.1	85	13.4	82	13.3	84	TPMT*1/1
219579	11.2	32	13.9	35	12.6	34	11.4	48	12.3	48	11.9	48	TPMT*1/1
219580	11.9	35	13.3	37	12.6	36	12.6	50	13.6	52	13.1	51	TPMT*1/1

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
219581	13.5	51	10.5	52	12.0	52	13.1	53	16.4	60	14.8	57	TPMT*1/1
219582	15.3	41	15.6	42	15.5	42	18.1	59	19.6	58	18.9	59	TPMT*1/1
219583	12.7	36	13.3	35	13.0	36	15.3	58	15.3	50	15.3	54	TPMT*1/1
219584	9.8	31	5.6	34	7.7	33	10.7	44	12.3	47	11.5	46	TPMT*1/1
219687	8.1	25	7.0	25	7.6	25	9.7	44	9.7	44	9.7	44	TPMT*1/1
219688	10.5	29	12.9	34	11.7	32	10.3	49	11.1	49	10.7	49	TPMT*1/1
219689	14.7	29	12.7	32	13.7	31	9.7	41	10.3	40	10.0	41	TPMT*1/1
219690	10.3	31	12.1	32	11.2	32	10.7	44	11.6	44	11.2	44	TPMT*1/1
219691	11.0	31	*	*	11.0	31	8.3	40	8.9	40	8.6	40	TPMT*1/1
219692	12.3	36	13.3	38	12.8	37	8.9	36	9.4	38	9.2	37	TPMT*1/1
219693	11.1	29	10.6	30	10.9	30	10.4	39	10.8	38	10.6	39	TPMT*1/1
219694	7.9	24	6.1	25	7.0	25	7.5	38	7.7	35	7.6	37	TPMT*1/1
219695	10.2	28	10.5	26	10.4	27	7.7	35	8.3	34	8.0	35	TPMT*1/1
219696	12.2	34	12.3	34	12.3	34	11.3	48	11.9	48	11.6	48	TPMT*1/1
219697	11.5	28	11.9	30	11.7	29	9.2	37	9.9	39	9.6	38	TPMT*1/1
219698	11.2	29	10.4	29	10.8	29	11.8	50	12.4	54	12.1	52	TPMT*1/1
219699	10.2	32	10.9	32	10.6	32	8.0	30	8.6	30	8.3	30	TPMT*1/1
219700	3.7	11	4.5	13	4.1	12	4.8	20	4.8	21	4.8	21	TPMT*1/3
219701	8.8	24	9.2	25	9.0	25	11.6	44	12.0	41	11.8	43	TPMT*1/1
219702	9.3	27	9.8	28	9.6	28	9.8	41	10.6	42	10.2	42	TPMT*1/1
219703	10.2	30	9.4	32	9.8	31	8.3	48	7.4	48	7.9	48	TPMT*1/1
219704	9.1	29	9.3	27	9.2	28	10.3	38	12.1	42	11.2	40	TPMT*1/1
219705	8.5	25	9.3	26	8.9	26	10.5	50	10.4	48	10.5	49	TPMT*1/1
219706	6.4	19	6.7	20	6.6	20	9.3	38	10.3	39	9.8	39	TPMT*1/1
219707	11.0	35	12.0	38	11.5	37	11.4	48	11.7	50	11.6	49	TPMT*1/1
219708	7.1	27	7.4	27	7.3	27	8.0	42	9.1	40	8.6	41	TPMT*1/1
219709	5.0	13	7.2	23	6.1	18	4.3	19	5.1	20	4.7	20	TPMT*1/3
219710	10.1	35	6.6	38	8.4	37	7.4	45	7.8	45	7.6	45	TPMT*1/1
219711	15.6	42	16.6	46	16.1	44	14.6	53	18.3	54	16.5	54	TPMT*1/1
219712	13.6	39	14.1	43	13.9	41	11.9	57	13.5	55	12.7	56	TPMT*1/1
219713	10.0	42	10.7	39	10.4	41	9.7	44	9.1	48	9.4	46	TPMT*1/1

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
219714	15.0	59	15.3	56	15.2	58	15.2	67	15.6	75	15.4	71	TPMT*1/1
219715	11.9	41	12.0	41	12.0	41	13.4	55	12.9	55	13.2	55	TPMT*1/1
219716	8.0	25	11.4	26	9.7	26	8.5	42	8.7	43	8.6	43	TPMT*1/1
219717	5.5	20	6.3	21	5.9	21	7.3	36	8.0	38	7.7	37	TPMT*1/1
219718	8.4	23	9.3	24	8.9	24	10.5	39	9.6	39	10.1	39	TPMT*1/1
219719	13.8	56	19.5	56	16.7	56	12.2	84	12.8	83	12.5	84	TPMT*1/1
219720	5.7	17	7.0	19	6.4	18	5.6	21	6.1	21	5.9	21	TPMT*1/3
219721	11.7	35	12.1	34	11.9	35	11.7	43	11.0	45	11.4	44	TPMT*1/1
219722	5.3	25	9.3	27	7.3	26	9.5	37	10.2	37	9.9	37	TPMT*1/1
219723	11.7	33	12.3	33	12.0	33	11.3	46	12.2	48	11.8	47	TPMT*1/1
219724	9.3	29	9.3	29	9.3	29	8.3	40	8.4	40	8.4	40	TPMT*1/1
219725	12.5	43	12.1	43	12.3	43	13.6	53	14.7	54	14.2	54	TPMT*1/1
219726	7.5	26	10.1	25	8.8	26	8.9	43	8.5	37	8.7	40	TPMT*1/1
219727	9.8	33	12.1	35	11.0	34	12.5	55	13.7	56	13.1	56	TPMT*1/1
219728	4.9	15	6.0	15	5.5	15	4.8	23	4.9	22	4.9	23	TPMT*1/3
219729	10.7	36	9.9	39	10.3	38	10.3	47	11.7	44	11.0	46	TPMT*1/1
219730	14.5	39	16.6	42	15.6	41	11.8	52	12.4	49	12.1	51	TPMT*1/1
219731	10.3	28	12.7	30	11.5	29	10.2	42	10.7	42	10.5	42	TPMT*1/1
219732	10.9	33	8.2	25	9.6	29	11.9	59	12.8	52	12.4	56	TPMT*1/1
219733	10.9	29	12.3	31	11.6	30	10.2	42	11.1	42	10.7	42	TPMT*1/1
219734	11.8	36	12.1	33	12.0	35	11.4	50	12.2	50	11.8	50	TPMT*1/1
219735	7.7	24	7.0	24	7.4	24	7.1	34	7.1	34	7.1	34	TPMT*1/1
219736	8.1	29	7.8	29	8.0	29	6.9	45	7.5	43	7.2	44	TPMT*1/1
219738	10.1	31	12.1	33	11.1	32	9.3	43	9.8	41	9.6	42	TPMT*1/1
219737	7.8	50	10.7	56	9.3	53	14.3	66	14.1	70	14.2	68	TPMT*1/1
219739	8.0	46	9.6	46	8.8	46	12.3	59	13.2	63	12.8	61	TPMT*1/1
219740	4.3	43	6.2	36	5.3	40	9.0	50	9.4	49	9.2	50	TPMT*1/1
219741	4.4	48	5.6	44	5.0	46	12.2	52	11.9	48	12.1	50	TPMT*1/1
219743	3.9	43	4.2	39	4.1	41	10.8	50	10.6	47	10.7	49	TPMT*1/1
219744	16.1	52	16.8	58	16.5	55	16.8	64	18.0	66	17.4	65	TPMT*1/1

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
219745	11.2	49	7.1	60	9.2	55	13.8	72	14.4	72	14.1	72	TPMT*1/1
219746	10.5	41	10.8	44	10.7	43	9.1	56	10.6	65	9.9	61	TPMT*1/1
219747	12.7	42	12.3	45	12.5	44	13.1	53	12.1	56	12.6	55	TPMT*1/1
219748	9.4	45	11.3	44	10.4	45	10.0	58	10.7	59	10.4	59	TPMT*1/1
219749	4.8	23	4.9	20	4.9	22	7.4	41	7.2	38	7.3	40	TPMT*1/3
219750	9.0	47	12.3	45	10.7	46	17.4	74	18.5	73	18.0	74	TPMT*1/1
219752	13.4	48	13.0	48	13.2	48	10.9	52	11.7	56	11.3	54	TPMT*1/1
219753	12.9	46	15.1	55	14.0	51	13.8	63	12.3	59	13.1	61	TPMT*1/1
219754	9.0	66	10.4	60	9.7	63	14.6	64	17.1	70	15.9	67	TPMT*1/1
219755	10.8	48	10.1	50	10.5	49	13.9	53	14.0	57	14.0	55	TPMT*1/1
219756	9.5	39	8.7	43	9.1	41	12.6	53	14.0	59	13.3	56	TPMT*1/1
219757	7.2	27	*	*	7.2	27	10.3	42	10.7	51	10.5	47	TPMT*1/1
219759	1.2	4	1.3	4.9	1.3	4	1.6	7.7	1.3	5.9	1.5	7	TPMT*3/3
219760	8.0	28	5.4	33	6.7	31	9.7	44	11.3	46	10.5	45	TPMT*1/1
219761	8.5	31	9.0	33	8.8	32	10.4	44	11.2	44	10.8	44	TPMT*1/1
219762	8.7	34	11.2	37	10.0	36	10.8	48	14.7	60	12.8	54	TPMT*1/1
219763	10.3	49	12.0	48	11.2	49	10.4	60	13.0	55	11.7	58	TPMT*1/1
219764	10.2	36	10.6	42	10.4	39	12.3	52	12.3	50	12.3	51	TPMT*1/1
219765	10.3	45	6.3	53	8.3	49	14.5	59	15.9	58	15.2	59	TPMT*1/1
219922	9.2	56	9.6	59	9.4	58	11.9	57	13.8	58	12.9	58	TPMT*1/1
219925	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	TPMT*3/3
219926	9.3	33	10.8	33	10.1	33	9.3	43	9.5	44	9.4	44	TPMT*1/1
219927	11.0	34	12.8	39	11.9	37	10.1	40	10.8	44	10.5	42	TPMT*1/1
219928	20.8	67	21.2	67	21.0	67	13.9	80	13.9	85	13.9	83	TPMT*1/1
219929	11.0	42	11.4	36	11.2	39	11.3	48	12.5	51	11.9	50	TPMT*1/1
219930	20.9	62	21.7	68	21.3	65	13.5	57	13.6	62	13.6	60	TPMT*1/1
219931	12.4	40	13.6	40	13.0	40	11.4	45	11.6	49	11.5	47	TPMT*1/1
219932	13.3	38	10.5	40	11.9	39	13.3	56	13.0	49	13.2	53	TPMT*1/1
219933	12.5	38	11.1	41	11.8	40	12.2	52	13.5	62	12.9	57	TPMT*1/1
219934	8.2	50	12.5	53	10.4	52	10.9	57	11.2	65	11.1	61	TPMT*1/1
219935	11.1	39	11.1	37	11.1	38	11.8	50	11.3	48	11.6	49	TPMT*1/1

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
219936	8.6	50	11.7	50	10.2	50	10.2	49	11.0	53	10.6	51	TPMT*1/1
219937	8.5	36	7.8	34	8.2	35	6.9	40	7.8	43	7.4	42	TPMT*1/3
219938	7.9	24	8.2	25	8.1	25	8.0	32	8.5	34	8.3	33	TPMT*1/3
219939	14.3	48	17.3	58	15.8	53	12.0	63	31.1	72	21.6	68	TPMT*1/1
219940	11.5	38	14.9	40	13.2	39	12.3	59	12.5	60	12.4	60	TPMT*1/3
219941	14.2	49	14.6	50	14.4	50	10.5	64	11.4	66	11.0	65	TPMT*1/1
219942	13.5	46	16.2	45	14.9	46	11.9	69	12.7	74	12.3	72	TPMT*1/1
219943	15.7	48	11.9	44	13.8	46	14.8	54	16.7	56	15.8	55	TPMT*1/1
219944	13.8	45	13.9	45	13.9	45	13.2	56	15.5	55	14.4	56	TPMT*1/1
219945	11.3	37	11.3	34	11.3	36	12.4	55	14.1	60	13.3	58	TPMT*1/1
219946	5.5	25	5.4	25	5.5	25	7.2	42	8.3	48	7.8	45	TPMT*1/3
219947	11.4	38	12.4	40	11.9	39	13.8	61	14.9	68	14.4	65	TPMT*1/1
219948	10.2	32	10.7	29	10.5	31	11.3	46	12.3	52	11.8	49	TPMT*1/1
219949	17.6	55	18.8	63	18.2	59	17.1	94	19.5	107	18.3	101	TPMT*1/1
219950	11.9	48	11.6	47	11.8	48	14.2	62	14.8	63	14.5	63	TPMT*1/1
219951	10.8	29	7.7	37	9.3	33	11.1	64	12.2	58	11.7	61	TPMT*1/1
219360	10.5	40	11.5	40	11.0	40	11.6	53	11.6	51	11.6	52	TPMT*1/1
219953	5.9	25	5.5	25	5.7	25	11.4	50	11.7	50	11.6	50	TPMT*1/1
219955	11.6	40	12.7	40	12.2	40	13.5	59	12.3	61	12.9	60	TPMT*1/1
219956	8.7	29	8.8	29	8.8	29	9.5	42	10.0	44	9.8	43	TPMT*1/1
219957	10.6	39	11.5	38	11.1	39	13.0	65	12.7	66	12.9	66	TPMT*1/1
219958	10.3	44	11.5	44	10.9	44	12.1	70	12.5	65	12.3	68	TPMT*1/1
219959	6.2	24	6.3	22	6.3	23	4.1	50	4.8	48	4.5	49	TPMT*1/1
219952	5.8	38	7.0	41	6.4	40	11.2	47	13.7	46	12.5	47	TPMT*1/1
219961	9.5	52	8.7	48	9.1	50	13.9	53	15.0	53	14.5	53	TPMT*1/1
219962	9.7	41	9.8	40	9.8	41	12.1	63	12.8	61	12.5	62	TPMT*1/1
219963	8.1	37	8.1	34	8.1	36	10.3	42	11.0	42	10.7	42	TPMT*1/1
219964	7.5	33	9.1	33	8.3	33	10.6	65	11.1	64	10.9	65	TPMT*1/1
219965	8.1	34	8.3	34	8.2	34	12.5	51	13.2	50	12.9	51	TPMT*1/1
219973	11.3	40	10.3	38	10.8	39	9.9	47	10.0	48	10.0	48	TPMT*1/1
219974	5.5	30	6.1	32	5.8	31	8.1	36	8.9	39	8.5	38	TPMT*1/1

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
219975	8.2	31	8.5	35	8.4	33	12.0	49	12.5	62	12.3	56	TPMT*1/1
219976	5.6	39	5.0	37	5.3	38	14.3	63	15.2	64	14.8	64	TPMT*1/1
219977	11.7	54	14.0	53	12.9	54	13.6	68	15.4	74	14.5	71	TPMT*1/1
219978	6.6	38	8.4	37	7.5	38	11.4	48	12.1	49	11.8	49	TPMT*1/1
219979	5.0	42	7.1	46	6.1	44	13.3	56	13.8	58	13.6	57	TPMT*1/1
219980	3.3	33	5.2	34	4.3	34	11.4	52	11.7	51	11.6	52	TPMT*1/1
219981	3.7	34	8.6	31	6.2	33	7.3	47	7.6	46	7.5	47	TPMT*1/1
219960	2.9	15	3.6	16	3.3	16	5.3	21	5.3	21	5.3	21	TPMT*1/3
219971	11.1	41	11.5	42	11.3	42	13.6	53	12.5	51	13.1	52	TPMT*1/1
219966	9.6	38	10.6	38	10.1	38	9.6	75	10.4	76	10.0	76	TPMT*1/1
219968	5.8	32	7.6	33	6.7	33	11.2	44	12.4	52	11.8	48	TPMT*1/1
219969	9.8	40	7.2	40	8.5	40	10.5	44	11.6	51	11.1	48	TPMT*1/1
219982	7.6	31	8.7	31	8.2	31	10.8	38	11.6	40	11.2	39	TPMT*1/1
219983	9.5	28	7.6	28	8.6	28	9.4	41	9.7	43	9.6	42	TPMT*1/1
219984	5.3	25	7.2	28	6.3	27	13.9	42	15.3	47	14.6	45	TPMT*1/1
219985	3.8	14	4.7	16	4.3	15	5.2	21	5.9	24	5.6	23	TPMT*1/3
219986	4.1	15	4.8	17	4.5	16	5.9	22	5.8	21	5.9	22	TPMT*1/3
219987	4.5	18	5.4	17	5.0	18	7.4	26	7.7	26	7.6	26	TPMT*1/3
219988	5.8	30	8.8	35	7.3	33	11.4	46	11.5	45	11.5	46	TPMT*1/1
219989	8.6	31	8.6	32	8.6	32	8.1	45	8.9	49	8.5	47	TPMT*1/1
219990	4.2	17	4.6	17	4.4	17	8.2	28	8.5	29	8.4	29	TPMT*1/3
219991	6.4	24	6.6	27	6.5	26	12.2	67	13.1	72	12.7	70	TPMT*1/1
219992	8.8	32	9.1	33	9.0	33	12.4	52	13.0	55	12.7	54	TPMT*1/1
219993	7.8	32	8.2	32	8.0	32	9.5	50	11.9	50	10.7	50	TPMT*1/1
219994	7.3	27	4.6	23	6.0	25	8.5	42	8.9	44	8.7	43	TPMT*1/1
219995	13.0	43	15.7	44	14.4	44	13.5	59	11.9	59	12.7	59	TPMT*1/1
219996	14.7	43	8.7	46	11.7	45	13.0	55	12.1	53	12.6	54	TPMT*1/1
219997	6.1	21	6.6	19	6.4	20	6.3	26	6.4	28	6.4	27	TPMT*1/3
219998	11.5	38	15.3	41	13.4	40	14.5	47	14.0	48	14.3	48	TPMT*1/1
219999	15.9	46	14.8	43	15.4	45	13.3	50	12.6	51	13.0	51	TPMT*1/1
210000	13.4	41	17.9	50	15.7	46	15.8	58	15.3	58	15.6	58	TPMT*1/1

Sample	RBC Lysate 1 Peak Ht.	Activity	RBC Lysate 2 Peak Ht.	Activity	Mean RBC Lysate Peak Ht.	Activity	Whole Blood 1 Peak Ht.	Activity	Whole Blood 2 Peak Ht.	Activity	Mean WB Peak Ht.	Activity	Genotype
229001	15.9	44	16.7	46	16.3	45	14.6	59	16.6	55	15.6	57	TPMT*1/1
229002	15.6	52	16.3	50	16.0	51	12.7	74	13.2	73	13.0	74	TPMT*1/1
229003	12.2	35	12.3	35	12.3	35	11.2	49	13.0	53	12.1	51	TPMT*1/1
229005	13.6	36	15.3	40	14.5	38	13.4	59	14.4	59	13.9	59	TPMT*1/1
229006	14.3	41	10.5	41	12.4	41	13.5	67	13.5	62	13.5	65	TPMT*1/1
229007	8.4	36	12.4	43	10.4	40	12.9	55	17.0	53	15.0	54	TPMT*1/1
229008	13.6	58	15.3	50	14.5	54	15.3	70	15.4	68	15.4	69	TPMT*1/1
229009	12.1	35	10.3	49	11.2	42	12.2	52	13.3	50	12.8	51	TPMT*1/1
229010	9.7	40	16.5	47	13.1	44	16.1	61	16.9	62	16.5	62	TPMT*1/1
229011	12.9	43	15.4	42	14.2	43	13.2	54	13.9	53	13.6	54	TPMT*1/1
229012	15.1	45	17.2	46	16.2	46	13.5	57	14.0	55	13.8	56	TPMT*1/1
229013	11.8	33	12.8	35	12.3	34	11.7	54	12.2	54	12.0	54	TPMT*1/1
229014	11.3	32	11.4	32	11.4	32	12.7	56	13.6	58	13.2	57	TPMT*1/1
229015	13.8	37	14.7	38	14.3	38	13.0	51	13.0	51	13.0	51	TPMT*1/1
229016	13.6	48	15.8	54	14.7	51	14.2	74	14.6	73	14.4	74	TPMT*1/1
229017	6.4	31	4.7	30	5.6	31	11.3	48	12.4	49	11.9	49	TPMT*1/1
229018	6.5	34	8.9	34	7.7	34	12.0	55	14.5	61	13.3	58	TPMT*1/1
229019	7.3	28	7.1	27	7.2	28	7.1	37	7.5	36	7.3	37	TPMT*1/3
229020	7.7	37	8.8	36	8.3	37	11.3	44	12.3	45	11.8	45	TPMT*1/1
229021	5.0	25	6.2	30	5.6	28	11.9	45	11.6	43	11.8	44	TPMT*1/1
229022	9.2	36	9.9	38	9.6	37	11.4	46	12.0	47	11.7	47	TPMT*1/1
229024	4.5	38	7.2	33	5.9	36	8.5	47	10.6	58	9.6	53	TPMT*1/1
229025	9.0	33	9.9	34	9.5	34	11.0	55	11.3	54	11.2	55	TPMT*1/1
229026	9.0	34	9.4	36	9.2	35	13.2	52	13.6	50	13.4	51	TPMT*1/1
229027	7.9	30	7.3	31	7.6	31	8.9	47	9.1	44	9.0	46	TPMT*1/1
229028	7.2	26	7.4	27	7.3	27	11.3	41	11.9	42	11.6	42	TPMT*1/1
229029	10.5	40	11.2	41	10.9	41	11.4	46	11.4	48	11.4	47	TPMT*1/1
229030	6.0	33	7.0	31	6.5	32	11.7	44	13.4	46	12.6	45	TPMT*1/1
229036	14.2	50	14.5	50	14.4	50	13.3	50	15.6	55	14.5	53	TPMT*1/1
229038	12.1	44	12.3	47	12.2	46	12.9	51	14.2	50	13.6	51	TPMT*1/1
229039	16.5	61	17.6	61	17.1	61	15.0	69	16.1	68	15.6	69	TPMT*1/1

	RBC Lysate 1		RBC Lysate 2		Mean RBC Lysate		Whole Blood 1		Whole Blood 2		Mean WB		
Sample	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Peak Ht.	Activity	Genotype
229040	8.5	42	13.9	42	11.2	42	12.6	53	13.0	49	12.8	51	TPMT*1/1
229041	4.8	29	6.3	30	5.6	30	10.9	50	10.5	44	10.7	47	TPMT*1/1
229042	11.2	32	13.5	34	12.4	33	11.2	47	11.3	48	11.3	48	TPMT*1/1
229043	10.0	32	9.5	32	9.8	32	6.7	37	6.8	34	6.8	36	TPMT*1/3
229044	11.9	31	12.8	34	12.4	33	10.1	38	11.2	42	10.7	40	TPMT*1/1
229045	14.0	44	15.1	40	14.6	42	13.6	55	13.7	54	13.7	55	TPMT*1/1
229046	10.1	25	11.2	26	10.7	26	10.5	46	11.0	45	10.8	46	TPMT*1/1
229047	11.2	40	12.6	42	11.9	41	10.4	60	10.8	59	10.6	60	TPMT*1/1
229048	8.0	28	11.6	39	9.8	34	12.4	47	13.3	47	12.9	47	TPMT*1/1
229049	5.2	25	6.1	22	5.7	24	6.7	31	6.7	29	6.7	30	TPMT*1/1
229050	15.7	47	18.0	51	16.9	49	13.7	75	12.5	76	13.1	76	TPMT*1/1
229051	11.2	32	13.0	38	12.1	35	11.1	53	18.1	42	14.6	48	TPMT*1/1
229052	8.1	25	9.2	26	8.7	26	9.6	56	10.2	59	9.9	58	TPMT*1/1
229053	8.0	26	10.5	32	9.3	29	11.9	48	10.9	43	11.4	46	TPMT*1/1
229054	3.6	13	5.8	18	4.7	16	5.2	20	4.6	22	4.9	21	TPMT*1/2
229055	11.1	39	10.8	35	11.0	37	9.0	50	9.4	49	9.2	50	TPMT*1/1
229031	11.0	38	11.8	38	11.4	38	11.0	50	10.5	48	10.8	49	TPMT*1/1
229033	10.4	41	10.9	40	10.7	41	12.6	48	10.3	47	11.5	48	TPMT*1/1
229034	5.7	26	6.2	27	6.0	27	10.0	48	9.2	51	9.6	50	TPMT*1/1
229035	11.3	44	12.6	43	12.0	44	13.1	53	8.7	53	10.9	53	TPMT*1/1
229056	9.0	38	12.0	38	10.5	38	9.2	51	4.0	49	6.6	50	TPMT*1/1
229057	6.7	29	7.3	28	7.0	29	10.2	51	11.2	51	10.7	51	TPMT*1/1
229058	9.7	43	8.4	42	9.1	43	11.7	58	12.0	53	11.9	56	TPMT*1/1
229059	3.9	21	9.2	39	6.6	30	11.7	51	12.3	50	12.0	51	TPMT*1/1
229060	5.4	20	4.7	18	5.1	19	4.0	22	4.3	24	4.2	23	TPMT*1/3
229061	4.8	16	4.9	15	4.9	16	6.2	25	6.7	29	6.5	27	TPMT*1/3
229062	5.0	15	5.0	17	5.0	16	5.4	28	5.3	29	5.4	29	TPMT*1/2
229063	10.2	36	9.2	35	9.7	36	10.1	53	10.6	58	10.4	56	TPMT*1/1
229064	9.9	36	5.8	34	7.9	35	9.6	46	69.0	40	39.3	43	TPMT*1/1
229065	10.6	38	11.6	39	11.1	39	12.7	58	12.7	63	12.7	61	TPMT*1/1
229066	29.5	83	32.4	81	31.0	82	19.4	101	18.6	102	19.0	102	TPMT*1/1

Sample	RBC Lysate 1 Peak Ht.	Activity	RBC Lysate 2 Peak Ht.	Activity	Mean RBC Lysate Peak Ht.	Activity	Whole Blood 1 Peak Ht.	Activity	Whole Blood 2 Peak Ht.	Activity	Mean WB Peak Ht.	Activity	Genotype
229067	13.9	40	15.9	43	14.9	42	21.4	67	21.8	69	21.6	68	TPMT*1/1
229068	16.1	45	15.6	48	15.9	47	13.8	61	13.4	61	13.6	61	TPMT*1/1
229069	13.0	38	13.4	35	13.2	37	12.0	60	12.7	66	12.4	63	TPMT*1/1
229070	13.2	44	15.9	45	14.6	45	16.2	59	15.3	60	15.8	60	TPMT*1/1
229071	5.8	49	9.3	49	7.6	49	16.2	61	16.0	61	16.1	61	TPMT*1/1
229072	8.0	28	11.8	35	9.9	32	17.6	61	17.3	61	17.5	61	TPMT*1/1
229073	13.8	41	14.0	41	13.9	41	12.8	59	12.9	62	12.9	61	TPMT*1/1
229074	13.3	39	13.4	40	13.4	40	11.7	50	8.6	59	10.2	55	TPMT*1/1
229076	17.4	48	12.8	49	15.1	49	18.5	70	18.0	71	18.3	71	TPMT*1/1
229077	12.6	42	13.1	44	12.9	43	16.1	61	11.8	65	14.0	63	TPMT*1/1
229078	14.7	40	14.3	41	14.5	41	11.6	61	11.3	62	11.5	62	TPMT*1/1
229079	6.2	30	9.4	29	7.8	30	10.3	47	10.6	51	10.5	49	TPMT*1/1
229080	10.8	34	11.6	38	11.2	36	13.6	60	13.0	62	13.3	61	TPMT*1/1
229081	13.3	56	11.3	56	12.3	56	19.5	65	18.9	69	19.2	67	TPMT*1/1
229082	13.8	39	15.9	42	14.9	41	14.0	57	13.5	57	13.8	57	TPMT*1/1
229083	5.7	39	13.0	38	9.4	39	16.0	53	14.8	51	15.4	52	TPMT*1/1
229084	13.2	45	15.9	43	14.6	44	15.6	57	15.5	61	15.6	59	TPMT*1/1
229086	19.2	62	21.6	61	20.4	62	17.3	73	16.9	69	17.1	71	TPMT*1/1
229087	14.6	46	13.4	42	14.0	44	15.0	66	15.7	72	15.4	69	TPMT*1/1
229088	9.2	28	10.0	28	9.6	28	9.1	39	8.4	37	8.8	38	TPMT*1/3
229089	15.3	50	16.4	49	15.9	50	20.1	74	18.6	71	19.4	73	TPMT*1/1
229090	13.6	50	16.3	45	15.0	48	16.2	59	15.9	65	16.1	62	TPMT*1/1
229091	16.0	48	16.1	47	16.1	48	17.6	81	17.5	77	17.6	79	TPMT*1/1
229094	14.2	56	15.2	54	14.7	55	17.0	72	16.3	72	16.7	72	TPMT*1/1
229095	18.0	57	17.4	53	17.7	55	14.7	67	14.3	68	14.5	68	TPMT*1/1
229096	14.5	46	16.0	45	15.3	46	16.6	65	15.6	64	16.1	65	TPMT*1/1
229097	23.0	63	24.2	61	23.6	62	18.5	73	17.3	70	17.9	72	TPMT*1/1
229098	16.3	45	19.7	46	18.0	46	13.4	61	13.4	61	13.4	61	TPMT*1/1
229099	18.0	52	19.1	51	18.6	52	17.2	79	16.5	83	16.9	81	TPMT*1/1
229100	21.9	67	21.8	63	21.9	65	16.5	73	16.8	74	16.7	74	TPMT*1/1
229101	13.6	38	13.6	36	13.6	37	15.6	64	14.7	62	15.2	63	TPMT*1/1

Appendix 3

Whole-blood thiopurine S-methyltransferase activity with genotype concordance: a new, simplified phenotyping assay
Loretta Ford, Valerie Graham and Jonathan Berg

http://acb.rsmjournals.com/cgi/content/abstract/43/5/354

Appendix 4

A Poster

A simplified assay for determination of red blood cell 6-thioguanine nucleotides; early evidence suggestions UK patients are receiving sub-optimal doses of thiopurine drugs

V Graham, Dr L T Ford, Dr J Berg
Department of Clinical Biochemistry, City Hospital, Birmingham, B18 7QH

Appendix 5

Sandwell and West Birmingham Hospitals
NHS Trust

Improving a test that has a growing clinical demand; blood 6TGN and it's role in pharmacogenomics

Valerie Graham, Dr. Jonathan Berg
Department of Clinical Biochemistry, City Hospital, Birmingham B18 7QH

Introduction

Pharmacogenetics links differences in gene expression to drug response. Advances in this field are helping to predict drug efficacy and toxicity. These developments have major medical benefits as they aid optimisation of drug dosage.

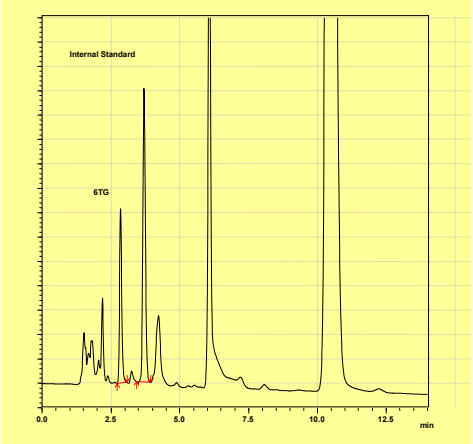
Currently, the measurement of thiopurine methyl transferase (TPMT) is the best clinically applied example of pharmacogenetics. This enzyme is involved in the metabolism of the widely used Thiopurine drugs. The quantitative determination of the active metabolites of these drugs, the 6-thioguanine nucleotides (6TGN), helps monitor a patient's response to treatment.

6TGN monitoring has not yet become routine practice in the UK, in part because methods have involved time consuming washed cell preparations, as conventionally 6TGN has been estimated in erythrocytes.

Method

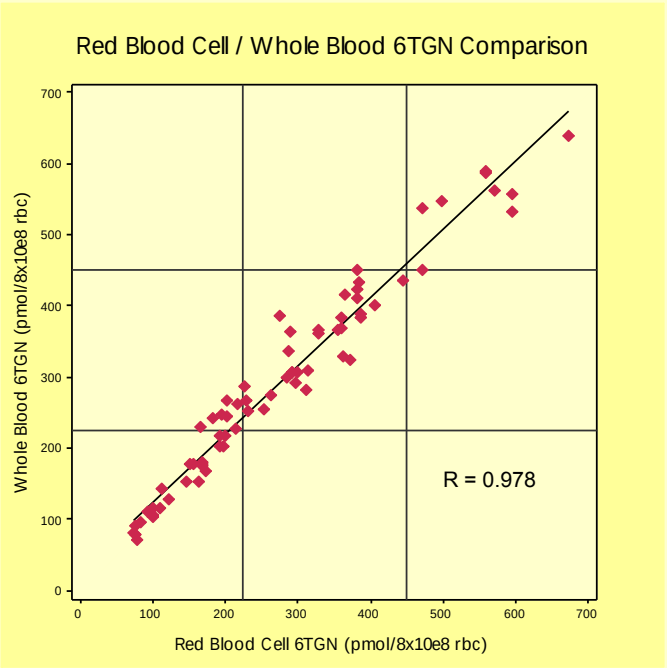
We have improved our method for 6TGN, estimating metabolite levels in whole blood rather than washed cells, making it more suitable for routine clinical use. The method liberates 6TGN from red blood cells (RBC). Perchloric acid at 95ºC hydrolyses the nucleotides back to their parent thiopurine, 6-thioguanine (6TG) which is measured by HPLC.

Example of Chromatography



Results

Our new whole blood method results were compared to those from conventional RBC analysis in 70 patient samples.



Conclusions

Our whole blood 6-TGN method shows excellent correlation between whole blood and washed RBC. The whole blood assay is less labour intensive and cell counting and pipetting errors are minimised. The sample volume required is also reduced. This quick, cost effective, simplified assay is now being introduced into routine clinical use.

This quantification will allow more confident escalation of dose in non-responders and dose reduction for those with high, potentially harmful, 6TGN concentrations. It will be particularly useful in assessing patients heterozygous for TPMT activity, who receive a reduced dose and for which achieving therapeutic levels can be problematic.

Appendix 6

Patients with high-normal TPMT activity identified during routine phenotypic testing: What is going on?

L T Ford, V Graham, J D Berg (Focus 2006 Poster)
Department of Clinical Biochemistry, City Hospital, Birmingham B18 7QH

In addition to individuals with undetectable and low TPMT activity at risk of adverse reactions to thiopurine drug treatment, there is also evidence of a group of patients with very high TPMT activity. These patients may not respond to standard doses of thiopurine drugs, and are at risk of hepatotoxicity from increased production of methylated-thiopurine metabolites and it is important to be able to identify this patient group. Approximately 2% of the patient samples sent to our TPMT phenotyping service have very high TPMT activity defined as greater than 60 nmol/gHb/hour. To determine if there is a pre-analytical cause in 2005 we sent out audit forms with all high TPMT activity results and received 42 returns.The mean patient age was 46 years and there were an equal number of males and females. The mean TPMT activity was 71 nmol/gHb/hour (range 60-92 nmol/gHb/hour). The mean patient haemoglobin concentration at the time of analysis was 10.5 g/dL (range 7-14 g/dL). Prior to measuring TPMT activity a red blood cell lysate is prepared. The haemoglobin concentration of this lysate is used to determine the concentration of TPMT enzyme present, and could result in over-estimation of TPMT activity. However, no correlation was found between haemoglobin concentration and TPMT activity. On average patients were receiving 5 different drugs at the time of testing, one patient was on 12 different drugs. The most common drug treatment was prednisolone with 79% of patients receiving therapy of between 10-100 mg od daily. No correlation was found between prednisolone dose and TPMT activity. Thiopurine drug treatment can itself induce TPMT activity but only 5 patients were receiving azathioprine at the time of testing. We could identify no pre-analytical factors responsible for the high TPMT activity measured in some patientsamples using TPMT phenotyping.

Appendix 7

Routine thiopurine drug monitoring by rapid determination of whole blood metabolite levels

V Graham, L Ford, J Berg (2008) Focus Poster
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Thiopurine drugs are widely used to treat auto-immune disease.Quantitative determination of the thiopurine active metabolites, 6-thioguanine nucleotides (6TGN), can guide treatment, increasing drug efficacy and reducing likelihood of side effects. Monitoring is particularly useful for individuals heterozygous for TPMT, who usually require a lower than normal dose to achieve therapeutic levels. Despite the potential benefits of 6TGN monitoring, it has not yet become an established test. Complex methods involving time consuming manufacture of washed cell preparations have clearly not been easy to apply in the routine laboratory.
Method: We have compared levels of 6TGN in both wholeblood and washed red blood cells after conversion of thiopurine metabolites back to 6TGN. The lystate method starts by preparing washed RBC. The whole blood assay does not
undertake this preparative step. The 6TGNs are hydrolysed to 6-thioguanine (6TG) by incubation with perchloric acid and dithiothretiol at 95°C for 1 hour. The concentration of 6TG is then measured using isocratic HPLC with UV detection at 304 nm.
Results: A comparison was made between the conventional washed red blood cell method and the new whole blood method. Results from whole blood were significantly higher than lysate preparations with a Mann Whitney U Test giving p=0.61. Regression analysis for patient samples prepared by the two methods gave the regression line: Whole Blood TGN = 16.4 + Lysate TGN x 0.977. Conclusion: The whole blood procedure is much less labour intensive and errors in cell counting and pipetting are also minimised. Our whole blood approach offers a simplified assay, which is quick and cost effective making it suitable for routine clinical use. Whole blood results are slightly higher but as application of the 6TGN assay has been limited, establishing reference intervals for the whole blood assay would be ideal at this stage.

Appendix 8 6TGN / Mean cell volume correlation data

6TGN Therapeutic range 235-450 pmol 6TG/8x108 rbc	**MCV** Normal range 77-99 fl
<50	83.0
<50	94.5
<50	107.5
<50	99.1
<50	91.6
97	100.6
109	107.7
115	67.0
116	91.4
132	102.1
147	88.5
147	107.1
157	92.1
161	105.7
174	98.4
178	76.2
186	93.2
186	114.2
191	101.9
197	107.0
210	96.9
215	110.2
217	102.7
230	115.7
261	99.4
282	82.9
292	109.2
297	113.4
302	109.8
304	103.0
312	87.7
325	103.3
327	113.3
327	113.4

6TGN Therapeutic range 235-450 pmol 6TG/8x108 rbc	**MCV** Normal range 77-99 fl
335	107.0
336	92.8
339	108.7
357	96.8
408	112.6
432	99.4
436	85.2
460	98.8
472	103.8
479	106.2
481	90.4
482	101.7
483	88.0
485	107.2
501	81.6
508	78.3
508	113.0
513	111.8
541	90.0
567	108.8
581	104.9
581	89.6
620	121.0
620	111.8
665	79.8
672	89.5
705	102.6
789	110.3
802	109.0
888	123.6
911	113.1
1043	90.0
1116	101.0

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