SCREENING FOR THIOPURINE S-METHYLTRANSFERASE (TPMT) GENE MUTATIONS IN SOUTH AFRICA

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This dissertation is submitted for the award of Master of Medicine (M.Med., UCT) in the Department of Chemical Pathology, Medical School, University of Cape Town, Cape Town, South Africa

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ABSTRACT

Thiopurine s-methyltransferase (TPMT) catalyses the s-methylation (inactivation) of purine analogue drugs including 6-mercaptopurine, azathioprine and thioguanine. These drugs are used for the treatment of a variety of clinical conditions such as acute leukemia, rheumatoid arthritis, autoimmune hepatitis, inflammatory bowel disease, multiple sclerosis, and in prevention of rejection in organ transplantation. Several studies have shown that patients with low TPMT activity are at risk for severe and potentially fatal haematopoietic toxicity when treated with conventional doses of the thiopurine drugs. Genetic polymorphism in the TPMT gene is an important determinant of mercaptopurine toxicity. Patients with mutations in the TPMT gene have a less efficient methylation process, and are therefore, predisposed to severe myelosuppression. Three common mutations have been reported in the general population, and these include, TPMT*2 (G238C), TPMT*3A (G460A and A719G mutations on the same allele) and TPMT*3C (A719G). TPMT*3A is the most common haplotype in Caucasians while TPMT*3C is the most common haplotype among African, African American and Asian population groups. The object of this study was to determine the frequency of these mutations in the various population groups in the Western Cape, and thereby help to avert the danger of myelosuppression in heterozygote as well as homozygote patients.

Exons 5, 7 and 10 of the TPMT gene were amplified using the polymerase chain reaction and characterised by restriction enzyme analysis and in some cases sequencing.

A total of 250 subjects, comprising 50, 100 and 100 Caucasians, coloureds and Africans respectively have been screened for the common TPMT mutations. 28 patients from Groote Schuur and Red Cross Children's Hospitals currently on purine

analogues were also screened for the common TPMT haplotypes. Allele frequencies of 6%, 0.5% and 0% for the TPMT*3A haplotype were found in the Caucasian, coloured and African population groups respectively. By contrast the allele frequency of the TPMT*3C haplotype was 3.5% in Africans, 3% in Coloured and 2% in Caucasians. No G238C (TPMT*2 haplotype) mutation was detected in the subjects screened. Out of the 28 patients currently on purine analogues screened, only one patient had a positive genotypic result. She was found to be doubly heterozygote for both the TPMT*3A and TPMT*3C haplotypes.

Conclusion: Results confirmed that TPMT*3A is the most common TPMT haplotype in Caucasians in South Africa, whereas TPMT*3C is the most common haplotype in the coloured and African population groups. Because of these ethnic differences, we therefore suggest that genotypic screening be carried out before the commencement of thiopurine drugs in all population groups in South Africa, and this study will assist this by defining the incidences of the various mutations in the different population groups.

This research work is dedicated to my best friend and beloved wife, Jay and our darling little boy Folusho, for their perseverance and unwavering support.

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ABBREVIATIONS

TPMT	Thiopurine S-Methyltransferase
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
RA	Rheumatoid Athritis
SLE	Systemic Lupus Erythromatosus
6-MP	6-mercaptopurine
AZA	Azathioprine
Kb	Kilobase
Sp1	Stimulating Protein 1
AMP	Adenosine Monophosphate
IMP	Inosine Monophosphate
GMP	Guanosine Monophosphate
5'NT	5'Nucleotidase
APRT	Adenine Phosphoribosyl Transferase
HGPRT	Hypoxanthine-Guanine Phosphoribosyl Transferase
PNP	Purine Nucleoside Phosphorylase
ADA	Adenosine Deaminase
XO	Xanthine Oxidase
VNTR	Variable Number of Tandem Repeats

DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetra-acetic Acid
PCR	Polymerase Chain Reaction
DNTPs	Dinucleotide Triphosphate
HPLC	High-Performance Liquid Chromatography
SNPs	Single Nucleotide Polymorphisms
Km	Michaelis-Menton Constant
USA	United States of America
RBC	Red Blood cell

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

Azathioprine, 6-mecaptopurne and 6-thioguanine are all purine analogues with a very narrow therapeutic index that can sometimes result in severe life threatening toxicity. These drugs are used in the treatment of acute lymphoblastic leukemia, chronic rheumatoid athritis, autoimmune hepatitis, inflammatory bowel disease, dermatological diseases, and as an immunosuppressant in prevention of rejection in organ transplantation. These purine analogues are prodrugs with no intrinsic activity, but only become active after undergoing metabolic conversion to thioguanine nucleotides. These nucleotides are subsequently incorporated into DNA, this being one of the principal mechanisms for the antiproliferative activity of these drugs.

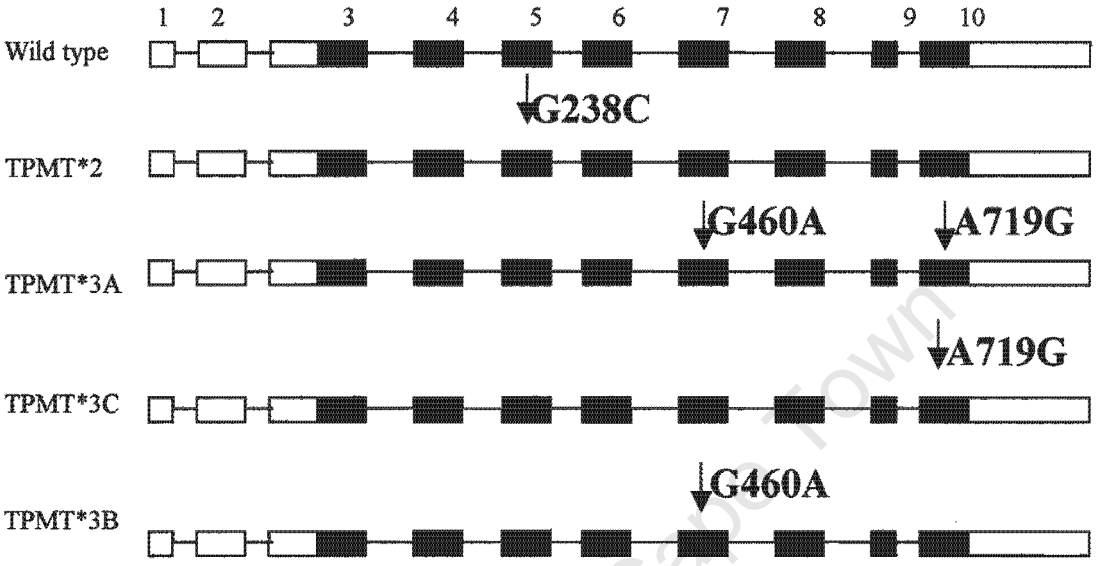
Prolonged use of these drugs is associated with certain untoward effects, which includes gastrointestinal disturbances, bone marrow suppression, hepatotoxicity and very infrequently severe hypersensitivity reaction. Gastrointestinal disturbance is a rare but known side effect of thiopurine drugs. There is very little in the literature about this uncommon adverse effect. One author reported that this side effect is known to adversely affect compliance with therapy in some patients on thiopurine drugs (8). However, by far the most dangerous side effect is that related to

haematopoietic toxicity (1) with a tendency to developing anaemia, predisposition to infection and bleeding. Frequent deaths have been reported due to these complications.

TPMT is the major detoxificating enzyme for thiopurine drugs, hence partial or absolute deficiency of this enzyme results in severe and life threatening toxicity. As a result of these grave consequences, it has been strongly recommended that genotypic screening be carried out in all patients prior to the commencement of purine analogue therapy. Alternatively, prospective measurement of erythrocyte TPMT activity could also be carried out to identify those patients at risk of severe drug toxicity. As a preventative measure, knowledge of genotype and or erythrocyte TPMT activity would help stratify patients into 3 clinical groups. In patients homozygous for the wild type allele in whom erythrocyte TPMT activity is high, use of purine analogues is infrequently associated with side effects, and is therefore relatively safe. However, patients heterozygous as well as homozygous for the common TPMT mutant alleles are associated with severe and life threatening side effects especially fatal myelosuppression. In these groups of patients, thiopurine drugs are either absolutely contraindicated (as in patients homozygous for the mutant TPMT alleles) or there should be considerable reduction in dosages in those patients heterozygous for mutant TPMT alleles. Side effects tend to occur very early during the course of therapy in homozygote patients.

THIOPURINE S-METHYLTRANSFERASE (TPMT) GENE MUTATIONS

Figure 1.1: Spectrum of the commonTPMT gene mutations



This depicts the TPMT gene in the wild type and the spectrum of common mutations of this gene. Exons are depicted as boxes (solid where translated into protein) and introns as single lines, not to scale.

Thiopurine s-methyltransferase deficiency is inherited as an autosomal co-dominant condition. About 10 different kind of mutations have been described; all are point mutations but only some are directly associated with enzyme deficiency; others are silent with some being in exons and a few in introns (10).

The three most common TPMT haplotypes described in the literature are TPMT*3A, TPMT*3C and TPMT*2 (Fig 1.1). These haplotypes account for approximately 95% of all known mutations. These mutations are located on exons 7, 10 and 5. Rare mutations located on exons 4, 6, 7, 8 and in the promoter region of the TPMT gene have also been reported (4,10,20). The TPMT*2 haplotype is located on exon 5 of

the TPMT gene. This haplotype is characterized by a G238C substitution with a change in the amino acid encoded from alanine to proline at codon 80 (Ala80Pro). This amino acid change has been reported to lead to a 100 fold reduction in TPMT enzyme catalytic activity compared to the wild type. This has been variably attributed to accelerated proteolytic degradation and change in the folding pattern of the TPMT enzyme protein as a consequence of the amino acid substitution.

The TPMT* 3A haplotype is located on both exons 7 and 10 of the TPMT gene. This haplotype has been reported to be the most prevalent mutant allele among Caucasians. It has been estimated that 85% of all subjects with the TPMT mutant allele have the TPMT*3A haplotype. This haplotype consist of both a G460A and an A719G mutation occurring together on the same allele. As a result of these mutations, the G460A leads to an alanine to a threonine substitution at codon 154, and the A719G leads to the tyrosine being substituted with a cysteine codon 240. As a consequence of these mutations, there is virtually no immunodetectable enzyme protein with approximately 400 fold less TPMT enzyme activity in patients or subjects homozygous for the TPMT*3A haplotype compared to the wild type.

By contrast, TPMT* 3C haplotype is the most prevalent mutant TPMT alleles among Africans, including African-Americans, and Asian population groups. In some reports especially from Japan, Thailand and the USA, all the patients with positive results were exclusively TPMT*3C haplotype. The TPMT*3C haplotype consists of an A719G mutation alone on one allele, and is located on exon 10. As a consequence of this mutation, there is also an amino acid substitution at codon 240 from tyrosine to cysteine. This mutation is associated with a 50% fold reduction in immunodetectable

TPMT enzyme protein and activity (27). As a result of the tyrosine to cysteine substitution, the TPMT*3C enzyme protein undergoes conformational changes which adversely affect the function and stability of this enzyme (27).

TPMT*3B haplotype is very rare in all population groups but a few cases have been reported in the literature especially among Caucasians (29,30). This haplotype is characterized by a G460A mutation occurring alone, and resulting in an amino acid substitution at codon 154 from alanine to a threonine. This mutation is also associated with a 9 fold reduction in immunodetectable TPMT protein.

The overall prevalence of these mutations is about 10% in Caucasians, both in Europe as well as in North America(2,4,5,20). The allele frequencies of these mutations in the various population groups (4,5,19) is shown in table 1.1

Table 1.1: Allele frequencies in the various population groups

POPULATION GROUPS	TPMT*2	TPMT*3A	TPMT*3C	OVERALL INCIDENCE
CAUCASIANS	1%	8.5%	0.5%	10%
SOUTH WEST ASIANS	0%	0%	2%	2%
CHINESE	0%	0%	4.7%	4.7%
AFRICAN-AMERICANS AND GHANAIANS	0%	1.3%	2.4%	3.7%

The allele frequencies of the various TPMT haplotypes differ from one population group to the other. For example, in a major review from France on TPMT gene

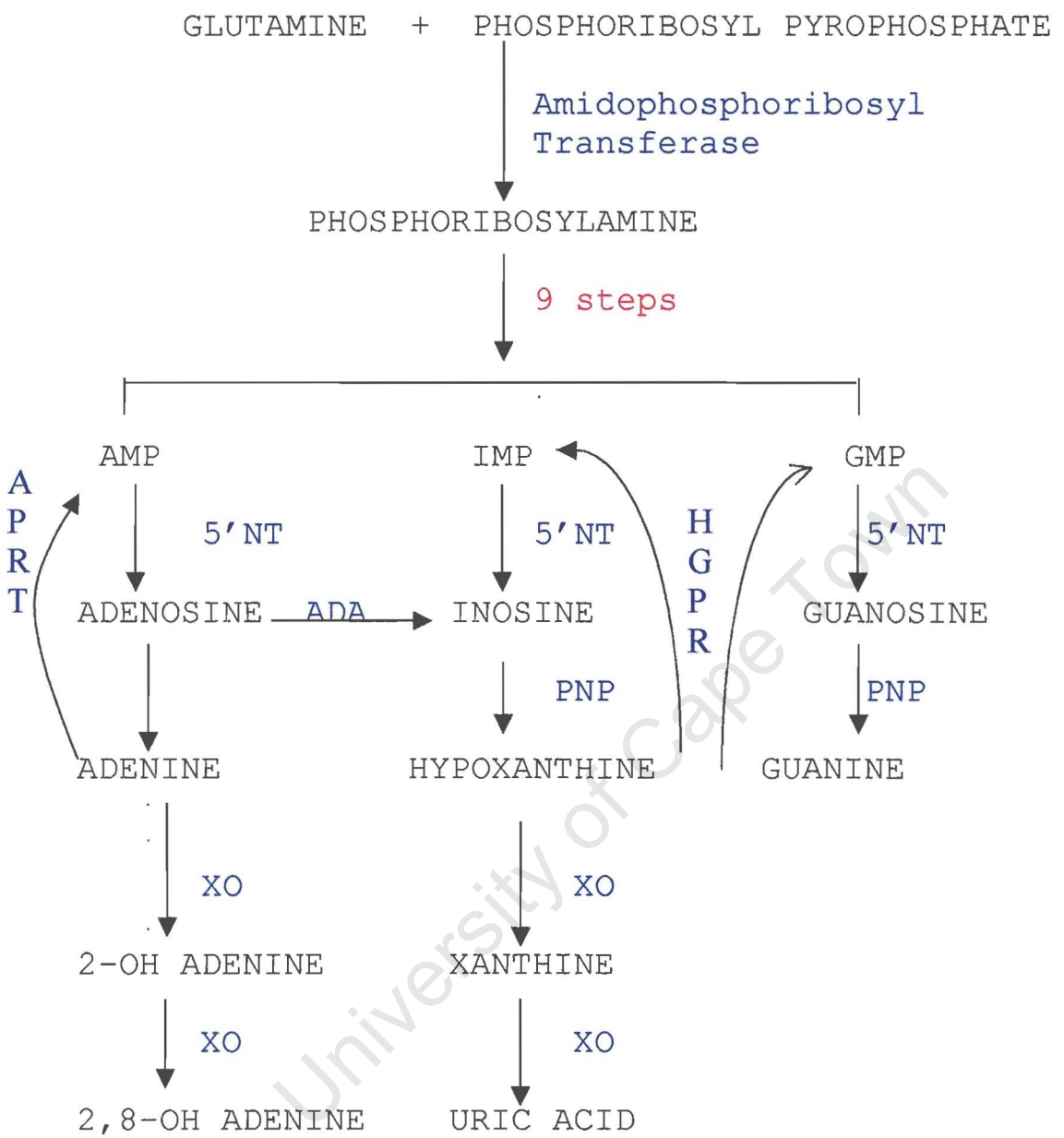
polymorphism in Europeans, out of 191 unrelated subjects screened, only 28 (15%) were reported to have positive results for the common mutations (25). 85% were reported to be normal while 22 out of 28 subjects with positive results (5.3% alelle frequency of the total subjects screened) were reported to be heterozygous for the TPMT*3A haplotype and one was homozygous for this haplotype. Three out of twenty eight (0.8% alelle frequency of the total subject screened) had the TPMT*3C haplotype, 2 out of 28 (0.5% allele frequency) had the TPMT*2 haplotype. Only one of the subjects was reported to have the rare TPMT*7 (T681G) haplotype.

METABOLISM OF PURINE AND PURINE ANALOGUES

The following key enzymes are important in the metabolism of purine and purine analogues. These are:

- Hypoxanthine-Guanine Phosphoribosyl Transferase (HGPRT)
- 5'Nucleotidase (5'NT)
- Purine Nucleoside Phosphorylase (PNP)
- Xanthine Oxidase (XO)
- Thiopurine S-methyltransferase (TPMT)

Figure 1.2: Biochemical pathways of Purine Metabolism

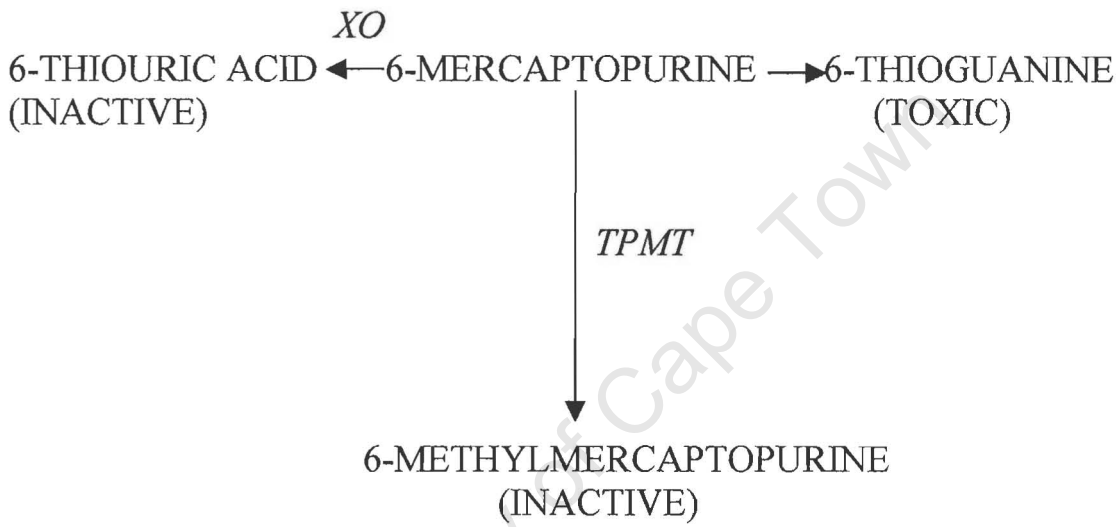


The roles and steps catalysed by these enzymes are shown in the biochemical pathways illustrated in Fig 1.2 and 1.3

Thiopurine S-methyltransferase is a cytoplasmic enzyme which catalyses the intracellular s-methylation (inactivation) of heterocylic and aromatic sulfhydryl compounds including purine analogues. These drugs which include 6-mercaptopurine, azathioprine and thioguanine, in the presence of TPMT enzyme, undergo metabolic conversion to their respective inactive methylated metabolites

either as a nucleotide, nucleoside or as a base. Methylated analogues are not incorporated into DNA, and are therefore non-toxic. For example, the inactive metabolites include 6-methyl thioinosine monophosphate, 6-methylmercaptopurine riboside and 6-mercaptopurine.

Figure 1.3: The role of Thiopurine s-methyltransferase in the metabolism of purine analogues



6-mercaptopurine is a purine analogue drug used commonly in clinical practice especially in the treatment of childhood leukemia. The anti-neoplastic action is as a result of the antiproliferative effect of 6-thioguanine. 6 mercaptopurine is first converted to 6-thioguanine, and this is then incorporated into DNA. 6-thioguanine is toxic to the cell. This metabolic conversion is employed therapeutically for killing malignant cells by 6-mercaptopurine in the treatment of certain cancers.

The metabolic conversion of 6-mercaptopurine to 6-thioguanine is critical for the therapeutic efficacy of 6-mercaptopurine in the treatment of certain malignancies, especially acute lymphoblastic leukemia. However, excessive conversion to 6-thioguanine could result in severe adverse drug effects particularly fatal and life

threatening myelosuppression. As shown in Figure 1.3 above, 6-mercaptopurine could also be shunted by thiopurine s-methyltransferase (TPMT) to its inactive metabolite, 6-methylmercaptopurine, and to a lesser extent by xanthine oxidase to another inactive metabolite, 6-thiouric acid. Deficiency of these inactivating enzymes results in increased levels of 6-thioguanine nucleotides in the cells, and therefore, is usually associated with severe drug toxicity. The level of 6-thoguanine in the cell is inversely proportional to TPMT enzyme activity (9,15,16,33). For example, individuals with intermediate TPMT activity have been reported to accumulate at least 50% more thioguanine nucleotide compared to those with normal (often referred to in the literature as high) activity (27,28).

Azathioprine, used commonly in clinical medicine as an immunosuppressant, is structurally very similar to 6-mercaptopurine. The only slight difference being that it is first converted in the body to 6-mercaptopurine and subsequently metabolized in a similar way as 6-mercaptopurine.

ERYTHROCYTE TPMT ACTIVITY

It is possible to measure erythrocyte TPMT activity as a screening test for subjects with TPMT gene polymorphism. It is advisable to carry out this measurement before the commencement of purine drugs in order to identify patients carrying the mutant alleles. However, the major drawback of this assay is in patients who have already received red cell transfusions, which would normally, of course, be from individuals with the wild type allele. In these patients, constitutive TPMT activity could only be measured after 8-12 weeks of such transfusions (13,14,15,32).

The explanation for the reduced TPMT enzyme activity in the subjects or patients with these mutant TPMT alleles has been ascribed to rapid degradation of the TPMT enzyme protein encoded by these mutant alleles (27). For example, the half life of wild type TPMT protein is about 18 hours, compared to only 15 minutes for TPMT*2 and TPMT*3A encoded enzyme proteins (27). The half life of TPMT*3B enzyme protein is 6 hours (27). This accelerated degradation will ultimately lead to significantly reduced enzyme activity. In the same report, the Michaelis-Menton constant (Km) for mercaptopurine methylation was significantly higher for the TPMT*3B encoded enzyme protein. High Km is associated with low substrate affinity (27). There was no change in Km reported for the TPMT*3C enzyme protein.

CHAPTER 2

MATERIALS AND METHODS

SAMPLING

A total of 250 subjects were screened for the common thiopurine s-methyltransferase (TPMT) gene mutations. These mutations included the TPMT*2, TPMT*3A and TPMT*3C haplotypes. These patients were a random selection of patients attending Groote Schuur Hospital out-patients clinics, and therefore should be representative of the local Western Cape Communities of South Africa. This subject set consists of 50 patients of Caucasian descent, 100 of mixed-race ("coloured") descent and 100 of African (black) descent.

In addition to the above subjects, 28 patients, from Groote Schuur and Red Cross Children's Hospitals, who are currently on purine analogue drugs for a variety of clinical conditions, were also screened for this polymorphism. Twenty three of these were paediatric patients from Red Cross Children's hospital and were on treatment with 6-mercaptopurine for acute lymphoblastic leukemia. Similarly, 3 out of the 5 adult patients from Groote Schuur Hospital were also on treatment with 6-mercaptopurine for acute leukemia, while one each of the two remaining adult patients, was on azathioprine for the treatment of systemic lupus erythromatosus and for rheumatoid athritis.

Prior to the commencement of this project, written approval from the ethics committee of the University of Cape Town had been obtained. Thorough explanation and pre-investigation counselling were carried out, and if the patient was willing to participate in this study, informed consent was then obtained. With regard to the subjects with banked DNA, prior informed consent had also been obtained. A copy of the informed consent form which were signed by all patients, or parent/guardian in case of minor subjects, is shown below

Consent form:

UNIVERSITY OF CAPE TOWN
Department of Chemical Pathology

Consent for Molecular Genetics of candidate genes associated with the metabolism of thiopurine drugs.

We are undertaking a study to determine the frequency of susceptibility in the community to thiopurine drugs used in the treatment of several common diseases. This susceptibility takes the form of a decreased ability to metabolise (process) the drug and hence causes a possible toxic effect. You are not required to take any drugs, but simply to give some blood.

For this study we require 2 x 5ml EDTA blood samples. The blood will not be used for any other purpose other than this investigation.

If for any reason you do not wish to participate in this study, you have every right to refuse and this will not affect the standard of treatment and care that you will receive.

I, the undersigned..... (Print Name), Hospital Number.....

.....

hereby confirm that I understand the nature and purpose of the above procedure and am aware that the results may be used for research purposes. I agree to participate voluntarily in the above investigation.

All the banked DNA samples were frozen at a temperature of -20 °C in a separate fridge at the Inherited Metabolic Disease laboratory, Medical School, University of Cape Town. Alternatively, for fresh samples, 2 X 5ml of EDTA blood were obtained on ice after informed consent was obtained. All the samples from the Red Cross Hospital were kept frozen, and were later transported on ice from the hospital to the Inherited Metabolic Disease laboratory in the medical school. DNA was subsequently extracted from the sample by an overnight extraction method .

DNA EXTRACTION

DNA was then extracted from the blood samples received using the *Whole Blood DNA Extraction Method described below:*

Reagents:

1. Lysis buffer, consisting of:
   - 0.32 M Sucrose
   - 10 mM Tris HCl
   - 5 mM $MgCl_2$
   - 1% Triton X

2. Normal Saline made up of 0.9g NaCl in 100ml distilled water.

3. 100µM EDTA made up of 3.72mg EDTA in 100ml distilled water.

4. 4 M Sodium Chloride made up of 33.37g NaCl in 100ml of distilled water.

5. Saline/EDTA (pH 8.0) made up of 50ml of 100μM EDTA and 3.7ml of 4M NaCl in 200ml of distilled water.

6. 10% SDS made up of 10g Sodium dodecylsulfate in 100ml of distilled water.

7. Proteinase K (20mg/ml) : 20mg Proteinase K in 1ml of distilled water.

8. 6 M Sodium Chloride : 35.1g NaCl in 100ml distilled water.

9. 70% Ethanol: 70ml absolute ethanol in 100ml distilled water.

PROCEDURE

A) 10ml of blood from each patient was transferred into a 50ml sterile conical tube.

B). To wash the white cells, 20ml of freshly prepared lysis buffer was added to each sample, and then mixed very well by inversion.

C) Each tube was spun for 10 minutes at 3000 rpm. The supernatant was carefully discarded to avoid dislodging the pellet of white cells in the base of the tube.

D) Steps B and C above (washing steps) were repeated 4 or more times until the pellet of white cells at the bottom of the tube was clean and free of red cells.

E) The following reagents were then added to the pellet in each tube:

- 2.25 ml Saline/EDTA: well mixed to re-suspend pellet,
- 250µl 10% SDS,
- 100µl Proteinase K (20mg/ml).

F) Each tube was mixed by inversion, and then vortexing.

G) All tubes were incubated overnight in a waterbath at 56°C.

H) After overnight incubation, the content of each conical tube was then transferred into an eppendorf tube to facilitate centrifugation.

I) 225µl of 6M NaCl was then added to each eppendorf tube, and each tube was then shaken vigorously for 15 seconds.

J) All tubes were then kept in the fridge for 30 minutes, and thereafter centrifuged in a microcentrifuge at 10,000 rpm for 10 minutes.

K) The supernatants were transferred into separate 12ml sterile glass tubes.

L) Absolute ethanol (ice cold) equal to 2 X the volume of the supernatant was then added to each tube.

M) Using a glass pipette, DNA was retrieved from each tube. 70% ethanol (in drops) was then used to wash DNA on the tip of the glass pipette loop, and then allow to air dry for a few minutes.

N) Dried DNA was subsequently washed off into a separate eppendorf containing 250µl of TE buffer. DNA was allowed to dissolve by gently shaking the tubes.

O) Each tube was then mixed well by inversion.

P) All tubes were then spun for 10 minutes at 3,000 rpm, and supernatants were carefully discarded to avoid loosing the DNA.

Q) The DNA pellet was washed twice with 1ml of 70% ethanol.

R) Finally, the pellet in each tube was allowed to air dry over 4-6 hours. Each pellet was subsequently reconstituted in 200 - 500µl of TE buffer (pH 8.0) depending on the amount of the DNA available.

POLYMERASE CHAIN REACTION

Screening for the various locations of the mutations was carried out at the Inherited Metabolic Disease Laboratory by amplifying various fragments of DNA using the polymerase chain reaction (PCR). Using the appropriate primers, the common TPMT gene mutations were screened.

In the case of the mutation located on exon 7, a 436 base pair fragment of DNA was amplified using the appropriate primer sequences. For mutation located on exon 10, a 179 base pair (bp) fragment of DNA was also amplified. A 464 bp fragment was amplified for mutation located on exon 5.

The respective primer sequences used for PCR amplification are shown below.

Mutation on exon 7:

Forward primer cacac ccagg tccac acatt c

Reverse primer cctta tagcc ttaca cccag gtctc

Mutation on exon 10:

Forward primer gcacc cagcc aattt tgagt a

Reverse primer ccatt acatt ttcag gcttt agcat a

Mutation on exon 5:

Forward primer gtatg atttt atgca ggttt g

Reverse primer taaat aggaa ccatc ggaca c

The mastermix for the PCR consists of the following:

Using the appropriate sized pipette, recommended volumes of the following were transferred into each PCR tube

- Appropriate forward primer : 0.4µl

- Appropriate reverse primer : 0.4µl
- dNTPs : 1.6µl
- PCR buffer : 4.0µl
- Magnesium chloride : 1.2µl
- Distilled water : 31.2µl
- Taq Polymerase : 0.2µl

Into each tube, 1µl of DNA sample was subsequently added, and 1µl of distilled water was added to a separate tube as a blank. During the process of pipetting, all PCR tubes were kept on ice.

All PCR tubes were then transferred to the thermal cycler (Eppendorf-Mastercycler Gradient). The thermal cycler program is shown in the table 2.1 below

Table 2.1: PCR thermal cycler program

TEMPERATURE IN °C	TIME	NUMBER OF CYCLES
94	1 minutes	1
94	1 minute	Repeat 35 cycles
54	30 seconds	
72	45 seconds	
72	10 minutes	1

Finally hold at 4 °C

TO CONFIRM PRESENCE OF PCR PRODUCT

After carrying out a PCR, a preliminary agarose (2%) gel electrophoresis was then carried out (checking gel) to confirm amplification. Blank, positive control sample (s) and suitable molecular size marker were included in all such analyses.

Reagents for construction of a 2% agarose gel:

1X Working TAE buffer

Agarose

Ethidium bromide

Loading buffer (dye)

Procedure

1. 2% solution of agarose was prepared (0.6g in 30ml of 1X TAE buffer)
   Dissolve by heating in the microwave checking regularly to avoid spill-over. Allow solution to slightly cool down, and then add 3.5µl of ethidium bromide.

2. The solution was then poured into a small gel casing into which a comb had been placed. The gel was then allowed to set (5-10 minutes). The comb was carefully removed in order to keep wells intact.

3. The gel was then placed in an agarose gel unit that has been filled with 1X TAE buffer.

4. 10µl of PCR product was then mixed with 5µl of loading buffer (dye). A blank, and a molecular size marker were also included to confirm that the fragment obtained is the correct size.

5. 15µl of sample was then loaded into an appropriate well.

ELECTROPHORESIS

The gel unit was then plugged into the power unit, and then run at 100 volts for 30 minutes. After running the gel for 30 minutes, the power unit was then switched off and the electrodes unplugged from the power source. If positive or PCR products were present, then the gel was photographed under ultraviolet light.

If amplification was successful then an overnight restriction enzyme digest was then carried out using the appropriate restriction enzyme (*Mwo*1, *Acc*1, *BsI*1) and results visualised using ethidium bromide stained agarose (digestion) gel electrophoresis.

RESTRICTION ENZYME DIGEST

The digestion reaction was set up using the appropriate restriction enzyme and corresponding buffer.

Incubate overnight at optimum temperature

Exon 7 : 60 °C

Exon 10 : 37 °C

Exon 5 : 55 °C

Known positive controls were included in each run/batch.

Before the main analysis a 4% final digestion gel was prepared (similar to the checking gel described above) to confirm effective restriction enzyme digestion.

After the restriction enzyme digestion, the samples were examined on a 4% agarose gel. A volume of 50µl consisting of the 40µl sample plus 10µl loading dye, was then loaded into the 4% agarose gel and run at 100 volts for 1 hour. The gels were then visualised under UV light and photographed.

CHAPTER 3

RESULTS

A total of 50, 100, and 100 Caucasians, Coloured and African subjects respectively were screened for the G238C, G460A, and the A719G mutations in the thiopurine -S-methyl transferase (TPMT) gene. Twenty eight patients from the Red Cross Children's and Groote Schuur Hospitals currently on thiopurine drugs were also screened for these mutations.

As previously indicated, the haplotypes containing these mutations are:

TPMT *2 - G238C on exon 5

TPMT*3A - G460A on exon 7 together with A719G on exon 10

TPMT*3B - G460A on exon 7 alone

TPMT*3C - A719G on exon 10 alone.

These 250 subjects were first screened for the G460A mutation. These subjects were a random sampling of patients attending out-patients clinics at Groote Schuur Hospital and should represent a reasonably unbiased cross section of the Cape Town population.

The appropriate restriction enzymes for analysis of the TPMT*3 haplotypes are *Mwo*1, which detects the G460A mutation, and *Acc*1, which detects the A719G mutation. Fragment sizes given by these enzymes are shown in Table 3.1. The results in combination allow for discrimination between the TPMT*3A, B and C

haplotypes.

Table 3.1: Fragment sizes given in the restriction enzyme digests

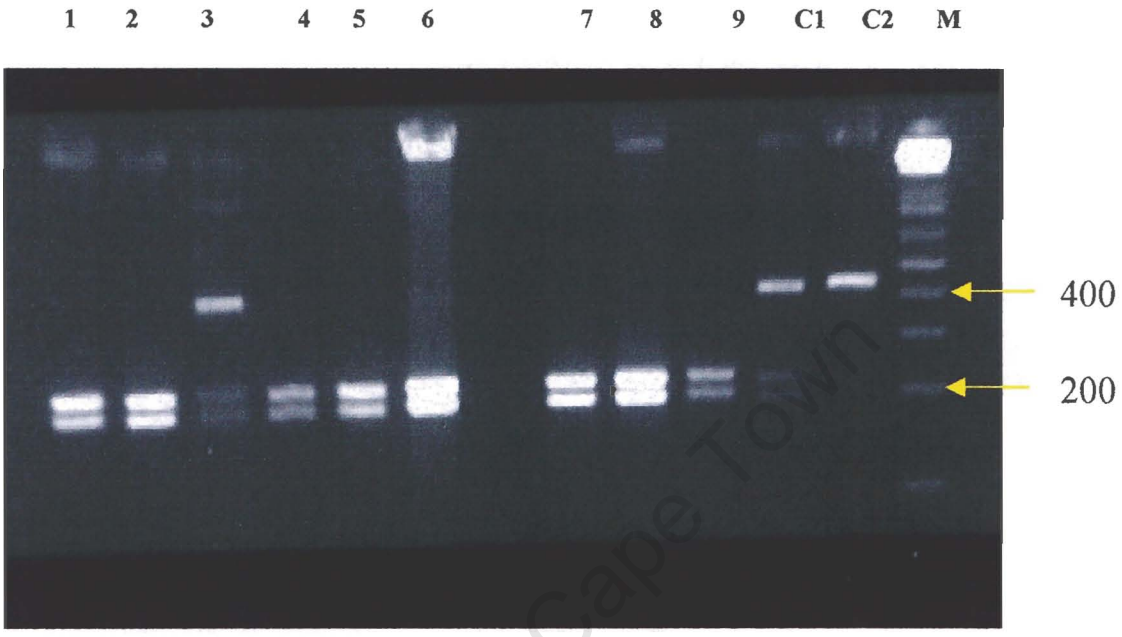
	***Mwo* 1**	***Acc* 1**
Normal	233,203	279
G460A	436	N/A
A719G	N/A	179,100

The restriction enzyme used for the analysis of the TPMT*2 haplotype was *Bsl*1. The fragment sizes given by this restriction enzyme is shown in table 3.2 below.

Table 3.2: Fragment sizes given in the *Bsl* 1 restriction enzyme digest

	***Bsl* 1**
Normal	464
G238C	238,226

Figure 3.1: Agarose gel electrophoresis of PCR amplified exon 7 of the TPMT gene digested with *Mwo*1 from leukaemic patients currently being treated with purine analogue drugs. Lanes 1-9 - patients; positive control - known heterozygote (C1) and known homozygote (C2) for the G460A mutation; M molecular size marker.



A typical gel electrophoretic analysis for the *Mwo*1 restriction enzyme digest is shown in figure 3.1. Eight of the patients illustrated are homozygous for the normal gene, and one (individual 3) is heterozygous for the G460A mutation, giving a picture similar to the positive control C1.

The overall results for analyses to detect the G460A mutation are documented in Table 3.3.

Table 3.3: Results of allele frequencies for the G460A mutation

Population Groups	Total Number of Subjects	Number of Normal Alleles	Number of Positive Alleles*	Allele Frequency (%)
Caucasians	50	94	6	6%
Coloured	100	199	1	0.5%
Africans	100	200	0	0%

* All were heterozygotes

The same group of 50, 100 and 100 Caucasians, Coloured and African subjects respectively were also screened for the A719G mutation (which is found in both the TPMT *3A and the TPMT*3C haplotypes).

A typical result obtained from an *Acc*1 restriction enzyme digest of the A719G mutation is shown in fig 3.2.

Figure 3.2: Agarose gel electrophoresis of PCR amplified exon 10 of the TPMT gene digested with *Acc*1 from seven individuals of the coloured population group. Lanes 2&3 are heterozygous for the A719G mutation. Lane C positive control - known homozygote

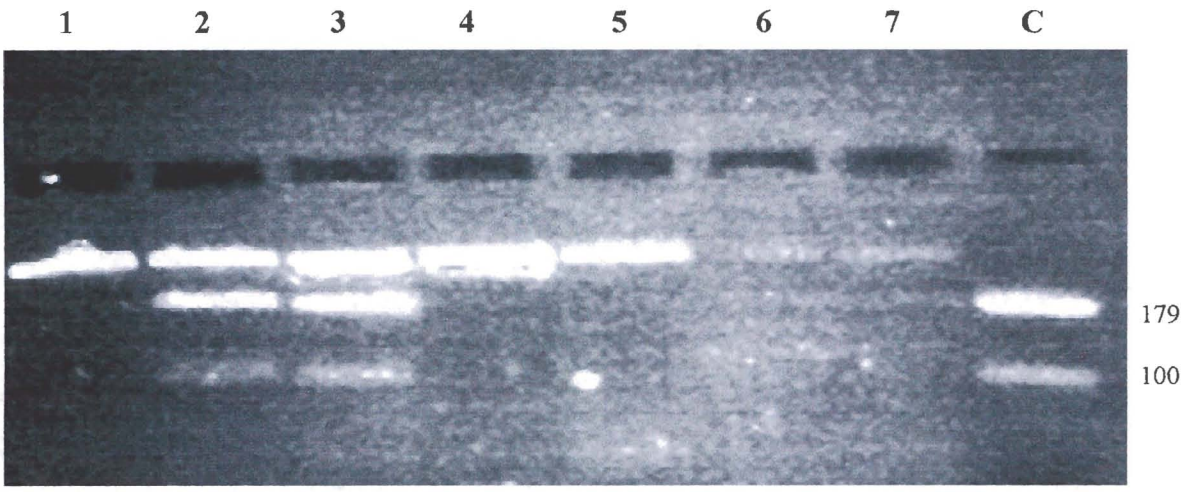


Table 3.4: Results of allele frequencies for the A719G mutation

Population Groups	Total Number of Subjects	Number of Normal Alleles	Number of Positive Alleles*	Allele Frequency (%)
Caucasians	50	92	8	8 %
Coloured	100	193	7	3.5 %
Africans	100	193	7	3.5 %

* All were heterozygotes

For clarity, it is useful to summarise the restriction fragments given by the various possible genotypes which might be observed in individuals carrying one or more of the two mutations: these are shown in Table 3.5.

It can be seen that all haplotypic combinations give unique patterns except for a heterozygote for TPMT 3A, which has an identical pattern to that given by the compound heterozygote TPMT 3B/TPMT 3C.

This is because individuals who possess both the G460A and the A719G mutations in *cis* on the same chromosome (haplotype TPMT 3A) will have the same combinations of restriction sites as individuals who have the G460A and the A719G mutations in *trans* on opposite chromosomes.

The likelihood of the latter can be estimated after analysis of the number of individuals with either G460A or A719G alone compared with the number who have the two together. These figures are documented in table 3.6a, with calculated haplotype frequencies in Table 3.6b

Table 3.5: Fragment sizes expected for various haplotypic combinations

Genotype			Fragment sizes	
Haplotype 1	Haplotype 2		*Mwo* 1	*Acc* 1
Normal	Normal		233 203	279
Normal	TPMT 3A		436 233 203	279 179 100
Normal	TPMT 3B		436 233 203	279
Normal	TPMT 3C		233 203	279 179 100
TPMT 3A	TPMT 3A		436	179 100
TPMT 3A	TPMT 3B		436	279 179 100
TPMT 3A	TPMT 3C		436 233 203	179 100
TPMT 3B	TPMT 3C		436 233 203	279 179 100

Table 3.6a: Numbers of individuals with different TPMT haplotypes

	G460A alone (TPMT 3B)	A719G alone (TPMT 3C)	G460A+ A719G (TPMT 3A)
Caucasians	0	2	6
Coloureds	0	6	1
Africans	0	7	0

Table 3.6b: Allele frequencies (%) for the TPMT*3A, TPMT*3B and TPMT*3C haplotypes in the various population groups

	Caucasian	Coloured	African
TPMT*3A	6%	0.5%	0%
TPMT*3C	2%	3%	3.5%
TPMT*3B	0%	0%	0%

Similarly, the same group of 50, 100 and 100 Caucasian, Coloured and African subjects respectively were later screened for the G238C mutation (TPMT *2 haplotype). No mutation was detected in any of the subjects screened.

The fact that both a heterozygote for the 3A haplotype and a compound heterozygote for 3B and 3C haplotypes would give identical restriction enzyme patterns (Table 3.5) raises the possibility of mis-assigning the genotype of some individuals. However, the total frequency of the G460A mutation alone (the 3b haplotype) in Caucasians is unlikely to be more than 1% since no individuals with this haplotype were seen in the 100 Caucasian alleles characterised. The frequency of Caucasian subjects with the A719G mutation alone (the 3C haplotype) is 2%. The chance therefore of a compound heterozygote should not be greater than 0.01 x 0.02 or (0.02%). Since there are only 6 individuals out of a total number of 50 (a frequency of 12%) Caucasian subjects with the pattern given by the genotypes of such a compound heterozygote as well as by the TPMT 3A / normal heterozygote, it can be concluded that the G460A + A719G patterns are much more likely to represent the TPMT 3A haplotype opposite a normal allele than a compound heterozygote of 3B and 3C.

The allele frequency calculated from subjects heterozygous for the TPMT*3A haplotype in Caucasians is 2 x 6/50 = 6%, while this haplotype was detected in only one subject out of the individuals sampled from the Coloured population group. This haplotype was not detected in any subject sampled from the African population group. This difference is statistically significant - at $p<0.001$ (chi square: 2 degrees of freedom =19.34)

In contrast to the results for TMPT*3A haplotype, the allele frequency for the TPMT*3C haplotype is higher in both the Coloured and African population groups than in the Caucasian group, but is not statistically significant: $p= 0.748$ (chi square 2 degrees of freedom =0.58)

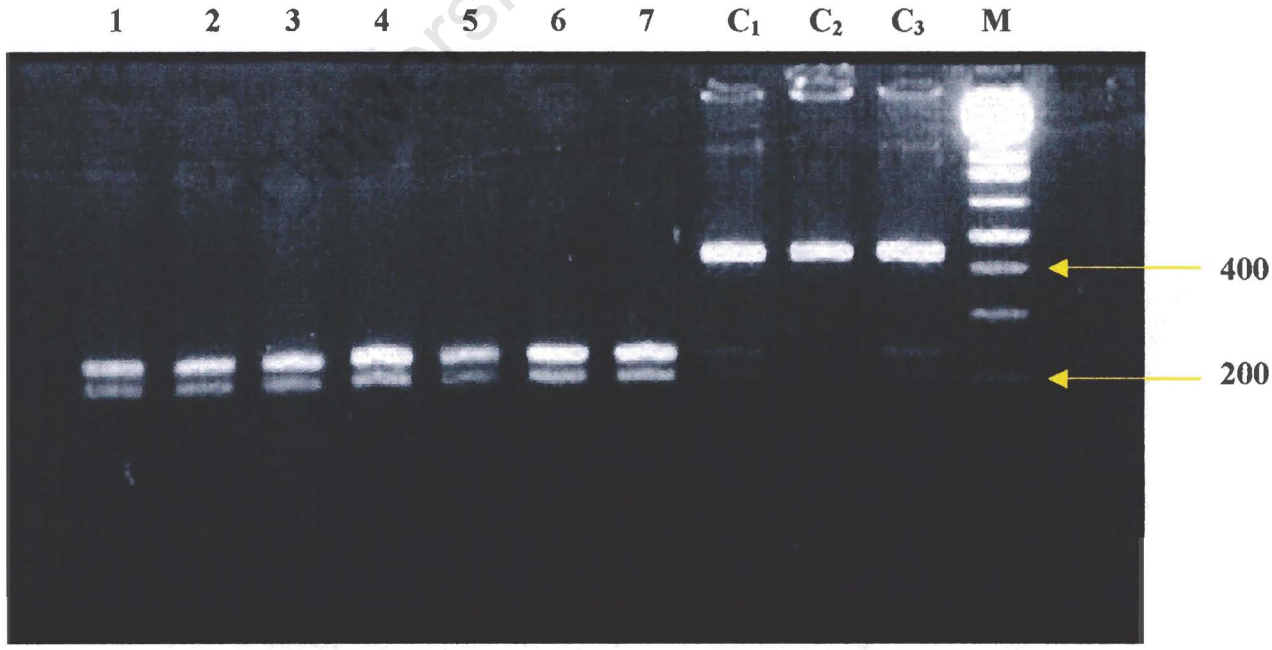
The TPMT 3B haplotype is generally rare, and was not detected in any of our local population groups. However, the TPMT*3C haplotype is reported to be the most common mutation among African-Americans and Ghanaians which is consistent with the results reported here.

PATIENTS RESULTS

A total of 28 patients from the Red Cross Children's and Groote Schuur Hospitals who are currently on purine analogue drugs were screened for the TPMT*3A, 3B and 3C haplotypes using the G460A and A719G mutations. These patients were also screened for the TPMT*2 haplotype (G238C mutation).

A typical gel electrophoretic analysis for the *Mwo*1 restriction enzyme digest of some of the patients is shown in figure 3.3. All the patients (1-7) illustrated are homozygous for the normal gene. C_2 is the homozygous positive control for the G460A mutation while C_1 and C_3 are heterozygote positive controls, and M is the molecular size marker.

Figure 3.3: Gel electrophoretic analysis for the *Mwo*1 restriction enzyme digest



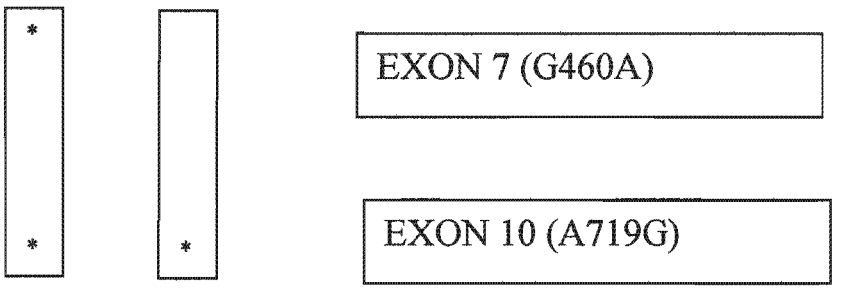
The results are documented in Table 3.7 below.

Table 3.7: Number of patients currently on thiopurine drugs with TMPT mutations

Population Groups	Total Number of Subjects	Number of Normal Results	Number of Positives Results
Caucasians	6	6	0
Coloured	16	15	1*
Africans	6	6	0

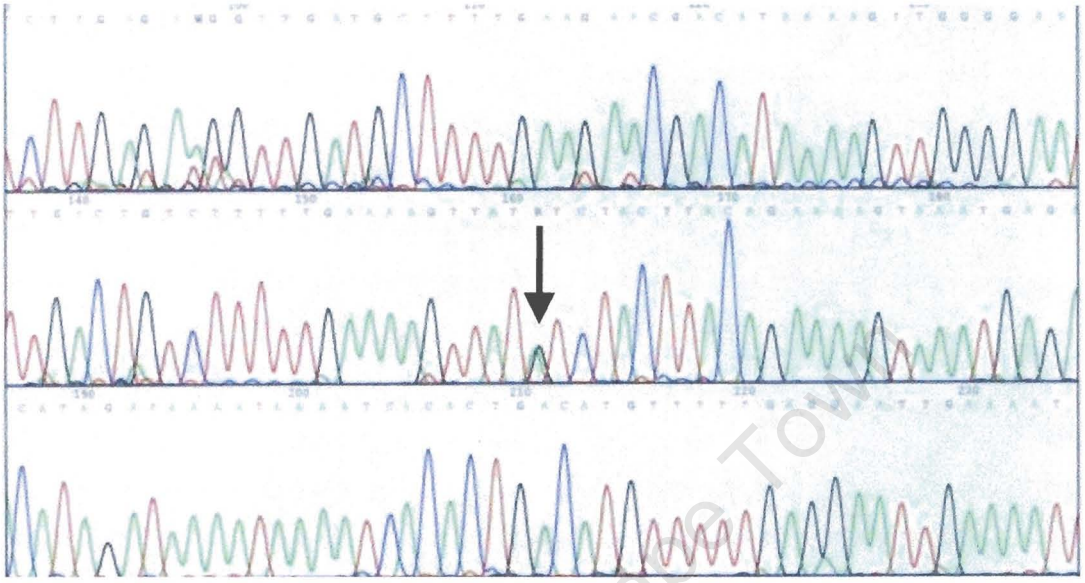
The only positive patient (*) is a 36 year old coloured female who is currently undergoing treatment for acute myeloid leukemia, at the Groote Schuur Hospital Haematology Unit, with 6-Mercaptopurine and Methotrexate. She was found to have restriction fragments of 436, 233, and 203 bp for *Mwo*1 and 179 and 100 bp for *Acc*1. These fragments were diagnostic for both the exon 7 (G460A) and exon 10 (A719G) mutations on one allele and the exon 10 (A719G) mutation alone on the second allele (see Table 3.4). This patient is therefore a compound heterozygote for the TPMT 3A and the TMPT 3C haplotypes. No G238C (TPMT*2 haplotype) mutation was detected in all the 28 patients screened.

Figure 3.4: Cartoon illustration of the patient with compound heterozygosity for both TPMT*3A and TPMT*3C haplotypes



Sequence of exon 10 of the TPMT gene

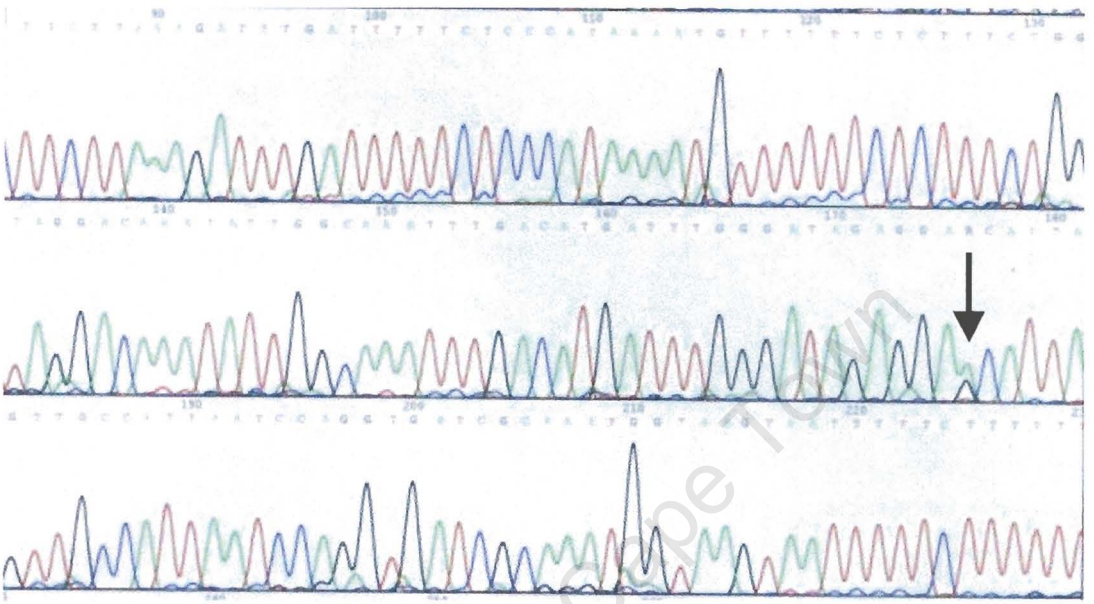
Fig 3.5. Nucleotide sequence of Exon 10 of the TPMT gene from a patient heterozygous for the A719G mutation.



*The sequence shown in the diagram above confirms the validity of the Acc*1 *restriction enzyme digest. The sequence is from one of the subjects who is a heterozygote for the A719G mutation located on exon 10 of TPMT gene. Specifically this point mutation is at position 161 in the nucleotide sequence (Figure 3.5). This shows a double peak (arrowed) of 2 bases (G &A) referred to in this sequence as R, which confirms the presence of both A in the wild type allele and G in the mutant allele.*

Sequence of exon 7 of the TPMT gene

Fig 3.6. Nucleotide sequence of Exon 7 of the TPMT gene from a patient heterozygous for the G460A mutation.



*Similarly the recognition sequence shown in Fig 3.6 confirms the validity of the Mwo*1 *restriction enzyme digest in one of the subjects heterozygote for the G460A mutation located on exon 7 of the TPMT gene. Specifically this point mutation is on position 176 in the nucleotide sequence in Figure 3.6 above. This shows a double peak of 2 bases (G and A) referred to in this sequence as R (arrowed), which confirms the presence of both G in the wild type allele and A in the mutant allele.*

Sequence of exon 5 of the TPMT gene

Fig 3.7: Nucleotide sequence of Intron 4 and part of Exon 5 of the TPMT gene from a known positive control patient heterozygous for the G238C mutation.

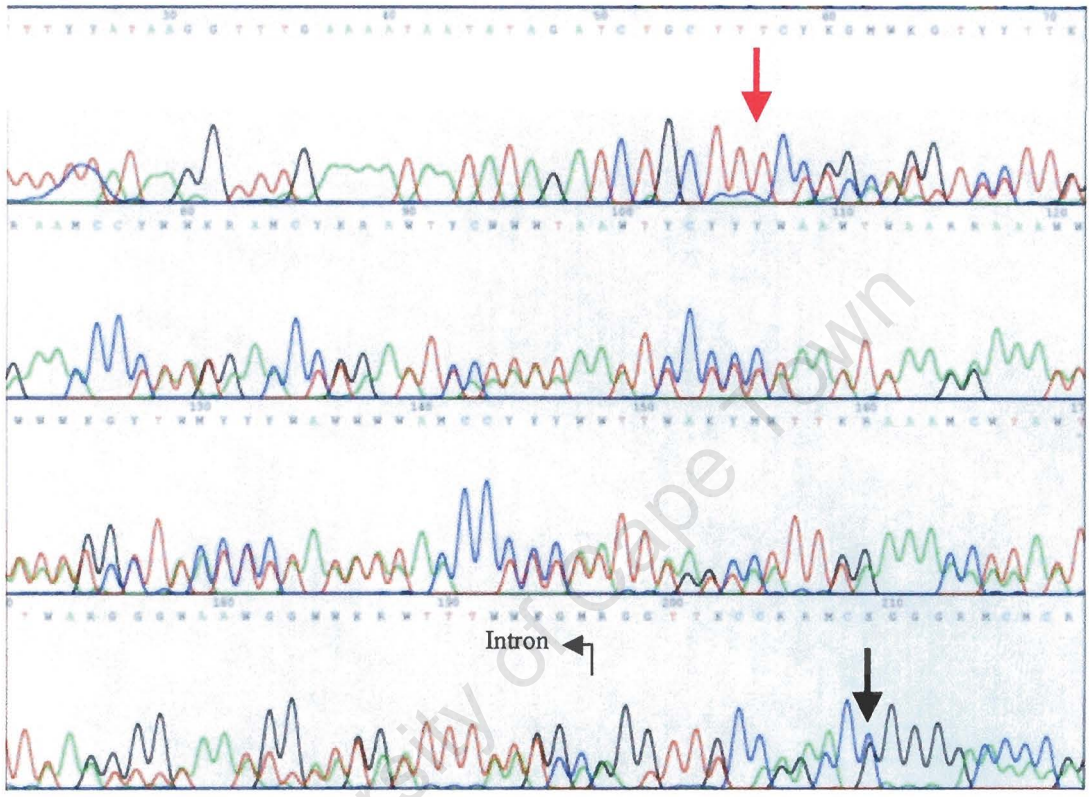


Figure 10 shows the nucleotide sequence of part of exon 5 of the TPMT gene from a reference patient (from Guy's hospital) which we sequenced to confirm presence of the G238C mutation. However, the sequence appears at first sight to have broken down, with double peaks in most positions. In addition, a sequential pattern of double peaks of various individual nucleotides often occur but in a haphazard fashion. Careful examination made it clear that this pattern was the consequence of a new mutation in the intron preceeding exon 4 in the form of a single base (C) deletion. Given this, it was also possible to observe in position 209 a double peak corresponding to both G and C, which would confirm heterozygosity for the expected G238C mutation.

This unexpected deletion of a single base (in red) in the sequence CCTGC in intron 4 presumably represents a polymorphism of no clinical consequence, since the shift produced only affects the nucleotide sequence and not the amino acid reading frame of the gene.

CHAPTER 4

DISCUSSION

OVERVIEW OF PURINE ANALOGUES

Purine analogues such as 6-mercaptopurine and azathioprine are used clinically in the treatment of a variety of clinical conditions including acute Lymphoblastic leukemia, chronic rheumatoid arthritis, inflammatory dermatological diseases, autoimmune hepatitis, inflammatory bowel disease and prevention of rejection in organ transplantation. Chronic administration of these drugs is associated with certain untoward effects, which include gastrointestinal disturbances, myelosuppression, hepatotoxicity and very rarely, severe hypersensitivity reaction. However, by far the most dangerous side effect is that related to severe and potentially fatal haematopoietic toxicity necessitating withdrawal of therapy in more than a third of patients (1,2,7,8,12,20).

In another review from France, approximately 10% of patients on treatment for inflammatory bowel disease developed severe side effects including pancreatitis, allergic reaction and myelosuppression (11). This also necessitated the withdrawal of therapy, and sometimes requiring hospitalisation (11). Frequent deaths have been reported (7,11,20). In a report from Japan, a patient who was found to have low TPMT activity died from severe sepsis following prolonged and recurrent leukopaenia (20). Myelosuppression is usually the first and most serious sign of drug toxicity, and usually occurs as a consequence of low or absent TPMT activity, (7) where

severe and life-threatening pancytopaenia can occur later. The enzyme deficiency results in the accumulation of toxic levels of 6-Thioguanine nucleotide (7). Myelosuppression tends to occur between the 4th and 10th week after commencement of therapy with the peak at around the 7th week (7). Bone marrow suppression tends to occur early in the patients who are homozygote for the mutant allele compared to the patients heterozygote for the mutant alleles (11).

GENETIC POLYMORPHISM

The existence of genetic polymorphism in TPMT activity in the various population groups has been reported. In Caucasians, the most prevalent non-functional mutant alleles are TPMT*3A and the TPMT*2, while TPMT*3C has been reported to be the most prevalent mutant allele among Africans, African-American and Chinese (2,4,5,16,18,20). In a large series from Japan, the authors also reported that the most prevalent mutant TPMT allele among the Japanese, is the TPMT*3C haplotype (18).

80% of Caucasians with low or intermediate TPMT activity have been reported to have inherited the mutant alleles of the TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C haplotypes (5,20). However, there are isolated reports of rare mutations such as TPMT*4 in a family of Northern European extraction (4,20), TPMT*6 in an individual from Korea (4,5,14,17,20), TPMT*7 from a European Caucasian (5,20), and TPMT*8 (G644A) in an African-American (4,5,20). By contrast, there was a reported case in the literature of TMPT*8 in a Caucasian from North Portugal with no known African ancestry (10). In another report from Japan by Hiratsuka and co-workers, none of these rare mutations were detected amongst the screened Japanese

subjects (24). Intronic and silent mutations have also been described (10,21). Subjects who inherit these mutant alleles are prone to severe thiopurine drug toxicities especially potentially fatal myelosuppresion.

In a separate report, 75 % of Caucasian patients with positive results have been shown to have inherited the mutant alleles of both the TPMT*2 and TPMT*3 haplotypes (12). 55-70% of these positive patients screened inherited the mutant alleles of the TPMT*3A haplotype, which is the predominant haplotype associated with low or absent TPMT activity in Caucasians (12,19). It has been established that both TPMT*2 and TPMT*3 haplotypes are associated with various degrees of reduction in TPMT activity and immunoreactive protein (12). The G460A mutation alone (TPMT*3B haplotype) leads to a modest 9 fold reduction in TPMT enzyme activity (9,12) while the A719G mutation alone (TPMT*3C haplotype) also results in 1.4 fold reduction in enzyme activity (9,12). However, a combination of the two mutations as in the TPMT*3A haplotype leads to virtual complete loss of TPMT enzyme activity (12), with about 400 fold less of immunodetectable protein compared to the wild type (9).

The TPMT*2 haplotype is also associated with a 100 fold reduction in enzymatic activity in the homozygous state relative to the wild type (12).

The G460A transition is also associated with a significant increase in Km of the TPMT enzyme for 6-mercaptopurine (46 fold) and the co-substrate, S-adenosylmethionine (208 fold), such that the intrinsic clearance of 6-mercaptopurine methylation was more than 10 fold lower than the wild type TPMT (9).

ERYTHROCYTE TPMT ENZYME ACTIVITY

Quantitative estimation of TPMT activity in erythrocytes has also been carried out. Subjects homozygous for the TPMT 3A haplotype have erythrocyte TPMT activity of less than 3nmol/hour/ml of red cells, while levels of between 3-10, and levels greater than 10nmol/hr/ml of red cells are found in subjects heterozygote for the mutant alleles and those homozygous for the wild type respectively (7). Approximately 89% of Caucasians and African-Americans have normal erythrocyte TPMT activity (referred to often in the literature as "high"), 11% have intermediate activity, while 0.3% of these populations have low activity (2,3,7,9,12,13,14).

In a separate case report from a dermatology clinic in Toronto, a higher prevalence rate was reported (13). Four (including 1 homozygote) out of twenty (20%) were shown to have inherited the mutant TPMT alleles (13). The author suggested that the prevalence of these mutations maybe higher in the patients with dermatological diseases (13). However, the few drawbacks of quantitative erythrocyte TPMT activity are that this measurement is laborious, expensive, and can only be performed in specialised laboratories (29,31).

GENOTYPE AND PHENOTYPE CORRELATION

There have been attempts elsewhere to correlate genotype and drug toxicity. Individuals with low TPMT activity are highly prone to severe drug toxicity especially bone marrow suppression (13,14,16,20). As alluded to previously, it is now well recognised that patients with TPMT deficiency are at a greater risk of fatal

haematopoietic toxicity while on treatment with conventional doses of purine analogues (16,18,20). According to this author, the conventional or standard dose of 6-mercaptopurine is 75mg/m^2 of the body surface area/day (15). This life-threatening side effect tends to occur more frequently and severely in the patients who are homozygote for the mutant TPMT alleles (16). Similarly, patients who are heterozygote for the mutant TPMT alleles are also at a higher risk of thiopurine-induced haematopoietic toxicity, although the frequency and severity are less than those patients homozygote for the mutant TPMT alleles (16).

There is enough evidence in the literature, that the patients with low or intermediate activity are prone to potentially fatal side effects especially myelosuppression (9,14,15,18,19,20). However, it has been suggested by several workers that the only way to avoid these dangerous side effects particularly bone marrow toxicity is to either reduce the dosage of the thiopurine drugs substantially (9,13,14,15) or use an alternative therapy (13). In one report from the United States, the median dose reduction of thiopurine drugs in patients who are homozygote for the mutant TPMT alleles compared to those homozygous for the wild type alleles, is approximately 90% (16). However, a 67% median dose reduction has been recommended in patients found to be heterozygote for the mutant TPMT alleles compared to the wild type (16); although in the author's previous experience, only a modest 15-30% dose reduction was recommended (16). The author suggested that the higher dose reduction in this current study, is probably because these patients had already developed haematopoietic toxicity, and may therefore represent the most sensitive subset of the heterozygous patients (16).

This significant dose reduction has not been shown to adversely affect treatment outcome because adequate erythrocyte thioguanine levels are still achieved at these lower dosages (16). In a previous report, the patients homozygote and heterozygote for the TPMT mutant alleles treated with lower doses of thiopurine drugs were shown to maintain erythrocyte thioguanine levels at or even greater levels than those achieved by patients homozygote for the wild type alleles (16). Thioguanine levels at or greater than 290*p*mol/8 x108 RBCs are critical for therapeutic efficacy, and this has been associated with a more favourable outcome in patients on treatment with thiopurine drugs for acute lymphoblastic leukemia (16).

The average prevalence rate for patients who were found to be intolerant to thiopurine drugs due to severe side effects especially myelosuppression is not exactly known (13). One author reported a 10% prevalence (11).

A much higher prevalence has been reported elsewhere. For example, in one report from the United States of America (16), a considerably higher prevalence has been reported. In this report, 65% of children reported to be intolerant to standard dosages of thiopurne drugs were later found to be homozygous as well as heterozygous for the mutant TPMT alleles. This was predominantly due to bone marrow toxicity alone (16) or in combination with other untoward effects especially hepatotoxicity (16), compared to other children homozygote for the wild type alleles on the same therapy for acute lymphoblastic leaukemia (16). In this study, 21 out of 23 patients found to be intolerant to thiopurine drugs (approximately 91%) presented with haematopoietic toxicity alone or in combination with other toxicities. Six out of twenty-three (26%)

were reported to be homozygous for the mutant TPMT alleles while 9 out of 23 (39%) were reported to be heterozygote for the mutant TPMT alleles. The rest of the patients except 2 who were not tested because the DNA was not available were all found to be homozygous for the wild type. In another report from Thailand, a considerably higher prevalence was shown (14). All the 8 children (100%) who were subsequently found to be heterozygote for the mutant allele of the TPMT*3C haplotype were reported to be intolerant to thiopurine drugs because of severe haemopoietic toxicity.

In the study described earlier from USA (16), only 2 out of 23 patients with side effects which represents approximately 9% of the patients studied, developed severe hepatic toxicity while on treatment with conventional doses of thiopurine drugs. These patients were found to be homozygote for the wild type TPMT gene. The author suggested that the methylated metabolites of the thiopurine drugs might be contributory to the hepatotoxicity. There was also a previous report (8), of patients with high TPMT activity, and hepatotoxicity, presumably due to these methylated metabolites; although a definitive causal relationship is yet to be established. It is therefore logical to speculate that hepatotoxicity would be an unlikely side effects in the patients with TPMT deficiency. It has also been reported in the literature that this group of patients with high TPMT activity also show some degree of resistance to purine analogues presumably also on the basis of increase metabolic conversion of the these drugs to their inactive metabolites (2,5,13).

It is strongly advisable to measure the TPMT activity in red cells before commencing purine analogue therapy to avert the danger of these toxicities. Unfortunately the

majority of these patients are only identified after experiencing severe toxicity, even though prospective measurements of erythrocyte TPMT activity has been advocated by some (9,12,15). TPMT enzyme assays and genotypic analysis are not widely available in many centres (13,14,15). This problem is further compounded by red blood cell transfusions because constitutive erythrocyte enzyme activity can accurately be determined only after 2-3 months following such transfusions (13,14,15,31,32). This was confirmed in one of these reports (15), where a patient's erythrocyte TPMT activity was found to be consistent with a heterozygous genotype (9.8 U/ml) after receiving 2 units of red cell transfusions. Four months following these transfusions, this patient was found to have virtually no TPMT activity in the red blood cells (15). The authors suggested that this particular patient most likely received transfusions from someone homozygous for the wild type allele (15). PCR based genotypic analysis is a more practical method for screening than red cell TPMT enzyme activity. It does not change, and only need to be determined once, it is quick, inexpensive, and it is not affected by donor erythrocytes (14,15,29,31).

In a case report from Germany, a child was discovered to be homozygote for the mutant TPMT*3A haplotype only after developing fatal pancytopaenia following severe myelosuppression while on a conventional dose of azathioprine for HLA-B27+ spondylarthritis (7). In another case report from Toronto, Canada, a 36 year old woman with bullous pemphigoid was also discovered to be homozygote for the TPMT mutant alleles only after experiencing severe azathioprine-induced myelosuppression (13). All subjects with "high" TPMT activity have been shown to be homozygous for the wild type allele, while the majority of subjects in the intermediate group are heterozygote for either the TPMT*2 or TPMT*3 haplotype

(7,13). Patients with low activity have been shown to be homozygote for the TPMT mutant alleles (7,13,31). A 98% concordance between genotype and phenotype has been reported in 47 patients who were investigated (13). The authors suggested that the lack of concordance in a small % of patients (2%) might have been caused by undiscovered or rare TPMT gene mutation(s) (13). At least 5% of mutations have not yet been isolated in Caucasians (15).

In another report, 4 African-Americans, which represents 17% of the group screened, had intermediate erythrocyte TPMT activity but were shown not to have inherited any known mutant alleles (4). The authors speculated that the mutation could be in the promoter region of the TPMT gene and that this could be a possible site for future mutation screening in subjects or patients with low or intermediate TPMT activity (4,16). One report stated that induced marrow suppression in the patients on azathioprine therapy tends to occur more frequently in those with intermediate activity, and who are on treatment for a dermatological condition (2). On the other hand, certain drugs including azathioprine (8,11,32), and diuretics (28) have been reported to induce TPMT activity.

Although 6-thioguanine nucleotide is toxic to the cell, a minimum intracellular concentration is required for therapeutic efficacy (11). For example a lower risk of recurrence of leukemia, relapse in Crohn's disease and rejection in organ transplantation have been reported in patients with 6-thioguanine nucleotides above a certain threshold (11). In another report, increased risk of treatment failure in children with acute lymphoblastic leukemia has been reported in those with high constitutive TPMT activity (26,31). Excessive or inadequate immunosuppression are

associated with complications including acute and opportunistic infections, and acute rejection respectively (12). A fine or delicate balance must therefore be achieved. This usually requires careful tailoring of immunosuppresive therapy to the specific needs of the individual patient (12,32). It is now clear why some patients with high TPMT activity show some degree of resistance to thiopurine drugs presumably on the basis of increased conversion to the inactive metabolites (2,5,13). This subset of patients sometimes require higher doses of thiopurine drugs to achieve therapeutic efficacy (13,32).

ETHNIC VARIATION IN ERYTHROCYTE TPMT ACTIVITY

Interethnic variation in erythrocyte TPMT activity has been reported in different population groups. Saami subjects (Norwegian Asian immigrants) have about 30% higher TPMT activity compared with whites from the same geographical area, while American Blacks have 17-33% lower TPMT activity compared to American white subjects (5). But in a recent article published in August 2001, no difference in TPMT activity was observed between the Saami population group and Caucasians in Northern Norway (17). The TPMT activity in African and White Americans was found to be considerably lower than that reported in 119 investigated Chinese subjects (5). In separate report (18), out of the 192 Japanese subjects screened for the common TPMT (TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C) haplotypes, only 3 subjects (less than 2%) were found to be heterozygote for the TPMT*3C haplotype (18). The author suggested that because of the low frequency of the mutant TPMT allele, individuals of Asian origin including Japanese, South West Asians and Chinese

population, have higher TPMT activity compared to European or American Caucasians (5,18).

In a recent review, the median erythrocyte TPMT activity was found to be similar in North American Caucasians, European Caucasians, and Asian population (14). Since no mutation analysis was performed in these studies, the underlying cause of these differences is still unresolved. Apart from interethnic variation, wide inter-individual variations in the TPMT activity and rate of methylation of thiopurine drugs have also been reported (13,20,31). These individual variations may partly explain individual susceptibility to purine drugs-induced bone marrow toxicity (11), or failure of response to therapy in some patients (13). This has been mainly attributed to the TPMT gene polymorphism with variation in the intracellular concentrations of the cytotoxic 6-thioguanine nucleotide (11). TPMT activity is said to be lower in females compared to males, and declines with increasing age (26,28).

CLINICAL IMPLICATIONS FOR GENE POLYMORPHISMS

The implications of the various polymorphisms, especially those associated with fatal and avoidable myelosuppression with tendencies to anaemia, sepsis and bleeding while on treatment with conventional or standard dose of purine analogue drugs, have been extensively reviewed in the literature (2,3,4,5,13,14). The mechanism for fatal haematopoietic toxicity has been shown to be due to excessive intracellular accumulation of 6-thioguanine nucleotide which is particularly toxic to cells undergoing rapid turn-over, including haematopoietic cells. This is a consequence of loss of TPMT activity (7,8,11). Thiopurine drugs including 6-mercaptopurine and

azathioprine are pro-drugs without any intrinsic activity but only become active after intracellular metabolic conversion to thioguanine nucleotide (9). The incorporation of 6-thioguanine nucleotide into DNA is thought to be one of the mechanisms for its antiproliferative effect (3,9). 6-methylmercaptopurine is also known to inhibit amidophosphoribosyltransferase: the 1st and rate limiting step in the de novo synthensis of purines. It is however not clear which of these is the principal mechanism of action of thiopurine drugs.

The level of 6-thioguanine is inversely proportional to the activity of TPMT; hence patients with low TPMT activity in the red cell, less efficiently methylate these thiopurine drugs, and therefore have more extensive conversion to thioguanine nucleotides (9,14,15,16). In a report from Thailand, patients heterozygous for the mutant TPMT alleles accumulate about 50% more thioguanine nucleotide than patients who have "high" TPMT activity, thereby placing these patients at an intermediate risk for toxicity (14,15). These patients are prone to severe drug toxicity especially myelosuppression (9,14,15). However, some have advocated that the only way to avert this potentially fatal side effect is to substantially reduce (8-15 folds) the dosage of these drugs (9,13,14,15). It is widely believed that azathioprine or 6-mercaptopurine are contraindicated in patients homozygote for the mutant TPMT alleles (11,13). There are however differing opinions as regard the safety of these drugs in patients heterozygote for these mutations (13). Some authors have recommended alternative treatments for azathioprine such as mycophenolate mofetil in the patients heterozygote for the mutant TPMT alleles (12,13) or alternatively the drugs could be used cautiously at significantly reduced dosages in heterozygote patients (11,13). Mycophenolate mofetil could be used safely in the patients with

TPMT deficiency because it is not metabolized via the TPMT inactivating pathway (12).

THIOPURINE S-METHYLTRANSFERASE ENZYME

The TPMT enzyme plays a critical role in the s-methylation (inactivation) of purine analogue drugs. It acts by rapidly shunting these drugs to their inactive (non-toxic) metabolites including 6-methyl-mercaptopurine or 6-methyl-thioguanine (9). Alternatively, these thiopurine drugs are converted to a lesser extent to another inactive metabolite, 6-thiouric acid, by xanthine oxidase (9,16). However, in haemopoietic tissues, xanthine oxidase activity is virtually absent, therefore the main inactivation pathway for thiopurine drugs in these tissues is via the TPMT pathway (16).

The TPMT protein encoded by the TPMT*2, and TPMT*3 mutant alleles have been shown to undergo accelerated proteolytic degradation with significantly reduced thiopurine S-methyltransferase enzyme activity in the body (4,16). Patients who are heterozygote for the mutant alleles of the TPMT*3A haplotype have erythrocytes TPMT protein 20-30 fold less compared to individuals homozygous for the wild type (9). It has been demonstrated or shown by immunotitration studies that immunoreactive TPMT protein levels correlate well with erythrocyte TPMT enzyme activity (9). The above findings were also confirmed in a recent report by Weinshilboum but these were thought to be due to the presence of 2 separate single nucleotide polymorphisms (SNPs) located on exon 7 and 10 (33). In those subjects homozygous for the TPMT*2 haplotype (G238C) where alanine is substituted for proline, the TPMT activity is also about a 100 fold less compared to the wild type.

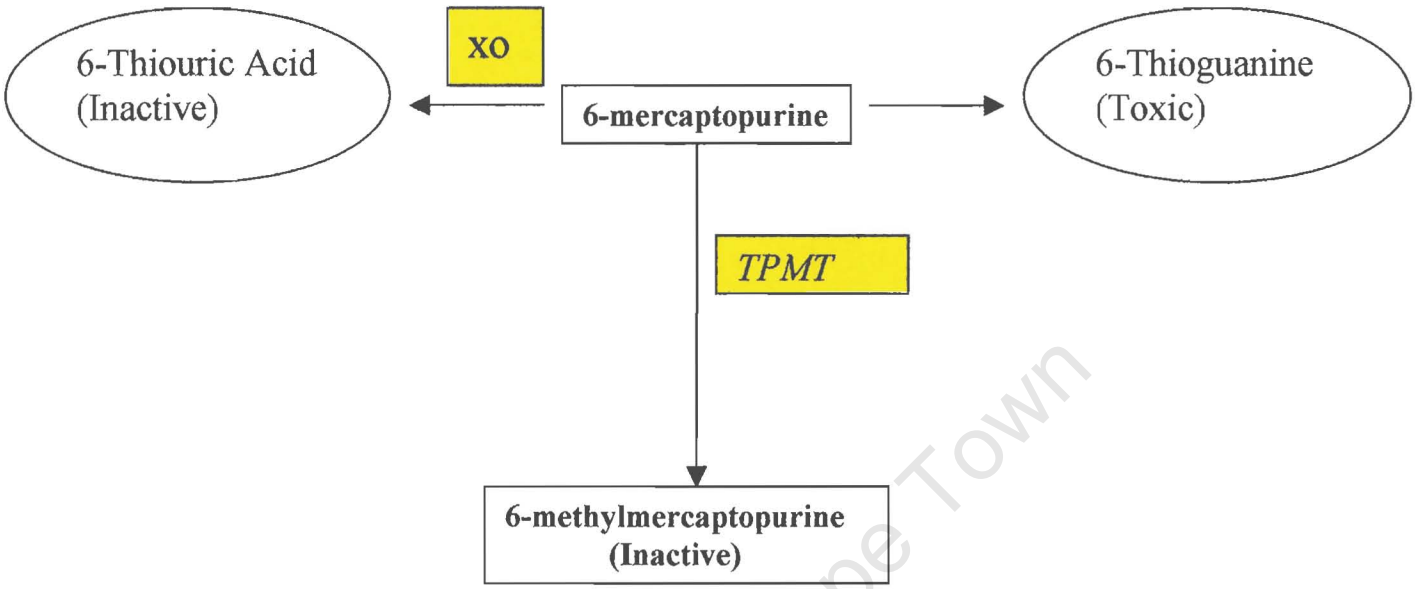
The measured immunodetectable protein in the different TPMT haplotypes is shown in the table 4.1 below.

Table 4.1: Immunodetectable proteins in the various TPMT haplotypes

MUTATION	*FRACTION OF IMMUNODETECTABLE TPMT ENZYME PROTEIN REMAINING IN HOMOZYGOTES*
TPMT*3A	1/400
TPMT*3B	1/360
TPMT*3C	1/40
TPMT*2	1/100

The reason for the loss of enzymatic activity could also be attributed to disruption of the active site or changes in the folding pattern of the protein (3). Proline is known to cause distortion in protein structure, and the region of protein with the Alanine to Proline substitution has an additional turn compared to the wild-type TPMT protein (3). In another report from Tennessee, United States, the TPMT protein encoded by mutant cDNA containing either the G460A mutation alone (TPMT*3B haplotype) or A719G mutation alone (TPMT*3C haplotype) have been shown to be associated with marked enzymatic catalytic instability (15). The central role of TPMT is shown in figure 4.1 below.

Fig 4.1: Role of TPMT in purine drugs metabolism



OTHER FACTORS AFFECTING PURINE ANALOGUES TOXICITIES

A recent report (11), described several features affecting purine analogue toxicity, and these will be described below in point form:

1) Two patients on purine analogues were reported with severe neutropenia and thrombocytopaenia, but these patients were discovered to have co-existing infections due to parvovirus B19 and varicella viruses.

2) Some other patients were simultaneously receiving purine analogues together with other medications known to interfere with Azathioprine metabolism: allopurinol, a xanthine oxidase inhibitor, and sulfasalazine and 5'aminosalicylate which have been shown to inhibit TPMT activity

3) One patient had severe bone marrow suppression following concomitant administration of olsalazine and Azathioprine. The red cell TPMT activity was in the

lower range of normal, and her genotype was found to be homozygous for the wild type. The authors concluded that the myelosuppression was due to accumulation of 6-thioguanine resulting from inhibition of TPMT by olsasalazine.

4) The literature review by these authors showed anecdotal cases of marrow suppression reported in the patients concomitantly taking commonly prescribed drugs such as trimethoprim-sulfamethoxazole, metronidazole and captopril.

In another report from England (28), significant neutropenia was reported in the patients with systemic lupus erythromatosus (SLE) on treatment with azathioprine. These patients were reported to be homozygote for the wild type allele. The author concluded that the neutropenia was as a result of SLE and not due to TPMT deficiency. It is therefore important to note that other factors apart from genetics can influence TMPT activity, and this possibility should be carefully considered in patients on thiopurine drugs with myelosuppression before deciding on withdrawal of therapy.

GENOTYPIC SCREENING RESULTS

Our overall results showed that A719G is the most common mutation in all population groups in South Africa. However, in Caucasians, this mutation is found to frequently co-exist with a G460A mutation on the same allele (5); this combination constitutes the TPMT*3A haplotype. By comparison, we have shown here that G460A mutation alone (which constitutes the TPMT*3B haplotype) is very rare in all population groups in South Africa. No TPMT*3B haplotype was detected in either the Caucasian or Coloured population groups. There was also no subject from the

African population group in South Africa with the G460A mutation alone (TPMT*3B) or with G460A in combination with the A719G on the same allele (TPMT*3A). Similarly, there were no Ghanaians reported to have the TPMT*3A or TPMT*3B haplotype (5). Our evidence, together with others, therefore indicates that these haplotypes may be rare in Africans.

Twenty- two subjects from the three ethnic groups in South Africa, which accounted for 8.8% of total subjects screened, were found to be heterozygous for the A719G mutation. However, seven of these subjects (six Caucasians and one coloured) have a co-existing G460A mutation on the same allele which constitute the TPMT*3A haplotype. This therefore translates into a frequency of 6 and 0.5% for the TPMT*3A haplotype among Caucasian and coloured population groups in South Africa respectively. From our results, 75% of all Caucasians in South African with a TMPT mutation were found to be heterozygote for the TPMT*3A haplotype. This is considerably lower than 84.9% reported among British Caucasians (5), but similar to the approximately 77% (17 out of 22) Caucasians heterozygote for the TPMT*3A haplotype in the United States (9). We did not find any subject from the African population group with the TPMT*3A haplotype. In the coloured population group, only 1% of the subjects screened were found to be heterozygote for the TPMT*3A haplotype. This result (0.5% allele frequency) is however surprising especially in our coloured/mixed ancestry population group in view of 17% allele frequency for the TPMT*3A haplotype reported in African-Americans (4).

In contrast, the most prevalent mutant allele in the South African Coloured and African population groups is the A719G mutation in isolation (TPMT*3C haplotype)

representing a frequency for this haplotype of 3 and 3.5% respectively in these populations.

Out of all the positive results among the coloured population group, 85.7% (6 out of 7) were found to be heterozygote for the TPMT*3C haplotype (the seventh was TMPT *3A), while all the positive results (100%) from the African population group were found to represent heterozygotes for this mutation. A similar finding was reported in Ghanaians (5), Japanese (18,20) and Thai children with acute leukemia (14) where TPMT*3C accounted for 100% of the mutant alleles studied.

In Japan, the allele frequency was reported to be considerably lower than that in South Africa (18). Only 3 out of 192 subjects screened had an A719G mutation and in all cases this was the TPMT*3C haplotype, the frequency therefore translates to 0.8% i.e 3 out of 384 alleles. In a recent report by Kubota et al. also from Japan, a similar allele frequency of 0.8% was reported (20). This reported allele frequency for the TPMT*3C haplotype in Japanese is comparable to those reported for British Caucasians (0.3%), American Caucasians (0.2%), and European Caucasians (0.8%) (20). These allele frequencies are however considerably lower than those reported among the Chinese (2.3%), African-American (2.4%), and Ghanaians (7.6%) (20). By comparison, the allele frequency in our Coloured population group is slightly higher than those reported in the Asian population group. In contrast, the allele frequency in the Ghanaians is considerably higher than the African population group in South Africa.

In a separate report, only 44.4% of the positive African Americans were reported to be heterozygote for the TPMT*3C haplotype (5). Similarly, in another report, 52%

of the positive African American were shown to be heterozygote for the TPMT*3C haplotype (4). There is abundant evidence therefore that TPMT*3C is the most common haplotype among African, African American and Asian population groups (5,14,16,18). This haplotype is relatively rare among Caucasians (4,5,14). For example, only 5.7% of the positive British Caucasians studied were reported to have inherited the mutant allele of the TPMT*3C haplotype (5). Similarly, only 5% of the positive American Caucasians were reported to be heterozygous for the TPMT*3C haplotype (4). By contrast, our results showed that 25% of Caucasians with positive results in South Africa (2 out of 8) were heterozygous for the TPMT*3C haplotype. This could be a simple consequence of our relatively small sample size. On the other hand, these "Caucasian" subjects were not personally visited at home, and may have been mis-classified as Caucasians by the medical record department, so the possibility cannot be excluded that some of these subjects may be of mixed ancestry.

ANCESTRAL TPMT GENE MUTATION

The TPMT*3C haplotype is likely to represent the ancestral TPMT mutant allele since it was present in both Caucasian and African subjects and has also been described in South West Asian and Chinese populations (5). An article from Norway on TPMT gene polymorphisms in the Saami population group (17), the author also supported the idea that the TPMT*3C haplotype is the ancestral TPMT mutant allele because this mutation is present in all the population groups studied in Norway. From gene evolution studies, it is considered that the most common allele in the combined population groups is usually the ancestral allele. The TPMT 3A haplotype reported in African Americans may then have been acquired as a result of

interbreeding with Caucasians. Our results are also consistent with the view that the TPMT*3C haplotype may be the ancestral allele since this haplotype was the most common mutant allele in Africans and coloureds, and although not the most common, was observed in Caucasians in South Africa.

SIGNIFICANCE OF PRE-TREATMENT SCREENING FOR TPMT ACTIVITY

The most dangerous but preventable untoward effect of thiopurine drugs is haematopoietic toxicity (1). This side effect was previously thought to be idiosyncratic, but can now be predicted by knowledge of metabolism of these drugs (13). The exact proportion of patient with the TPMT haplotypes who experience myelosuppression while on treatment with conventional doses of these drugs is not known (13).

Variable statistics have been reported from all over the world. For example only 1.2% of 739 patients on treatment with azathioprine for inflammatory bowel disease experienced myelosuppression (13). On the other hand, in another review from France, a considerably higher proportion (approximately 10%) of their patients, also on treatment for inflammatory bowel disease, developed side effects including myelosuppression (11). Several studies have shown that patients with low or intermediate TPMT activity (homozygous or heterozygous for the TPMT mutant alleles respectively) are at high risk of myelosuppression if treated with conventional doses of azathioprine (13).

Pre-treatment screening to avoid this life threatening side effect has been advocated by many workers (1,8,13,14,31). Recent reviews have shown that patients prone to

severe drug toxicity from conventional doses of thiopurine drugs could be identified before commencement of therapy (13). This could be achieved by either measuring the erythrocyte activity of the TPMT enzyme (1) or by carrying out a genotypic screening for the common haplotypes associated with decreased TPMT activity (13). Another alternative method of screening for the TPMT gene mutation is by denaturing HPLC (22,32). The advantages of this high-throughput method over established screening methods are easy automation, rapidity and reproducibility. It is also an efficient and convenient genotypic method for screening known mutations, and in this case, TPMT*2, TPMT*3A and TPMT*3C haplotypes. Because the above screening methods are not widely available, one report suggested weekly peripheral blood counts monitoring during the first 5 weeks of therapy (7).

COST IMPLICATIONS

The cost of regular monitoring of peripheral blood counts and management of complications is more than PCR-based genotypic screening (2). In a case report from Toronto, it was estimated that about $8,000 (Canadian) dollars would be required for the management and follow up of any patient who develops complications while on treatment with Azathioprine (13). A large percentage of this cost is incured from transfusions, use of granulocyte colony stimulating factor and hospitalisation (13). A similar patient on Azathioprine but without experiencing myelosuppression would require approximately $710 (Canadian) dollars for 6 months treatment with Azathioprine (13). By contrast, it costs merely $100 (Canadian) dollars in the same centre to screen a patient for the common TPMT gene mutations (13).

Pre-treatment screening could also be carried out by measuring erythrocyte TPMT activity (13), but TPMT genotyping is a simpler and less expensive test compared to this enzyme assay (13).

It is therefore evident that prevention is far cheaper and better than treatment.

We therefore recommend that all patients due to commence purine analogues should be genotypically screened for the common mutations of the TPMT gene.

PATIENT RESULTS

A total of 28 patients from Red Cross Children's and Groote Schuur Hospitals currently on treatment with thiopurine drugs were screened for the G460A, A719G and G238C mutations. Out of the total number of 28 patients screened, 16 patients were from the coloured population group, while 6 patients each were Africans and Caucasians. The majority of these patients (82%) were less than 16 years of age from the Red Cross Children's Hospital, and were on treatment with 6-mercaptopurine for acute lymphoblastic leukemia.. The race category, age, sex, diagnosis and treatment of the individual patients are shown in the table 4.2 below.

Table 4.2: Patient's diagnosis and relevant vital statistics

NO	AGE (YEARS)	SEX	RACE	DIAGNOSIS	TREATMENT
1.	31	Female	Coloured	SLE	Azathioprine
2.	18	Male	Caucasian	ALL	Azathioprine
3	36	Female	Coloured	AML	6-mp
4	35	Female	Coloured	RA	Azathioprine
5	46	Male	African	AML	6-mp
6	5	Male	Caucasians	ALL	6-mp
7	3	Male	Coloured	ALL	6-mp
8	12	Male	Coloured	ALL	6-mp
9	8	Female	African	ALL	6-mp
10	18	Male	Coloured	ALL	6-mp
11	15	Male	African	ALL	6-mp
12	5	Male	Caucasian	ALL	6-mp
13	7	Male	Coloured	ALL	6-mp
14	4	Male	Coloured	ALL	6-mp
15	13	Male	Coloured	ALL	6-mp
16	8	Male	African	ALL	6-mp
17	4	Male	Caucasian	ALL	6-mp
18	11	Female	Caucasian	ALL	6-mp
19	7	Female	Caucasian	ALL	6-mp
20	7	Male	Coloured	ALL	6-mp
21	6	Male	Coloured	ALL	6-mp

22	3	Male	Coloured	ALL	6-mp
23	15	Male	African	ALL	6-mp
24	9	Female	Coloured	ALL	6-mp
25	11	Male	Coloured	ALL	6-mp
26	6	Female	African	ALL	6-mp
27	8	Male	Coloured	ALL	6-mp
28	5	Male	Coloured	ALL	6-mp

ALL : Acute lymphoblastic leukemia

AML : Acute myeloid leukemia

RA : Rheumatoid Arthritis

SLE : Systemic lupus erythromatosus

6-mp : 6-mercaptopurine

All the 28 patients screened except one were found to be homozygote for the wild type allele. The only positive patient, a 36 year old coloured female, was on treatment with 6-mercaptopurine for acute myeloid leukemia. She was discovered on routine screening to be a heterozygote for both the G460A & A719G mutations on the same allele (TPMT*3A haplotype). She was also found to be a heterozygote for the A719G mutation alone on the second allele (TPMT*3C). The combination of these mutations therefore makes this patient a compound heterozygote for both the TPMT*3A and TPMT*3C haplotypes. The summary of individual patients and mutation(s) detected is shown in table 4.3 below.

Table 4.3: Results of DNA analysis in the patients currently on purine analogues at the Red Cross Children's and Groote Schuur Hospitals

Patient Number	*Allele 1*	*Allele 2*
1.	Wild type	Wild type
2.	Wild type	Wild type
**3.	TPMT*3A	TPMT*3C
4.	Wild type	Wild type
5.	Wild type	Wild type
6.	Wild type	Wild type
7.	Wild type	Wild type
8.	Wild type	Wild type
9.	Wild type	Wild type
10.	Wild type	Wild type
11.	Wild type	Wild type
12.	Wild type	Wild type
13.	Wild type	Wild type
14.	Wild type	Wild type
15.	Wild type	Wild type
16.	Wild type	Wild type
17.	Wild type	Wild type
18.	Wild type	Wild type
19.	Wild type	Wild type
20.	Wild type	Wild type

21.	Wild type	Wild type
22.	Wild type	Wild type
23.	Wild type	Wild type
24.	Wild type	Wild type
25.	Wild type	Wild type
26.	Wild type	Wild type
27.	Wild type	Wild type
28.	Wild type	Wild type

** 3 : Compound Heterozygote

As discussed earlier, the only positive patient, a 36 year old coloured female on 6-mp for acute myeloid leukaemia, developed pancytopaenia even before commencement of therapy. After commencement of therapy, this patient became persistently neutropaenic with J-line sepsis and recurrent mucositis. As a result of this, the dose of 6-mercaptopurine was reduced considerably. Because her genotype was only determined 6 months after onset of therapy, her previous worsening pancytopaenia could either be due to the continued effect of malignant cell infiltration of the marrow, or consequent to the TPMT deficiency or both. The attending haematological physician was of the opinion that it would be difficult to ascertain which of these two causes predominated. The clinical course of this patient reinforces the significance of prospective genotypic analysis to identify those at risk of severe toxicity.

It has been established by several workers that subjects who are heterozygote for one of the common TPMT mutant alleles have intermediate erythrocyte TPMT activity

(7,13,14,15), the reference range for subjects with intermediate activity being 3-10 nmol/hour/ml of RBC. However, homozygotes for the TPMT*3A haplotype have 1/400 of the normal levels of immunologically detectable enzyme protein whereas homozygotes for the 3C mutation have 1/40 of the normal levels of immunologically detectable enzyme protein. Since our patient is a double heterozygote for these two mutant TPMT alleles, about (0.05+0.0025)/2 = 0.026 times the level of enzyme protein would be expected, i.e. somewhat more than a homozygote for the 3A mutation. It is not therefore surprising that our patient was persistently pancytopaenic.

It is of interest therefore that the physician considered it appropriate to continue therapy, albeit at a much reduced dose.

Another explanation for her relative tolerance for 6-mercaptopurine may be as a result of multiple transfusions which she received while on therapy. The blood donors were very likely to have been homozygote for the wild type allele, and the patient would therefore be receiving red blood cells with TPMT activity similar to the wild type. In one report (15), a patient homozygous for the TPMT*3A alleles had erythrocyte TPMT activity found to be consistent with a heterozygous genotype (9.8 U/ml) after receiving 2 units of red cell transfusions. Four months following these transfusions, this patient was found to have virtually no TPMT activity. This is consistent with the transfused blood having supplied significant TPMT activity to the patient.

In another report, a 23 year old woman was on treatment with a standard dose of azathioprine (2mg/kg/day) for Crohn's disease, and subsequently developed severe

and life threatening pancytopaenia during the 5th week of therapy (23). This also necessitated immediate withdrawal of therapy, and the patient was immediately transfused with 4 units of packed red cells. Erythrocyte TPMT activity of the patient following these transfusions was reported to be compatible with those subjects with the wild type allele. It was subsequently discovered that the patient was homozygote for the TPMT*3A haplotype. Eight weeks after these transfusions, the constitutive TPMT activity in the red cell of this patient confirmed very low activity consistent with her genotype. Since genotype can predict phenotype in almost all cases, it is advisable to carry out genotypic screening prior to commencement of therapy to avoid erroneous misclassification.

It is not surprising that none of our patients were positive for the G238C (TPMT*2 haplotype) mutation because this mutation has been reported in the literature to be rare in the various population groups studied across the world. For example, one study reported that of the American Caucasians carrying a mutant TPMT allele, only 5% of these corresponded to the TPMT*2 haplotype (15). In another report, a relatively higher allele frequency of 9.4% in British Caucasians was reported for the TPMT*2 haplotype (5). No instances of the TPMT*2 haplotype were reported amongst all the Ghanaian, South West Asian and Chinese subjects in other studies, the authors concluding that the TPMT*2 haplotype is either very rare or specific to Caucasians (5).

CHAPTER 5

CONCLUSION

We have so far screened 250 subjects attending the out-patient clinics at the Groote Schuur Hospital. In addition, we screened 28 patients from the Red Cross Children's and Groote Schuur Hospitals currently on thiopurine analogues for a variety of clinical conditions. The majority are on treatment for acute lymphoblastic leukemia.

Our results showed an overall allele frequency of 8% for the TPMT (TPMT*3A and TPMT*3C) haplotypes among Caucasians in South Africa. This is slightly lower but similar to the approximately 10% allelic frequency reported among Caucasians in USA and Britain. Among the African (Black) population group in South Africa, the TPMT*3C allele frequency was 3.5% in the subjects investigated. Similarly, the allele frequency of the TPMT*3C haplotype in the coloured population group (mixed ancestry) was 3%. By contrast, the allele frequency of the TPMT*3C haplotype in the local Caucasians was 2% (i.e. present in 25% of positive cases). This value is considerably higher than allele frequencies for the TPMT*3C haplotype among Caucasians reported elsewhere. However, the higher allele frequencies in Africans and Coloureds are not statistically significant, so our finding may be a result of small sample size. The TPMT*2 haplotypte was not detected in any of the subjects or patients screened.

Out of the 28 patients presently on treatment with thiopurine drugs, only one patient, a

36 year old female with acute myeloid leukemia, was positive for TPMT mutation. She was found to be a compound heterozygote (TMPT*3A/*3C) and was reported to be intolerant to Purine analogues. She suffered from severe and protracted myelosuppression, and the dosage of the drugs was as a consequence reduced considerably.

Several workers have emphasised the advantages of pre-treatment genotypic screening for the TPMT polymorphisms. This can avert the danger of fatal but potentially preventable myelosuppression. Pre-treatment screening is quick, inexpensive and not affected by previous red blood cell transfusions. There is an economic merit to preventing this complication, as the cost of the treatment for any patient with this complication can be 100 times more expensive than pre-treatment screening. The allele frequency for the TPMT haplotypes (TPMT*3A and TPMT*3C) in the South African Caucasians investigated is 8%, and we therefore strongly recommend that a PCR based screening be carried out in all patients before the commencement of purine analogue therapy.

CHAPTER 6

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