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Identification of single nucleotide polymorphisms in inflammatory bowel disease patients on azathioprine therapy

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ABSTRACT

Azathioprine, an immunosuppressant used for many years in the medical sector to maintain long term disease remission in inflammatory bowel disease has a side effect profile that has raised many concerns over the years with numerous studies into the risk, cause and prevention of these adverse events. It is estimated that 18 in every 100 000 South Africans suffer from Crohn's disease, while 5 in every 100 000 suffer from ulcerative colitis. Of these inflammatory bowel disease patients at least 20% will suffer from leukopenia and eventually bone marrow suppression. Much of the side effect profile of Azathioprine can be linked to a single nucleotide polymorphism in the *thiopurine methyltransferase* gene which ensures the breakdown and efficacy of Azathioprine. The *thiopurine methyltransferase* single nucleotide polymorphism profiles of various American, Asian and European populations have been studied, but little literature is available for the South African population.

The aim of this study was to determine if it is essential to include "early warning" single nucleotide polymorphism testing in South African health care before treatment with Azathioprine is initiated. This was performed by evaluating 40 patients suffering from inflammatory bowel disease who met the inclusion/ exclusion criteria and who were on continuous Azathioprine therapy. The prevalence of *3A, *3B or *3C *thiopurine methyltransferase* gene SNP's were determined to assess the efficacy of Azathioprine in reducing therapeutic markers and to compare the frequency of SNP's within the Azathioprine dosing groups.

This study showed an increase in the allelic frequency of *thiopurine methyltransferase* *3B and a statistically significant presence ($p<0.001$) of homozygous *thiopurine methyltransferase* *3B alleles in South African inflammatory bowel disease patients when compared to healthy South African individuals- this has not previously been reported in published literature.

Thus, in future, the enzymatic effect of *thiopurine methyltransferase* *3B/*3B should be studied in a larger sample size prior to recommending early warning single nucleotide polymorphism testing in inflammatory bowel disease patient using Azathioprine as these results cannot be ignored.

DECLARATION

I, Lyla Adam, hereby declare that this research dissertation is my own work and has not been presented for any degree at another University.

Signed: 

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And to lastly, I am grateful to God, for carrying me through it all.

What a beautiful thing it is to be able to stand tall and say; I fell apart, but I survived.

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ABBREVIATIONS

6-me-MP	6-methyl-mercaptopurine
6-MP	6-mercaptopurine
TG	Thioguanine
6-TGN	6-thioguanine nucleotides
6-TITP	6-thio-inosine triphosphate
ADR	Adverse drug reaction
AE	Adverse event
AZA	Azathioprine
BMI	Body mass index
CD	Crohn's disease
CDAI	Crohn's disease activity index
CPIC	Clinical Pharmacogenetics Implementation Consortium
CRP	C-reactive protein
DD	Dose-dependent
DI	Dose-independent
DMARD	Disease modifying antirheumatic drug
DNA	Deoxyribonucleic acid
DRO	Delayed release oral formulation
ECCO	European Crohn's and Colitis Organisation
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
ESR	Erythrocyte sedimentation rate
FDA	Food and Drug Administration of the USA
HBF	Hydrophilic foam
HPF	Hydrophobic foam
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
IBD	Inflammatory bowel disease
IMP	Inosine monophosphate
ITP	Inosine triphosphate
ITPASE	Inosine triphosphatase
MPC	Mean platelet count

MTX	Methotrexate
NMSC	Non-melanoma skin cancer
NRH	Nodular regenerative hyperplasia
PCR	Polymerase chain reaction
PDS	Prednisone
RCT	Randomised clinical trials
RFLP-PCR	Restriction fragment length polymorphism- polymerase chain reaction
RVX	Revelex
SNP	Single nucleotide polymorphism
SZN	Salazopyrin
TAE	Tris-acetate- ethylenediaminetetraacetic acid buffer
TDM	Therapeutic drug monitoring
TNF	Tumour necrosis factor
TPMT	Thiopurine methyltransferase
UC	Ulcerative colitis
WBC	White blood cells
WHO	World Health Organization
XO	Xanthine oxidase

CHAPTER 1: INTRODUCTION & LITERATURE REVIEW

1.1 Chapter Objective

The objective of this chapter is to give an overview of Azathioprine (AZA), including the history, pharmacology, dosing guidelines and role in the field of gastroenterology.

1.2 Introduction

Azathioprine (AZA) is a well-known immunosuppressant used for many years in the medical sector for its ability to maintain long term disease remission in inflammatory bowel diseases (IBD) at an affordable cost to the public. However, the side effect profile has raised many concerns over the years with numerous studies into the risk, cause and prevention of these adverse events. Adverse events include pancreatitis, malaise, hepatitis, nausea, brittle teeth, abdominal pain and flu-like symptoms.

It is estimated that 18 in every 100 000 South Africans suffer from Crohn's disease (CD), while 5 in every 100 000 suffer from ulcerative colitis (UC).[2] Of these IBD patients at least 20% will suffer from leukopenia, a common side effect, and eventually myelosuppression.[3]

Much of the side effect profile of AZA can be linked to a single nucleotide polymorphism (SNP) in the thiopurine methyltransferase (*TPMT*) gene which ensures the breakdown and efficacy of AZA[4]. Mutated *TPMT* alleles result in low levels or deficient *TPMT* activity which directly correlates to cytotoxity of the drug. Knowing a patient's *TPMT* status allows the prescribing doctor to make an informed decision about dosage and be more alert to the signs of cytotoxicity.[5] The *TPMT* SNP profile of various American, Asian and European populations have been studied, but little literature is available for the South African population.[6-8]

In this study, patients suffering from IBD who were on continuous AZA therapy, were tested for the presence of the common *TPMT* SNP. Therapeutic markers of AZA therapy (CRP and ESR) were analysed and adverse reactions to the AZA therapy were recorded to determine the effect of the *TPMT* SNP on effective AZA therapy in the patients.

Azathioprine therapy is the gold standard for IBD treatment in tertiary health care facilities in South Africa. However, genetic testing in South Africa is costly and not common practice, despite the potential it has to decrease the incidence of hospitalisation, medication and treatment costs caused by AZA cytotoxicity. The aim of this study was to determine if it is essential to include "early warning" SNP testing in South African health care before treatment with AZA is initiated. This was performed by determining the prevalence of *3A, *3B or *3C *TPMT* gene SNP's, to determine the efficacy of AZA in reducing therapeutic markers and to compare the frequency of SNP's within the AZA dosing groups.

1.3 Literature Review

1.3.1 Azathioprine

Azathioprine is an immunosuppressant drug that was first produced in 1957 and is included in the World Health Organization's (WHO) list of essential medicines[1]. Azathioprine is a purine analogue which interrupts the synthesis of purine ribonucleotides guanine and adenine, causing mis-incorporation of bases and preventing deoxyribonucleic acid (DNA) repair mechanisms [9-11]. It has a most notable effect on fast dividing cells such as T- lymphocytes; at low doses AZA works as an anti-inflammatory while at high doses it has immunosuppressant and cytotoxic characteristics [12, 13]. The thiopurines drug class is commonly used to treat dermatological conditions, malignancies, rheumatic diseases, prevention of rejection after organ transplant and for the treatment of inflammatory gastrointestinal disorders such as Inflammatory Bowel Disease (IBD)[14].

1.3.2 Mechanism of Action

As shown in Figure 1, AZA is a prodrug which once administered, is converted to the inactive drug compound 6-mercaptopurine (6-MP) by sulfhydryl-containing compounds such as glutathione[15]. Thereafter 6-MP undergoes methylation via the key enzyme *TPMT* to form an inactive methylated metabolite of 6-mercaptopurine (6-Me-MP)[14]. In the absence of this methylation by *TPMT*, 6-MP is converted into 6-thioguanine (6-TG) by xanthine oxidase, where after hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) converts 6-TG into 6-thioguanine nucleotide metabolites (6-TGN). This 6-TGN is the active metabolite that determines cytotoxicity

or efficacy. The *TPMT* competes with xanthine oxidase and HGPRT to determine how much of the 6-MP is converted to 6-TGN[16, 17] . The enzyme activity of *TPMT* is reported to vary greatly between patients due to the presence of polymorphic variation in the *TPMT* gene[10] .

At normal levels of *TPMT* activity, 6-TGN inhibits intracellular signalling pathways and induces lymphocytic apoptosis. An increase in *TPMT* enzyme activity above normal results in decreased 6-TGN and hence a decrease in drug efficacy. The decrease or absence of *TPMT* activity (such as that seen in the *TPMT* polymorphisms) results in increased levels of 6-TGN which incorporates into the DNA and trigger cytotoxicity[17-19] .

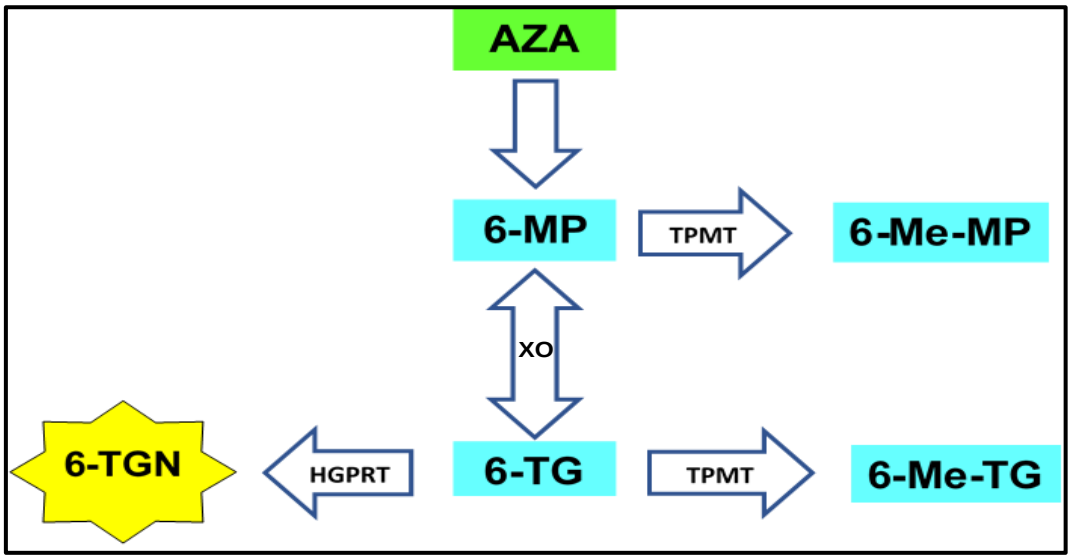

Figure 1: Proposed Metabolic Pathways of Azathioprine[1]

AZA (azathioprine), inactive **6-MP** (6-mercaptopurine), **HGPRT** (hypoxanthine guanine phosphoribosyl transferase), **XO** (xanthine oxidase), ***TPMT*** (thiopurine S-methyltransferase), **6-Me-MP** (6-methyl-mercaptopurine), **6-TG** (thioguanine), active **6-TGN** (6-thioguanine nucleotides) (active metabolite).

1.3.3 Pharmacology

Azathioprine can be administered intravenously or orally in both a delayed release oral (DRO) capsule, or tablet form. A study by Van Os *et al* (1996) compared the bioavailability of 50mg AZA when administered in the different forms such as a DRO tablet, oral capsule, rectal hydrophilic foam (HBF), rectal hydrophobic foam (HPF) and intravenously. The DRO bioavailability was 9.6%, oral bioavailability was 41.6%, HBF was 5.9% and HPF was 1.8%, assuming the intravenous bioavailability was 100%[20].

Oral tablets are considered to be a “localised” approach as it delivers a lower bioavailability and thus less risk of toxicity[21] .

Direct delivery to the large colon is ideal as it avoids first pass metabolism by the liver and directly targets the epithelial cells of the colon, however, direct delivery of rectal foam to the colon produced a lower bioavailability due to reduced absorption of the colon mucosa when compared to that of the gastric mucosa[22]. In a recent animal study by Helmy *et al* (2017) they proposed a solution by loading AZA into colon-targeted chitosan beads, and inserting the beads into an acid-resistant capsule[23].

The systemic elimination half-life of AZA is reported to be 26-80 min, or 3-5 hours if metabolites are included[24, 25]. The half-life of 6-TGN in erythrocytes is reported to be 5 days, and a minimum of 3 weeks may be needed to reach a steady state of 6-TGN concentration[26]. This may explain why a prolonged treatment period is needed before a clinical response occurs.

Therapeutic dosage concentrations range from 1-3 mg/kg bodyweight/day, depending on the severity of the disease and the side effect profile. On average it takes about 17 weeks for a therapeutic response in most patients, but it has been suggested that the response time can be reduced by administering an IV loading dose of 5 mg/kg body weight to achieve a greater cumulative concentration[10, 22, 27]. Van Os *et al* (1996) observed that the patient response time can also be decreased by administering a loading dose of AZA intravenously, thus providing a portion of the cumulative dose rapidly[20].

On average 47% of orally administered AZA reaches systemic circulation. Pharmacokinetic testing using IV AZA found that AZA absorption ranges between 16-50% and although more than 80% of AZA is converted to 6-MP, 6-MP only has a 16% bioavailability[15, 20, 21].

Although AZA has been proven to increase miscarriages by 20 fold in mice, conflicting studies and reports have been published with regards to the teratogenic nature of the drug in humans, with some studies[11, 28] reporting only low birth weight and premature birth, while other studies[29, 30] claim the drug is teratogenic. It is difficult to relate AZA directly to foetal abnormalities as majority of the females taking the medication are on combination therapy and are advised to stay on treatment during the pregnancy to prevent disease relapse. Many studies therefore conclude that birth abnormalities are due to underlying disease rather than the medication itself[21, 28]. Yet, AZA has been

listed as a class D teratogen according to the Food and Drug Administration (FDA), and is not recommended while breastfeeding[20] .

1.3.3.1 Adverse Events

Azathioprine has been associated with a variety of adverse events ranging from general nausea to myelosuppression, in very rare cases red-cell aplasia or death (in cases of TMPT polymorphisms)[31, 32]. Most adverse events can be separated into two reaction groups, namely dose-independent (DI) and dose-dependent (DD) reactions. Drug induced reactions tend to be hypersensitivity, allergy-like reactions which tend to occur within the first few weeks after initial dosing. Symptoms and signs such as pancreatitis, fever, joint pain, gastrointestinal disturbances and rash are common in DI reactions[4, 26, 33]. DD reactions tend to appear at later stages of therapy due to metabolite build up and often present as myelosuppression resulting in leukopenia, uncommon bacterial infections, cholestatic jaundice, hepatitis, and nausea. DD related adverse events will generally disappear if the dosage is decreased, while DI reactions will continue until therapy is discontinued[20, 21, 26]. Many studies have reported patients developing adverse effects long after therapy was initiated with two studies totalling 1135 patients reporting the onset of toxicity from immediately after first dose to developing toxicity after 11 years of therapy [26-28]. In a more recent study by Björnsson *et al* (2017), it was noted that nearly three-quarters of patients who had developed thiopurine-induced hepatotoxicity, also developed cholestatic hepatitis within 3 months of starting on the drug or after increasing the dosage[34] .

There are reported associations between thiopurine treatment for rheumatoid arthritis or renal transplant and the increased risk of developing a malignancy[35]. However, there are differing opinions regarding the association in IBD patients[36, 37]. Some studies claim no significant association between thiopurine therapy for IBD patients and the risk of malignancies[37], while other studies claim that there is a significant association with an increased risk of malignancies[38, 39], These malignancies include mostly urinary tract cancer in older men, non-melanoma skin cancer (NMSC) in younger patients and lymphoma in the general population[40, 41]. However, both study groups agree that there is limited data to establish the causality[9].

Kandiel *et al* (2005) argued that a 4-fold increase in lymphoma is observed in AZA treated IBD cases, but whether this is due to the underlying disease, or as a result of thiopurines or a combination of the two factors, remains unclear[9, 42, 43]. A study by

Lewis *et al* (2000) pointed out that despite the 4-fold increase in risk of lymphoma, AZA is still a vital component of immunomodulatory therapy in IBD management and that the increased risk would have to be greater than 9.8-fold for the benefit of alternative therapies to outweigh the benefit of AZA therapy[44, 45]. This statement has been reiterated recently in a study by Clowry *et al* (2017) on the relationship between thiopurine therapy and the risk of NMSC where, as previously concluded, due to the rising prevalence of IBD in young patients with little other alternative to immunosuppressive therapy, the benefit of this treatment outweighs the risk. It was additionally noted that combination therapy for long periods of time also increased the carcinogenic risk factors for……..[46].

Individuals using AZA are commonly on combination therapy with a corticosteroid; where the steroid is slowly phased out once disease remission status is achieved. A study by de Jong *et al* (2014) has found that patients who are on a combination therapy with a higher dosage of corticosteroid when starting the AZA tend to have fewer and less severe adverse events[47]. It is estimated that 20-30% of all patients taking AZA monotherapy will discontinue AZA use due to an adverse drug reaction (ADR) of some kind[17, 26].

1.3.4 Pharmacogenetics

The varying degrees of therapeutic response and potential toxicity can be attributed to genetic variations of genes which encode key enzymes for the metabolism of thiopurines. The key enzymes involved in the metabolism of AZA include *TPMT*, HGPRT, inosine triphosphatase (ITPase) and xanthine oxidase. Allelic polymorphisms in any of the genes coding for these enzymes results in the formation of variable concentrations of active metabolites, of which ITPase and *TPMT* metabolites are the most studied[48]. Abnormalities in other AZA metabolizing enzymes are extremely rare[16].

1.3.4.1 Inosine Triphosphate and Azathioprine

Inosine triphosphate (ITP), an intermediate in the purine metabolic pathway, is catalysed by ITPase to form inosine monophosphate (IMP). Deficiency of ITPase is due to an allelic variation on the ITP encoding gene on chromosome 20 and is characterised by irregular accumulation of ITP in erythrocytes, but it is a benign condition and under normal circumstances thought to be of no detriment to patient health[17]. However, in the presence of AZA or 6-MP, ITPase deficiency results in the

accumulation of 6-thio-inosine triphosphate (6-TITP). 6-TITP is described as a “rogue nucleotide” with the potential to cause cell toxicity [18]. Currently there are five known ITPase allele variations of which three are silent and two have been linked to a decrease in ITPase activity when present in a homozygous state. It has been estimated that 6% of the population is heterozygous for a decreased activity allele, and even less so for a homozygous allele variation[19]. Despite three studies [17, 18, 22] having reported an association between ITPase deficiency and thiopurine toxicity, no consistent toxicity pattern has been reported; thus a conclusion was made by Lennard (2002) that ITP genotype testing or observation does not indicate whether a patient is at risk for cell toxicity or not[4, 21]. This conclusion was supported by a more recent study by Citterio-Quentin *et al* (2017), in which it was shown that ITPase activity is rarely influenced by factors other than genetic parameters and has no influence on the concentration of AZA metabolites (6-TGN and 6-me-MP)[25].

1.3.4.2 Thiopurine methyltransferase and Azathioprine

Thiopurine methyltransferase is encoded by the *TPMT* gene which is located on chromosome 6[9]. This gene exhibits autosomal codominant genetic polymorphisms which can lead to an absent or low level of *TPMT* activity in individuals who are heterozygous or homozygous for this genetic polymorphism[24].

Updated recommendation for AZA dosing based on *TPMT* genotyping are annually published by the Clinical Pharmacogenetics Implementation Consortium (CPIC), an association created to meet the need for specific guidance of pharmacogenetic testing for both laboratories and clinicians. This dosing guideline is based on the phenotype and genotype of the individual[11].

Table 1: CPIC recommended AZA therapy, according to *TPMT* phenotype [26, 27]

Phenotype	***TPMT* Genotype**	**Example of Diplotypes**	**Recommendations: Therapeutic Dose**	**Recommendations: Steady State**
Homozygous wild-type -High enzyme activity	Two functional alleles	*1/*1	2-3 mg/kg/day	Allow 2 weeks to achieve steady state between each dose adjustment
Heterozygous -intermediate enzyme activity	One functional allele + One non-functional allele	*1/*2 *1/3A *1/3B *1/3C *1/4	Start at 30-70% of target dose, increase based on tolerance	Allow 2-4 weeks to achieve steady state between each dose adjustment
Homozygous variant -low/deficient enzyme activity	Two non-functioning alleles	*2/3A *3A/3A *3A/4 *3C/2 *3C/3A	Dose 3 x weekly or reduce daily dose by 10-fold. - Consider alternative treatment	Allow 4-6 weeks to achieve steady state between each dose adjustment

World-wide an average of 11% of Caucasian individuals have a *TPMT* polymorphism, of these 1/300 individuals will have a homozygous deficient variant allele and thus completely *TPMT* deficient [14]. Measuring the *TPMT* activity level is an effective method of determining an individual's risk of adverse effects and allows for dose adjustment or drug avoidance, to prevent the risk of the individual developing bone marrow suppression, leukopenia, thrombocytopenia or neutropenia[16, 28-32, 49]. *TPMT* polymorphisms have only been associated with overall thiopurine-induced adverse drug reactions and not with the less severe adverse events such as gastrointestinal upsets, skin reactions and pancreatitis; which are reactions often attributed to the underlying disease [33]. In 2003, the FDA recommended that *TPMT* status be determined prior to dosing [9] and made it compulsory to add this guideline to the AZA drug package insert

1.4 Gastrointestinal Therapy

1.4.1 Inflammatory Bowel Disease

One and a half million Americans suffer from IBD - an umbrella term used to represent a group of intestinal disorders affecting all or part of the digestive tract - which are comprised of 2 major diseases: ulcerative colitis (UC) and Crohn's disease (CD). IBD is estimated to affect 0.3% of the westernised world and has a rising prevalence in newly industrialised countries such as Africa, South America and the Middle East [50, 51]. It is estimated that 18 in every 100 000 South Africans suffer from CD, while 5 in every 100 000 suffer from UC [2]. While UC and CD are very similar in symptoms, they differ in anatomical location and treatment. UC only affects the inner lining of the large colon whereas CD can affect any component of the gastrointestinal tract from the oesophagus to the rectum, and may also affect the skin, eyes or liver.

Individuals suffering from IBD have a chronically inflamed gastrointestinal tract as the body perceives food or bacteria as a foreign substance and responds by activating T-lymphocytes, triggering inflammation. Leukopenia and eventual myelosuppression is a common haematological sign of AZA toxicity, and occurs in up to 20% of IBD patients due to the *TPMT* polymorphism [31, 52]. In 2012, 100,000 people were admitted to hospitals in the United States for various complications arising from IBD resulting in an annual hospitalization cost of over $3 billion [30].

1.4.2 Ulcerative colitis

Ulcerative colitis affects mainly the inner lining of the colon and may cause stunted growth in children suffering from the disease. Although thiopurine drugs are the gold-standard therapy for corticosteroid-dependent CD, there is still debate as to whether AZA is as effective in UC[53]. It has been argued that the only benefit AZA has in the treatment of UC is that it allows a large reduction or complete avoidance of corticosteroid use while maintaining the patient in remission[37, 49]. There have been very few randomised clinical trials (RCT's) related to use of AZA for UC treatment, therefore, although controversial, the use of thiopurine drugs to treat UC remains an accepted practice[36, 38].

1.4.3 Crohn's Disease

Crohn's disease is a chronic inflammatory disease affecting the lining of the abdominal cavity, resulting in relapse-remitting episodes of cramping of the gastrointestinal tract. Over time the disease will progress, and complications arise due to the formation of abscesses and fistulas. Pharmacological treatment includes the use of mesalamine, budesonide, salicylates and systemic corticosteroids[39, 40]. However corticosteroids are not appropriate for long-term or maintenance therapy due to the adverse events and dependence nature of the drug[40]. Immunosuppressive agents such as AZA are frequently prescribed for patients whose first line therapy has failed due to no response or corticosteroid-dependency[41].

1.5 Azathioprine and its role in Inflammatory Bowel Disease

A meta-analysis was conducted by Gisbert *et al* (2009), to review the efficacy of AZA and mercaptopurine in the induction and maintenance of disease remission in UC[36]. This included a selection of RCT's comparing AZA to placebo or aminosalicylates. A total of 1065 patients were included from 15 studies, taken from the year 2000 and onward. The summary of these studies can be found in Table 2. Of the 1065 patients, efficacy was observed in 697 patients (65.45%), i.e. they were found to be in disease remission at the end of the respective study period. The majority of these studies made use of AZA for induction and maintenance purposes.

Table 2: Azathioprine monotherapy randomised clinical trials[36]

Author	Year of Publication	Use of Azathioprine	Efficacy = n/N (%)
Bastida *et al*[43]	2007	Induction / Maintenance	21/25 (80%)
Campbell & Ghosh[45]	2001	Maintenance	82/94 (87%)
Christodoulou *et al*[54]	2003	Induction / Maintenance	15/15 (94%)
Cuffari *et al*[55]	2001	Maintenance	14/19 (74%)
Falasco *et al* [48]	2002	Induction / Maintenance	25/58 (43%)
Fraser *et al*[56]	2002	Induction / Maintenance	201/346 (58%)
Gisbert *et al*[3]	2008	Induction / Maintenance	65/156 (42%)
Hibi *et al*[57]	2003	Maintenance	15/17 (88%)
Khan *et al*[58]	2000	Induction / Maintenance	38/53 (72%)
Kull & Beau[59]	2002	Induction / Maintenance	23/30 (77%)
Lopez-Sanroman *et al*[60]	2004	Maintenance	24/34 &71%)
Mantzaris *et al*[61]	2001	Induction	24/40 (60%)
Mantzaris *et al*[62]	2004	Maintenance	28/34 (82%)
Paoluzi *et al*[63]	2002	Induction / Maintenance	22/32 (69%)
Sood *et al*[64]	2006	Induction / Maintenance	101/111 (91%)

A review was published by Cassieri *et al* (2017), documenting the number of IBD patients undergoing AZA treatment at the IBD Outpatient Clinic at Cristo Fe Hospital in Italy over a 5-year period. There were 260 IBD patients receiving AZA treatment lasting over the observation period, 145 for CD and 115 for UC. After 5 years, 86 CD patients (59.3%) and 49 UC patients (42.6%) were reported to be in remission[65] .

Azathioprine can be used in conjunction with corticosteroids, methotrexate or biological agents to induce disease-remission and maintain remission, by reducing the white cell count[66]. In recent years biological agents such as infliximab and other anti-tumour necrosis factor (anti-TNF) therapies have proven effective as a treatment and maintenance care for CD, but given the cost and adverse events associated with this therapy, it is usually reserved for when conventional therapies have failed[40, 67] In 2017 it was observed in a study by Colombel *et al*, that a combination therapy of infliximab and AZA resulted in a significantly higher remission rate in CD patients when compared to AZA monotherapy or infliximab monotherapy[40, 49]

This observation was also reported by Cholapranee *et al* (2017) who released a systematic review of RCT's conducted to compare the efficacy of AZA monotherapy, infliximab monotherapy and a combination of AZA and infliximab in the treatment of CD. This review mentioned 12 RCT's which made use of monotherapy or combination therapy to attain disease remission induction and maintenance as well as mucosal healing[39]. Two studies worth mentioning were conducted by Lemann *et al*[68] (2006) and Colombel *et al* (2010)[40]. Both studies were double-blind and made use of a placebo.

The study by Lemann *et al* (2006) took into consideration that results may be altered in patients having previously used AZA or being treated with AZA at inclusion or commencement of the study, hence the study made use of two groups within the dosing arms; a failure stratum (previously exposed to AZA) and a naïve stratum[68]. A sum of 55 patients were part of the failure stratum and 58 of the naïve stratum. Patients were then given a treatment of AZA and a placebo or AZA and infliximab, the Crohn's Disease Activity Index (CDAI) was used to evaluate patients at 12 and 24 weeks. A score of CDAI <150 was used as the marker for disease remission. Of the 113 patients partaking in the study, 57% of the combination group had disease remission at 24 weeks in comparison to the 29% experienced by the monotherapy group. No significant effect was seen when comparing the stratum to the treatment group[68].

Colombel *et al* (2010) made use of three separate dosing arms in the double-blind study, comparing infliximab and placebo versus AZA and placebo versus Infliximab and AZA. A total of 508 patients were included in a 26-week study, which is summarised in Table 3 and where the combination therapy had more efficacy in reaching disease remission compared to the other treatment arms (56.8% vs 44.4% vs 30.0%). This pattern was also seen in the mucosal healing within the arms[40]. This study made use of the CDAI scoring.

Table 3: Details of the double-blind randomised clinical trials by Lemann *et al*[68] and Colombel *et al*[40]

Author	Year of Publication	Drug	Combination drug	n=arm	Efficacy = n/N (%)
Lemann *et al*[68]	2006	Azathioprine	Infliximab	57	32/57 (57%)
		Azathioprine	Placebo	56	16/56 (29%)
Colombel *et al*[40]	2010	Azathioprine	Placebo	170	51/170 (30.%)
		Infliximab	Placebo	169	75/169 (44.4%)
		Infliximab	Azathioprine	169	96/169 (56.8%)

1.6 Thiosix and Therapeutic Drug Monitoring

In recent years, thioguanine has been rediscovered as a drug with great potential in the treatment of IBD. Thioguanine was originally approved for the treatment of paediatric leukaemia in 1950 due to its immunosuppressant properties, but due to claims that thioguanine is associated with nodular regenerative hyperplasia (NRH) and other liver toxicities, many physicians are hesitant to prescribe it[69].

In 2001, it was proven to be successfully used to treat IBD patients who had previously failed AZA therapy, and in 2015 the drug was re-registered by the European Medicines Agency (EMA) under the brand name of Thiosix[70]. Thiosix has become the gold standard used in IBD treatment in the Netherlands, with the number of IBD patients using AZA dwindling each year[70]. As thioguanine is directly converted to 6-TGN and allows the omission of the *TPMT* pathways, it avoids the risk of *TPMT* SNP mutation and enables direct manipulation of the levels of the active metabolite 6-TGN, which as previously mentioned is what causes efficacy or cytotoxicity in the AZA pathway. The use of thioguanine has seen patients presenting with much fewer adverse events; in 2017, a study confirmed that while NRH is considered a dose-dependent side effect of thioguanine, thioguanine is not associated with other liver toxicities [70-72].

The use of Thiosix requires therapeutic drug monitoring (TDM); which combines clinical chemistry and pharmacology to measure the concentration of a certain drug in the blood, with a main focus on drugs with a narrow therapeutic window [19]. Optimal levels of 230 – 400 pmol x 10^8 6-TGN erythrocytes in the erythrocytes may be associated with disease remission and drug response, while elevated levels of 6-TGN in the erythrocytes greater than 400 pmol x 10^8 erythrocytes may be associated with

bone marrow suppression. Levels greater than 5700 pmol x 10^8 erythrocytes may be associated with liver toxicity [73, 74].

Medilabs in the United Kingdom is one of only two labs to produce a test kit to detect the 6-TGN levels in IBD patients. These kits are used to monitor the progress in IBD patients using Thiosix, where blood is tested at 4 weeks post initial dosing as this is when steady state should be achieved [75]. The drug concentration levels are shown in Table 4 below. The test costs R2 293,73 (as at 12 January 2019) excluding shipment and is seen as critical in the TDM process

Table 4: Reference Range For 6-TGN Testing[74, 76] *TGN levels are tested in the erythrocytes*

6TGN	<235 pmol.6-TGN x10^8 erythrocytes	Sub-optimal dose / non-compliance
	235 – 450 pmol.6-TGN x10^8 erythrocytes	Maximum drug efficiency (associated with clinical remission of IBD)
	>450 pmol.6-TGN x10^8 erythrocytes	Linked to increased risk of myelotoxicity

To date, Thiosix has yet to be approved by the FDA and hence is not available to the South African population. However, should Thiosix be approved - and the TGN testing be provided by a local lab- this drug has the potential to overtake AZA for the maintenance treatment of IBD. Due to the fact that Thiosix avoids any SNP related ADR's, it can potentially provide a huge cost reduction in the procedures, medication and hospital visits to both the patient and the health care facility[70].

In summary; AZA is a prodrug immunosuppressant which is metabolised by three key enzymes, most notably *TPMT*, to form the active metabolite 6-TGN. The most significant adverse side effect of the thiopurine drug class is myelosuppression; which can be either dose-dependent or dose independent. Individuals suffering from a polymorphism of the *TPMT* allele may lack *TPMT* enzyme activity resulting in increased levels of 6-TGN, which result in dose-independent cytotoxicity and myelosuppression. AZA is commonly used to treat IBD, with particular success as an inducer of disease activity remission (used concomitantly with corticosteroids) as well as for maintenance therapy after remission is achieved.

CHAPTER 2: PATHOPHYSIOLOGY AND TREATMENT OPTIONS IN IBD AND PATIENT PHARMACOLOGICAL TREATMENT

2.1 Patient Information

2.1.1 IBD Aetiology

In healthy patients, the mucosal immune system within the digestive tract induces antigen-specific non-responsiveness when micro-organisms and antigens are administered through the diet. This lack of immune responsiveness is a learned mechanism within the intestinal mucosa termed oral tolerance where the immune cells recognise and adapt to frequently consumed micro-organisms [77, 78]. There are multiple mechanisms involved in the suppression of antigen reactive T cells that in turn suppress inflammation in the gut.

However, patients suffering from IBD lack oral tolerance and instead suffer from a hyper responsive immune system [79]. This occurs when the parenchymal and hematopoietic cells within the intestinal mucosa react to micro-organisms, whether symbiotic or pathogenic, which results in excessive secretion of inflammatory cytokines and an unsuppressed inflammatory cascade [77]. This inflammatory cascade is a natural response to infection, however, IBD patients lack the natural ability to inhibit this response prior to tissue damage due to the dysregulation of proinflammatory and anti-inflammatory mediators [78].

A number of research based observations[80, 81] have been made regarding the genetic factors that may contribute to IBD susceptibility and while UC can result from a variety of genetic alterations in both the immune system and epithelial function, more than 85% of CD patients have no familial history of IBD[81].

Clinical studies involving monozygotic twins with IBD found that the concordance rate (chances of the same trait being present in a pair of monozygotic twins) was a lot higher in CD individuals than in UC (50% vs 19%)[82].

One study found that children with parents who both had IBD (UC or CD) had a 33% chance of developing IBD by the age of 28[83]. Additionally, having a sibling with CD,

increases the risk of developing CD by a factor of 30 when compared to the general population- and approximately two thirds of these sibling pairs will be diagnosed with 10 years of each other[84]. Hence having a family history of first-degree family members with IBD still presents that highest risk for developing IBD[80].

2.1.2 Diagnosis and Classification of IBD

While raised inflammatory markers such as ESR and CRP, blood in stool and abdominal pain might be indicative of IBD, according to the European Crohn's and Colitis Organisation (ECCO) 2018 guidelines, the gold standard for an accurate diagnosis is widely considered to be examination through colonoscopy or gastroscopy under anaesthesia. This would include all forms of colonoscopies and/or gastroscopies as well as any relevant biopsies and imaging needed[85]. Further blood test such as ferritin, serology, electrolytes and white blood cells test or stool tests such as calprotectin are also required to make a positive diagnosis[86, 87]. In up to 15% of cases patients may present with indeterminate colitis where patients present with CD symptoms but manifested in regions common to UC or with UC symptoms but in regions common to CD[78].

Clinical History

Patients may have previously presented with gastrointestinal symptoms; however, IBD may also manifest as extra intestinal symptoms and signs, including arthritis, skin diseases, osteoporosis, inflammatory ocular diseases, skin lesions or fractures. Often a family history of intestinal disorders like IBD, colorectal cancer or celiac disease may be present. Inflammatory Bowel Disease has the potential to be classified as a familial disease, where first-degree relatives of an IBD patient have a 5- 10% chance of also being diagnosed. In cases where both parents suffer from IBD, each child has a 36% chance of also developing IBD [78]. Patients who are currently or have previously smoked are at a greater risk of developing CD than patients who don't – although many patients also report that smoking does minimise the pain, but whether this is a physiological or placebo effect has not been studied [88, 89].

Symptoms

As IBD is a chronic disease with frequent acute flares, symptoms can range from mild to severe at any time but depend greatly on the disease activity and the section of the intestinal tract affected.

Symptoms of damage to the digestive tract include: blood in stools, incontinence,

diarrhoea, constipation, rectal bleeding, fatigue, weight loss, growth retardation in children, tenesmus especially at night, abdominal pain and cramping in the lower right quadrant of the abdomen, or around the umbilicus in the case of UC and in the lower left quadrant in CD [78, 89, 90].

2.1.2.1 Classification of Ulcerative Colitis

Ulcerative Colitis is classified by examining both the region of disease activity in the colon as well as the extent of the disease. Classification is commonly determined by performing a sigmoidoscopy; a colonoscopy performed to examine both the rectum and the closest part of the colon – the sigmoid colon. On average 40-50% of UC patients present with proctitis- inflammation of the rectum and anus, 30-40% have inflammation of the sigmoid colon, rectum and a section of the distal colon, while 20% present with pancolitis- inflammation of the entire large intestine including the rectum[78].

Disease activity is ranked as mild, moderate or severe- while a flare in disease activity may cause a rapid change from mild to severe disease activity. Mild UC is characterised by slight friability, mucosa with a granular surface resembling sandpaper, erythema, decreased visibility of the vascular pattern, with little to no blood in the stool[78]. Moderate disease is characterised by slight friability, defined erythema, no visibility of the vascular pattern and bleeding of the colon lining on contact. Severe disease activity is characterised by spontaneous bleeding of the colon lining as well as visible ulcerations in the colon. Disease classifications as described in Harrison's Principles of Internal Medicine[78] as shown in Table 5 below.

Table 5: Classification of Ulcerative Colitis Through Disease Activity[78]

	Mild	**Moderate**	**Severe**
Bowel movements	<4 per day	4–6 per day	>6 per day
Blood in stool	Small	Moderate	Severe
Fever	None	<37.5°C mean	>37.5°C mean
Tachycardia	None	<90 mean pulse	>90 mean pulse
ESR	<30 mm/hr		>30 mm/hr
Endoscopic appearance	Erythema, decreased	Marked erythema, coarse granularity, absent	Spontaneous bleeding,

	vascular pattern, fine granularity	vascular markings, contact bleeding, no ulcerations	ulcerations

2.1.2.2 Classification of Crohn's Disease

Compared to UC, CD is classified according to the disease activity and the region affected by the disease. However, in contrast to UC, CD manifests itself in one of two disease patterns and treatment will vary accordingly. Crohn's manifests in either a stricturing pattern, where fibrosis results in obstruction or restriction of an intestinal region, or in a penetration pattern, where fistulas penetrate deep into the bowel wall – a third of all CD suffers will develop fistula's[78, 89, 91]. Either of these patterns can be seen throughout the gastrointestinal tract and may occur individually or in overlapping segments.

Jejunoileitis

Inflammation of the jejunum is associated with a loss of absorption and digestion surface area resulting in malabsorption, nutritional deficiencies and secondary mineral deficiencies throughout the body. Hence, patients suffering from Crohn's jejunoileitis require oral and intravenous mineral and vitamin supplementation- most commonly vitamin B, C, D and iron. Diarrhoea is considered a sign of active disease and may be due to formation of fistulae, malabsorption of bile acid or inflammation of the intestine[78].

Ileocolitis

Inflammation is commonly seen in the terminal ileum and is often mistaken for appendicitis as the initial symptoms are similar and pain is pronounced in the lower right abdominal quadrant with fever and a palpable mass. Weight loss is common in Crohn's ileocolitis due to diarrhoea and abdominal pain after eating. Fibrosis of the bowel wall may cause postprandial pain in the early stages of the disease due to oedema and spasms. Later stages of the disease are concurrent with fibro-stenotic narrowing and restriction of the bowel wall resulting in chronic bowel obstructions which may require bowel resection. Severe inflammation of the ileum may result in wall thinning within localised region, causing perforation of the bowel wall and the formation of fistula's connecting to neighbouring skin, bowel or tissue[78].

Colitis

Inflammation of the colon is characterised by mild fever, diarrhoea, abdominal cramping, and 50% of patients with exclusively CD will experience bloody stools; however, only 2% will bleed excessively [78]. Movement of faecal matter through constricted and inflamed segments of the large bowel result in abdominal pain and symptoms of bowel obstruction. Patients suffering from Crohn's colitis may require a surgical resection should the symptoms of a chronic obstruction persist. Colitis may result in fistulas forming into the duodenum or stomach causing vomiting, or, the jejunum or mid-small bowel causing the intestinal tract to bypass the rest of the intestinal tract during digestion resulting in poor digestive absorption[78].

2.1.3 Medication Used to Treat Inflammatory Bowel Disease

As there is no complete cure for IBD, medication is used to treat the symptoms and to suppress the disease activity. Patients suffering from IBD will use a range of treatments or therapies before finding one that will ensure disease remission and pain relief. Typically, a corticosteroid will be used prior to and during use of AZA for a period, at the discretion of the treating physician, when the patient can successfully be weaned off the corticosteroid treatment. This trend of treatment pathways is depicted in Harrison's Principles of Internal Medicine as seen in Figure 2.

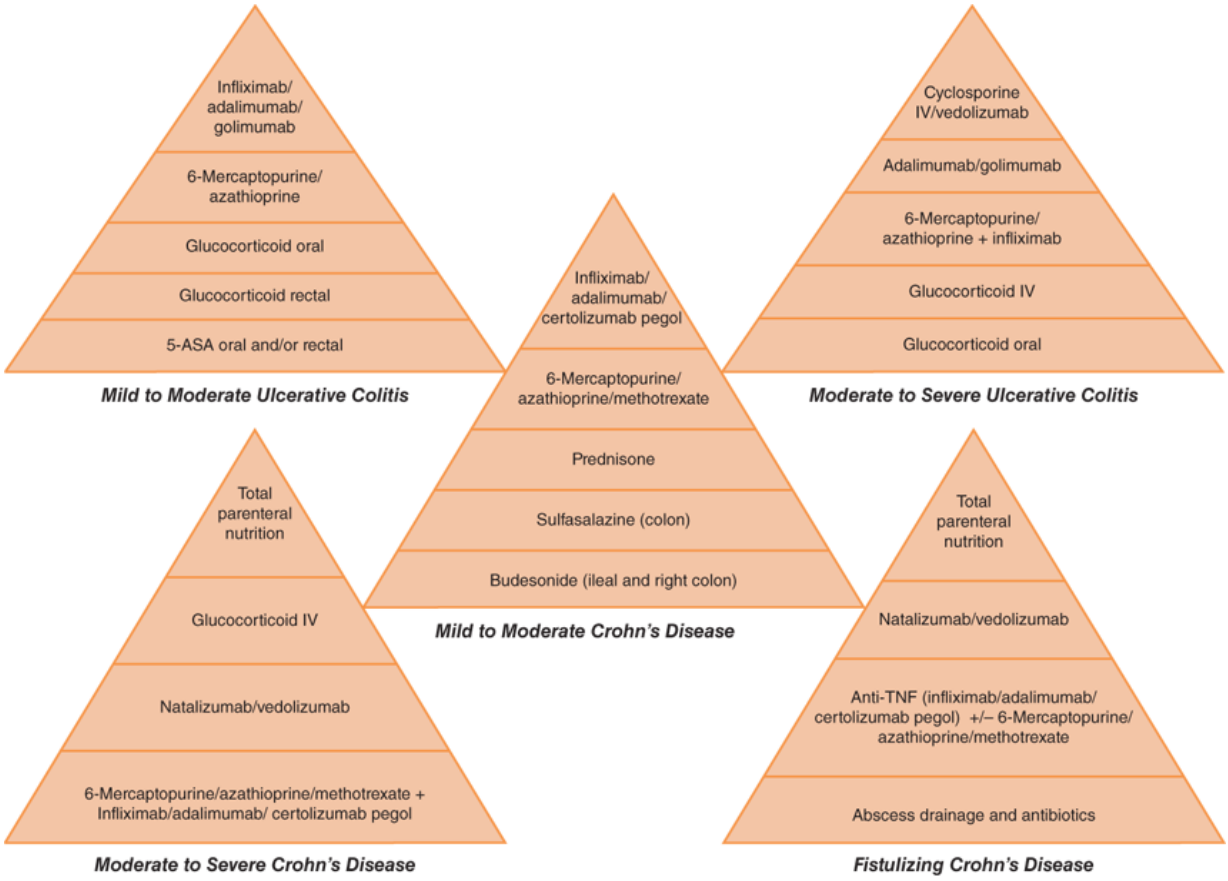

Figure 2: Treatment Pathways for UC and CD according to Harrison's Principles of Internal Medicine[78] **Natalizumab /vedolizumab is not available in South Africa*

A list of pharmacological treatments commonly used in combination or as a monotherapy in South Africa for IBD is summarised in Table 6 below.

Table 6: Other pharmacological therapies common to IBD- used as monotherapy or in combination with AZA[89, 92]

Active Ingredient	Brand Name	Drug Class	Route of Administration
Adalimumab	Humira	αTNF-blocker	Injection
Budesonide	Entocord	Corticosteroid	Tablet
Infliximab	Revelex (RVX)	αTNF-blocker	Infusion / Intravenous
Mesalazine	Pentasa, Asacol	5-aminosalicylate	Tablet, enema, suppositories

Methotrexate (MTX)	Methotrexate	Antimetabolite/ immunosuppressive	Tablet, injection
Prednisone (PDS)	Medrol	Corticosteroid	Tablet
Sulphasalazine	Salazopyrin (SZN)	Amino salicylate	Tablet

2.1.3.1 Adalimumab

Adalimumab is commonly sold under the brand name of Humira, an injectable monoclonal antibody that prevents the immune system's inflammatory response to alpha tumour necrosis factors (α-TNF)[92]. Adalimumab is used to treat moderate to severe active CD and UC either in combination with methotrexate or when methotrexate and other conventional therapies have failed. Patients will commonly inject themselves every second week[93].

2.1.3.2 Budesonide

Budesonide is a corticosteroid hormone, which reduces the immune system's response. Budesonide is available in delayed release capsules, extended release tablets for the treatment of IBD[92]. Budesonide is best used to treat UC where the rectum and sigmoid colon is affected and in the induction of disease remission in mild to moderate CD where the ileum or ascending colon is affected[94].

2.1.3.3 Infliximab

Infliximab is monoclonal antibody drug used to block the effects of the α-TNF. It used to treat autoimmune diseases such as arthritis, psoriasis and IBD[92]. Infliximab is effective in treating moderate to severe active CD and UC where response to other therapy is lacking. Revellex (RVX) is commonly administered via infusion over the course of 4hours and is repeated every 8 weeks[95].

2.1.3.4 Mesalazine

Is a small molecule drug based on salicylic acid used to control inflammation, most notable in the treatment of mild-moderate UC as well as in the maintenance of disease-remission in UC[92].

2.1.3.5 Methotrexate (MTX)

Is an antimetabolite with immunosuppressant properties, it inhibits the formation of active folate which then interrupts the synthesis of DNA. Commonly used in the treatment of rheumatoid arthritis and cancer as it blocks cells that reproduce rapidly[96].

2.1.3.6 Prednisone (PDS)

Prednisone, prednisolone and methylprednisolone are all glucocorticosteroids that suppress the immune system, they are used to treat autoimmune reactions such as allergies, lupus, arthritis and UC. Corticosteroids should not be used chronically although in some cases this is unavoidable; in these cases, secondary-osteoporosis tends to be a common side effect[97].

2.1.3.7 Sulphasalazine

Is an amino salicylate used to control pain and inflammation in cases of acute UC and CD and in the maintenance of disease remission in UC and CD[92]. Care should be taken while using Salazopyrin (SZN) as folic acid and iron deficiency are a common adverse events [98].

For each patient on this study, the medications used to treat IBD were noted as obtained from each patient's medical file – these are listed in Table 19 in the Appendix, these were all used in combination therapy alongside AZA. A total 25 of the 40 patients used mesalazine oral as an anti-inflammatory and pain therapy, while prednisone was the most commonly prescribed corticosteroid. The patients who showed severe adverse effects or had no efficacy to AZA were switched to adalimumab or infliximab as alternative treatments.

2.1.4 Supplements Commonly Used During IBD Treatment

2.1.4.1 Calcium and Vitamin D

Chronic use of high dose prednisone results in depletion in calcium and vitamin D, resulting in a decrease in bone density and eventually osteoporosis caused by the corticosteroid resulting in an increase in induced bone reabsorption[92, 94, 97] .

2.1.4.2 Folic Acid and Iron Supplementation

Many IBD patients develop anaemia due to chronic blood loss or folate deficiency caused by treatments such as Humira and Methotrexate as these medication suppress

T-lymphocytes [93, 99, 100].Folic acid plays a role in the control of homocysteine, an amino acid that in excess is hypothesised to result in blood clots and hearts attacks[101-103]. Hence folic acid and iron supplementation is often required- many patients opt for biannual iron transfusions which is effective in regulating their iron levels.

The list of vitamin and mineral supplements consumed or administered by patients on the study are found in Table 20 in the Appendix. Folic acid is the most commonly noted supplement and was mostly seen in patients also receiving prednisone. It should be noted that these patients were only prescribed vitamin or mineral supplements when their whole blood iron levels were clinically reported as low, however many patients did take self-prescribe supplements as a precautionary measure and in this case, it would not have been noted in the patient file.

CHAPTER 3: STUDY DESIGN

3.1 Inclusion & Exclusion

Inclusion criteria

A participant will only be eligible for participation in the study if all the following inclusion criteria are met:

1. Written Informed Consent for study participation prior to the start of any study related procedures.
2. Males or females > 18years
3. Diagnosed with inflammatory bowel disease
4. Be on continuous AZA treatment for a minimum of 12 months post initial dose

Exclusion criteria

A participant will not be eligible for participation in the study if any of the following exclusion criteria are met:

1. Diagnosed with autoimmune hepatitis, rheumatoid arthritis or acute lymphoblastic leukaemia
2. Presence of myelosuppression at baseline
3. Intermittent use of AZA at any point during the first 12 months post initial dosing
4. Individuals who are pregnant at any stage during the first 12 months post initial dosing
5. Unwilling to consent
6. Any other medical condition which in the opinion of the investigator could compromise the participant's ability to participate in the study

3.2 Objectives

1. To determine the presence or absence of *TPMT* allele mutations; *3A, *3B or*3C in IBD patients receiving AZA therapy, using Restriction Fragment Length Polymorphism- Polymerase Chain Reaction (RFLP-PCR).
2. To determine the efficacy of AZA in reducing the CRP and ESR count as well as to observe the change in leucocyte (WBC) count from baseline to at least month 12, in patients being treated for IBD at Steve Biko Academic Hospital and a private hospital
3. To compare the frequency of *TPMT* SNP's within dose groups

3.3 Statistics

A power calculation analysis was performed by a statistician using STATA 13 to determine an optimum sample size. Statistical significance was determined by a p-value of <0.05. As IBD is a rare disease effecting a small number of patients it was concluded that expected sample numbers should be between 30-40 patients. A copy of the sample size statistics can be found in the Appendix

3.4 Ethical Approval

Ethical approval was granted by the University of Pretoria Ethics Committee (approval number 357/2017), as well as the director of Steve Biko Academic Hospital for the access to their patient population. Ethical approval was also obtained from the research committee of the private hospital for access to their patient population. A copy of the ethics approval letters can be found in the Appendix.

3.5 Patient Procedure

The collaborating clinicians were Dr Mpho Kgomo and Dr Samuel Mashoeshoe at the Department of Gastroenterology at Steve Biko Academic Hospital in Arcadia, Pretoria, as well as with Dr Robert Bond, a physician gastroenterologist at a private hospital in Pretoria.

Dr Mashoeshoe, interacted with the patient, offering them information about the study and then asking if they would like to volunteer. Potential patients of Dr Bond's practice were contacted by his staff and patient permission obtained prior to patient contact details being made available to the investigator.

Informed consent was obtained from the patient prior to any study procedures or obtaining of patient information. The Patient Information Leaflet for Dr Bond as well as the Informed Consent can be found in the Appendix.

A total of 40 IBD patients, both male and female were enrolled. Each participant was assigned a randomisation number starting at 001, and the participant was only referred to by this number to ensure patient confidentiality. The investigator or available nurse drew 4.5ml of blood from each patient in a blue top citrate tube. CRP, ESR, leucocyte

counts, biopsy findings and concomitant medication for the first year post initial AZA dosing was collected from the patient file. All patient results and information were treated as confidential by using screening numbers.

3.6 Control Patients

A total of 40 healthy subjects were enrolled as control subjects, each subject received a control randomisation number to ensure participant confidentiality. In this context healthy implied a patient that did not suffer from IBD, was not undergoing AZA treatment and did not have any chronic illness.

CHAPTER 4: SINGLE NUCLEOTIDE POLYMORPHISMS

4.1 Abstract

4.1.1 Objective

To determine the presence or absence of *TPMT* allele mutations *3A, 3B* or*3C in IBD patients receiving AZA therapy, using Restriction Fragment Length Polymorphism- Polymerase Chain Reaction (RFLP-PCR).

4.1.2 Materials & Methods

Patients suffering from IBD who had previously or were currently using AZA for more than 12 consecutive months were recruited from both the Steve Biko Academic Hospital Gastroenterology Unit, and a private hospital in Pretoria. Once patient informed consent was obtained, a blood sample was drawn from each patient. Samples were tested for the presence of *TPMT* *3A, *3B and *3C SNP's using RFLP-PCR. Patient demographics and drug reaction data was collected from the patient file.

4.1.3 Results

Forty sets of data from individuals suffering from IBD and 40 sets of control data were collected. *TPMT* 1/*3A was detected in 8/40 (20%) controls and 6/40 (15%) IBD individuals, *TPMT* 1/*3B was detected in 20/40 (50%) controls and 1/40 (2.5%) IBD individuals. *TPMT* *3B/*3B was detected in 6/40 (15%) controls and 33/40 (82.5%) IBD individuals, with a significance of $p<0.001$.

4.1.4 Conclusion

This study showed a higher allelic frequency of the *TPMT* *3B SNP in the South African population in comparison to other global populations. A novel, significant ($p<0.001$) finding of homozygous *TPMT* *3B was observed, which has previously not been reported in published literature.

4.2 Introduction

Thiopurine Methyl Transferase (*TPMT*) is responsible for the methylation of thiopurine prodrugs, such as AZA, 6-mercaptopurine (6-MP) and thiopurine, which plays a critical role in the activity of the thiopurine drug metabolites. Metabolism of these prodrugs by *TPMT* produces both active and inactive metabolites which can be incorporated into deoxyribonucleic acid (DNA) causing replication inhibition leading to apoptosis, can prevent purine synthesis or can interfere with intracellular signalling pathways. These drug characteristics lead to the thiopurine drug class being utilized as antineoplastic and immunosuppressant agents[104].

A large variation in the enzyme activity of TMPT was first researched in 1980 by Weinshilboum and Sladek, who postulated that *TPMT* is inherited in an autosomal codominant manner with *TPMT* enzyme activity classified as either normal, intermediate or undetectable [105]. In later years the variation in enzyme activity was attributed to the presence of polymorphisms which[106], is defined as "the presence of genetic variation within a population, upon which natural selection can operate" within the exon coding sequences of the *TPMT* gene [104, 106]. Some polymorphisms are referred to as single nucleotide polymorphisms (SNP's), which are the result of a single base-pair change at a specific site within a gene found in the human DNA sequence and can result in a range of allele variations in the genome. Allele's are defined as "each of two or more alternative forms of a gene that arise by mutation and are found at the same place on a chromosome"[107]. SNP's differ from other genetic mutations in that they will only be defined as a SNP when the specific allele change is observed in more than 1% of the population [108].

In 2011, the Clinical Pharmacogenetics Implementation Consortium (CPIC) released a guideline to assist in the interpretation of *TPMT* genotyping results. To date there are 41 known *TPMT* SNP's, which have been scientifically named according to recognised nomenclature[104]. The genetic coding of these SNP's is also listed on the NCBI database[5, 109]. Twenty of the 41 variant alleles are associated with a decrease in enzymatic activity *in vitro* [5, 110], however only a few of these alleles have a known clinical effect, with studies observing that allele phenotype may be specific to race or ethnicity [109, 111]. This observation was highlighted in a paper by Appell *et al*, which stated that interethnic differences play a large role in the frequencies of certain alleles an may explain the low frequency of certain *TPMT* alleles. A study by Oliviera *et al*

found that the *TPMT** 8 polymorphism occurred in 1.5% of the Mozambican study population but was found to very rarely occur in other investigated populations [104, 112].

Globally, the most common functional allele is *TPMT** 1, while *TPMT** 2, *3A, *3B, *3C and *4 are well documented polymorphic alleles associated with heterozygous or homozygous variant genotypes as listed in Table 1. *TPMT** 2, *3A and *3C account for over 90% of the inactive alleles [113] in the populations that have been studied and reported to date.

TPMT *3A is a double mutant comprised of both *TPMT* *3B and *3C mutations. When the *TPMT* *3C and *3B mutations are found in cis (i.e. they are found on the same strand of DNA as the regulated gene[114]) the mutation is defined as *TPMT* *1/*3A, however when the *TPMT* *3A and *3B mutations are found in trans positioning (i.e. the gene regulation occurs a distance from where the gene transcription occurs[114]) the mutation is described as *TPMT* *3B/*3C. Occurrence of the trans mutation is rare and results in complete *TPMT* enzyme activity deficiency. The differentiation between cis and trans linkage can only be determined via haplotyping[115, 116].

Individuals can be homozygous normal, signifying that both inherited alleles are wild-type (normal metabolizing), or they can be homozygous variant signifying that both inherited alleles have the same SNP's and hence there is little to no *TPMT* activity. Lastly an individual can have a combination of different SNP's, or heterozygous, implying that they inherited two alleles of different functionality with the possibility of both a functional and a nonfunction allele resulting in a moderate enzyme activity level.

Table 7: Nomenclature of common *TPMT* SNPs [104, 117]. *The extent of the TPMT enzymatic effect of the SNP's is not fully known for most TPMT SNP's*

Common Allele Name	Nucleotide Changes in The *TPMT* gene	Protein Change
***TPMT** 1**	Wild type	
	474T	
***TPMT** 1A**	178C>T	
***TPMT** 1S**	474T>C	Ile158Ile
***TPMT** 2**	238G>C	Ala80Pro
***TPMT** 3A**	460G>A	Ala154Thr
	719A>G	Tyr240Cys
***TPMT** 3B**	460G>A	Ala154Thr
***TPMT** 3C**	719A>G	Tyr240Cys
***TPMT** 3D**	292G>T	Glu98Stop
	460G>A	Ala154Thr
	719A>G	Tyr240Cys
***TPMT** 3E**	140+114T>A	
	141-101A>T	
	366+58T>C	
	460G>A	Ala154Thr
	474T>C	Ile158Ile
	719A>G	Tyr240Cys
***TPMT** 4**	626-1G>A	
***TPMT** 5**	146T>C	Leu49Ser
***TPMT** 6**	539A>T	Tyr180Phe
***TPMT** 7**	681T>G	His227Gln
***TPMT** 8**	644G>A	Arg215His
***TPMT** 9**	356A>C	Lys119Thr
***TPMT** 10**	430G>C	Gly144Arg
***TPMT** 11**	395G>A	Cys132Tyr
***TPMT** 12**	374C>T	Ser125Leu
***TPMT** 13**	83A>T	Glu28Val
***TPMT** 14**	1A>G	Met1Val
***TPMT** 15**	495-1G>A	

***TPMT** 16**	488G>A	Arg163His
***TPMT** 17**	124C>G	Gln42Glu
***TPMT** 18**	211G>A	Gly71Arg
***TPMT** 19**	365A>C	Lys122Thr
***TPMT** 20**	712A>G	Lys238Glu
***TPMT** 21**	205C>G	Leu69Val
***TPMT** 22**	488G>C	Arg163Pro
***TPMT** 23**	500C>G	Ala167Gly
***TPMT** 24**	537G>T	Gln179His
***TPMT** 25**	634T>C	Cys212Arg
***TPMT** 26**	622T>C	Phe208Leu
***TPMT** 27**	319T>G	Tyr107Asp
***TPMT** 28**	349G>C2	Gly117Arg
***TPMT** 29**	2T>C	Met1Thr
***TPMT** 30**	106G>A	Gly36Ser
***TPMT** 31**	611T>C	Ile204Thr
***TPMT** 32**	340G>A	Glu114Lys
***TPMT** 33**	487C>T	Arg163Cys
***TPMT** 34**	244C>T	Arg82Trp
***TPMT** 35**	200T>C3	Phe67Ser
***TPMT** 36**	595G>A3	Val199Ile
***TPMT** 37**	648T>A	Cys216Ter
***TPMT** 38**	514T>C	Ser172Pro
***TPMT** 39**	218C>T	Ala73Val
***TPMT** 40**	677G>A	Arg226Q
***TPMT** 41**	719A>C	Tyr240Ser

There have been numerous studies on the *TPMT* genetic differences in Caucasian and Asian individuals but less so in the African population. A study by Fong *et al* (2017) found the *3A allele to be most common in the Caucasian and Indian population and the *3C allele to be most common in the Asian population [9]. Studies in Nigeria and Libya have shown majority of the African population to have the *3C allele, a finding supported in a study on *TPMT* activity in African-Americans by Hon *et al* (1999) [24, 118, 119].

TMPT status can be determined via phenotypic enzyme-level testing using red blood cells, or by creating a genetic profile using DNA extracted from white blood cells [1, 28]. Determining the *TPMT* genotype of the individual can enable the clinician to better understand and predict potential ADR's [120]. Monitoring of known SNP's indicating *TPMT* gene variation has is one of the best examples of incorporation of pharmacogenomics into routine clinical practice in many countries [104].

4.3 Materials and Methods

Upon collection of whole blood samples in citrate tubes, blood was aliquoted into microcentrifuge tubes with 1 ml per aliquot. Samples were then frozen at -80℃ until processing. Patient demographics and data pertaining to drug reactions were obtained from the patient files. In this study, ADR's were only reported as present if they were severe enough for patient to be withdrawn from AZA therapy.

DNA Extraction was conducted using the Reliaprep Blood gDNA Miniprep System (Promega, USA), which included all reagents required for the extraction. Blood samples were completely thawed at ambient temperature prior to processing, once thawed, samples were mixed using a shaker for 10 minutes. To prepare one sample; 20 µl Proteinase K was added to a microcentrifuge tube and 200 µl of the thawed blood added and mixed well. A volume of 200 µl of Cell Lysis Buffer was added to the solution and vortex mixed for 10 seconds. The solution was then incubated in a water bath at 56℃ for 10 minutes, where after 250 µl binding buffer was added and the solution was again vortex mixed for 10 seconds. Exactly 670 µl of the solution was added to a Reliaprep Binding Column placed in an empty collection tube. The binding column and collection tube were placed in a centrifuge for 1 minute at 2000 *x g* where after the collection tube was removed, pass through fraction disposed of and the binding column placed in a clean binding column. A volume of 500 µl of column wash solution was added to the binding column and the column was centrifuged for 5 minutes 2000 *x g* ensuring that the lysate had completely passed through the membrane. The wash process was repeated two more times. The binding column was then placed in a microcentrifuge tube and 70 µl of nuclease-free water was added to the binding column before centrifugation for 1 minute at 2000 *x g*. The remaining DNA was stored at -20℃.

DNA was quantified using a Nanodrop Spectrophotometer (Thermo Fisher, UK) to quantify isolated DNA in the eluent which was standardised to 50 ng/µl. Nuclease-Free Water (Promega, USA), was used as the blank during quantification.

*TPMT** 3B and *3C primer concentrations were optimised by preparing DNA samples with the respective *TPMT** 3B or *3C primer sequence, amplifying through a polymerase chain reaction (PCR) and viewing the products amplicons following agarose gel electrophoresis. The primer PCR products were tested at concentrations of 0.1 µM, 0.2 µM, 0.4 µM, 0.6 µM, 0.8 µM and 1.0 µM. It was determined that optimal concentrations for both the *3B and *3C primers were 0.4 µM. The primer sequences used to detect *TPMT* *3B and *3C are listed in Table 8 below, these were obtained from an article by Tantawy *et al*[5] and synthesised by Inqaba Biotec, the synthesis report can be found in the Appendix.

There is currently a primer available that specifically tests for *TPMT* *3A mutation, however many studies[5, 8, 116, 121] have made use of only the primers testing for *TPMT* *3B and *3C because it is more cost effective and provides more allelic data than just a *TPMT* *3A specific primer would. Hence, for the purpose of this study no *TPMT* *3A specific primer was used, instead the presence of both the *TPMT* *3B and *3C SNP in the same patient using individual *TPMT* *3B and *3C primers was reported as *TPMT* *3A.

Table 8: The primer sequences used to detect the *TPMT* SNPs[5, 122]

***TMPT* allele**	**Primer Sequence 5' – 3'**
*3A	*F:* AGG CAG CTA GGG AAA AAG AAA GGT G *R*: CAA GCC TTA TAG CCT TAC ACC CAG G *F*: GAG ACA GAG TTT CAC CAT CTT GG *R*: CAG GCT TTA GCA TAA TTT TCA ATT CCT C
*3B	*F:* AGG CAG CTA GGG AAA AAG AAA GGT G *R*: CAA GCC TTA TAG CCT TAC ACC CAG G
*3C	*F*: GAG ACA GAG TTT CAC CAT CTT GG *R*: CAG GCT TTA GCA TAA TTT TCA ATT CCT C

PCR was conducted using the GoTaq Hot Start Green Master Mix Kit (Promega, USA). Each assay tube was loaded with 23 µL of the Hot Start Kit and 2 µL of DNA from the

individual patients. The PCR amplification was run using the following program: an initial 2-minute denaturation at 94ºC hereafter all denaturation steps were 30 seconds. The annealing step was run for 30 seconds at 60.4ºC for *TPMT** 3B and 54.8ºC for *TPMT** 3C. The extension step was run at 72ºC for 60 seconds per 1 kb of DNA. The final extension step ran at 74ºC for 5 minutes. PCR products were stained with GR-Green -the stain was already included in the composition of the in the GoTaq Hot Start Green Master Mix Kit (Promega, USA).

A 500 ml working stock solution of TAE 1x (40 mM Tris, 20 mM Glacial Acetic Acid and 1 mM EDTA, pH8) was used to prepare the agarose gels and for electrophoresis.

Agarose gel for 1.8% gel electrophoresis was prepared by combining 1.8 g of Agarose powder (Promega, USA) with 100ml TAE 1x and heated for 1 minute in the microwave until the mixture was clear. A volume of 10 µL ethidium bromide was added to the solution before the heated agarose solution was poured into the gel casting tray and allowed to set. Once the gel was set the well casts were removed, TAE was added until the layer of gel was covered and the samples were pipetted into the respective wells. A total of 10 µL of each PCR sample was pipetted into each respective well.

Each gel contained 20 lanes. A DNA ladder (New England Biolabs) was added to the first lane of every gel to assist in fragment length determination. Instructions for the ladder can be found in the Appendix. Upon electrophoresis the ladder dye split into blue and yellow.

The gel electrophoresis was run at 100v until the dye line (yellow) was 80% down the length of gel. The gel was then viewed under an ultra violet light to confirm that PCR was successful.

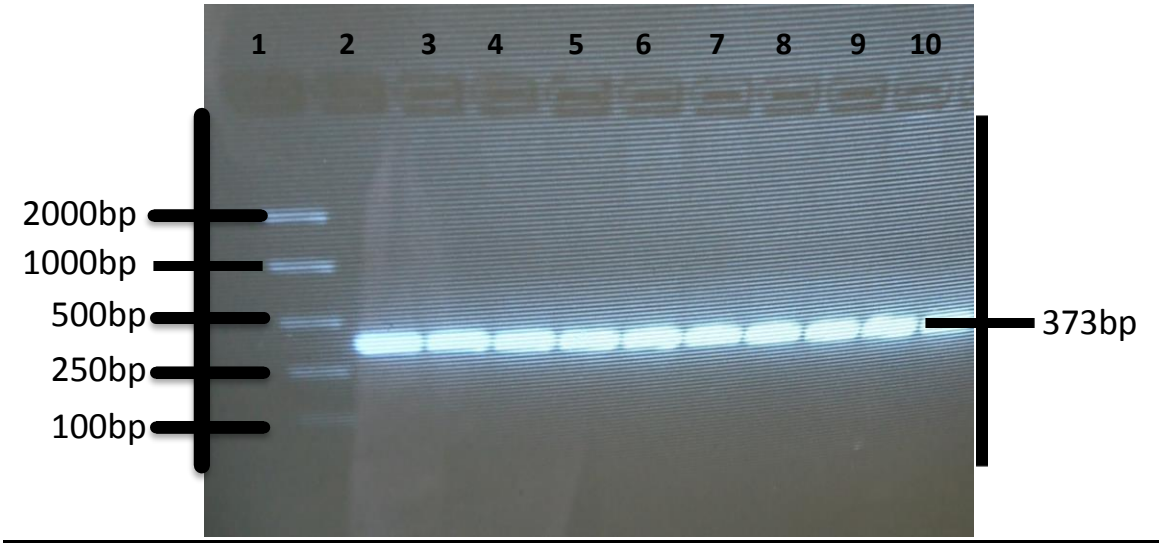

Figure 3:Gel electrophoresis patterns for *TPMT 3C on 1.8% agarose gel.** *The first column depicts the ladder, column 2- 10 contains unrestricted PCR product for IBD patients 01 -09.*

Restriction digestion of the PCR products was conducted using Cutsmart *MwoI* and *AccI* (New England Biolabs) restriction enzymes to identify *TPMT** 3B and *TPMT** 3C SNP's respectively. The same batch of restriction enzymes were used for all testing. A buffer was provided with the Cutsmart kit. To prepare one sample a 1 x buffer was prepared by diluting 100 µL of 10 x buffer with 900 µL of nuclease-free water. A volume of 5 µL of this 1 x buffer was then combined with 16 µL of nuclease-free water and 1 µL of the relevant restriction enzyme added. This solution was then vortex mixed. A total volume of 22 µL of the prepared enzyme was added to an aliquot and combined with 3 µL of PCR product. The final restriction PCR amplicon was then incubated. *MwoI* was incubated for 15 minutes at 60℃ while *AccI* was incubated at 37℃ for 15 minutes and further inactivated at 80℃ for 20 minutes.

The restricted PCR products were then separated on a gel electrophoresis on 3% agarose gel using TAE buffer to allow slow migration and visualisation of smaller fragments percentage of agarose gel used for the separation was determined by optimisation, where the best band separation was visualised. Agarose gel for 3.0% gel electrophoresis was prepared by combining 3.0 g of Agarose powder (Promega, USA) with 100ml TAE 1x and heated for 1 minute in the microwave until the mixture was clear. A volume of 10 µL ethidium bromide was added to the solution before the heated agarose solution was poured into the gel casting tray and allowed to set. Once the gel was set the well casts were removed, TAE was added until the layer of gel was covered and the samples were pipetted into the respective wells. A total of 10 µL of each PCR

sample was pipetted into each respective well.

A ladder (New England Biolabs) was added to the first lane of every gel to assist in fragment length determination. The instruction sheet for this restriction process can be found in the Appendix. Upon electrophoresis the ladder dye split into blue and yellow. The gel electrophoresis was run at 100V until the dye line (yellow) was 80% down the length of gel. The gel was then viewed under an ultra violet light to confirm that PCR was successful. The gel electrophoresis of each sample was conducted in duplicate.

The following cleavage was expected from each SNP; *TPMT* *3B should produce 443 and 251 kb fragments when digested by *MwoI* and provide and uncleaved 694 bp fragment in the wild type allele. While *TPMT* *3C polymorphism should produce fragments of 283 and 90 bp and a wild type fragment of 373 bp when digested by *AccI.*

As indicated in the article by Lee *et al*[123] "The leading model for DNA movement through an agarose gel is "biased reptation", whereby the leading edge moves forward and pulls the rest of the molecule along. The rate of migration of a DNA molecule through a gel is determined by the following: 1) size of DNA molecule; 2) agarose concentration; 3) DNA conformation(5); 4) voltage applied, 5) presence of ethidium bromide, 6) type of agarose and 7) electrophoresis buffer.[123]

For each gel electrophoresis run of the restricted amplicons, one column contained undigested PCR product as a positive control to confirm that a successful digestion was taking place.

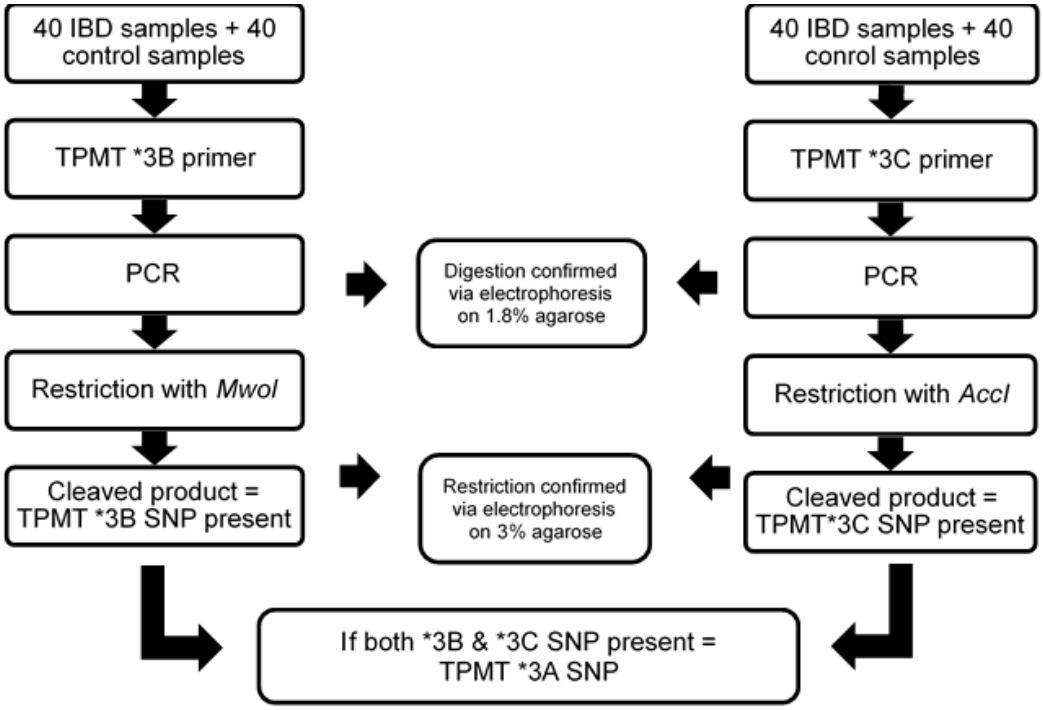

Figure 4: Overview of SNP detection method

All of the IBD and control samples were tested for the presence of the *TPMT* *3B or the *3C SNP's. i.e. 80 samples (40 IBD and 40 control) were combined with *TPMT* *3B primers, amplified through PCR and then restricted using *MwoI*. The same 80 samples were separately added to *TPMT* *3C primer, amplified through PCR and restricted using *AccI*. The presence of both SNP's meant a heterozygous variant *3A was present.

4.3.1 Statistical Analysis

The genotype class of both the control and IBD samples were tabulated in contingency tables and due to a sample size of < 1000 samples, a Fisher's Exact test of independence was calculated to determine if a non-random association existed between the variables- i.e. whether the fact that the sample was a control or IBD sample played a role in what the genotype would be.

4.4 Results

The actual gel electrophoresis patterns observed in the study can be seen the Appendix. Figure 5 & 6 schematics of the gel electrophoresis have been provided due to the poor quality of the actual gel images. In Figure 4, the *TPMT* *3B SNP schematic shows the presence of *3B in both heterozygous and homozygous form, electrophoresis of the *3B SNP was performed in duplicate for all 40 IBD patients to ensure optimal viewing of electrophoresis patterns as the bands often showed poor separation despite lengthy runs. Figure 5 depicts a schematic of the *TPMT* *3C gel electrophoresis patterns, showing both heterozygous and homozygous form.

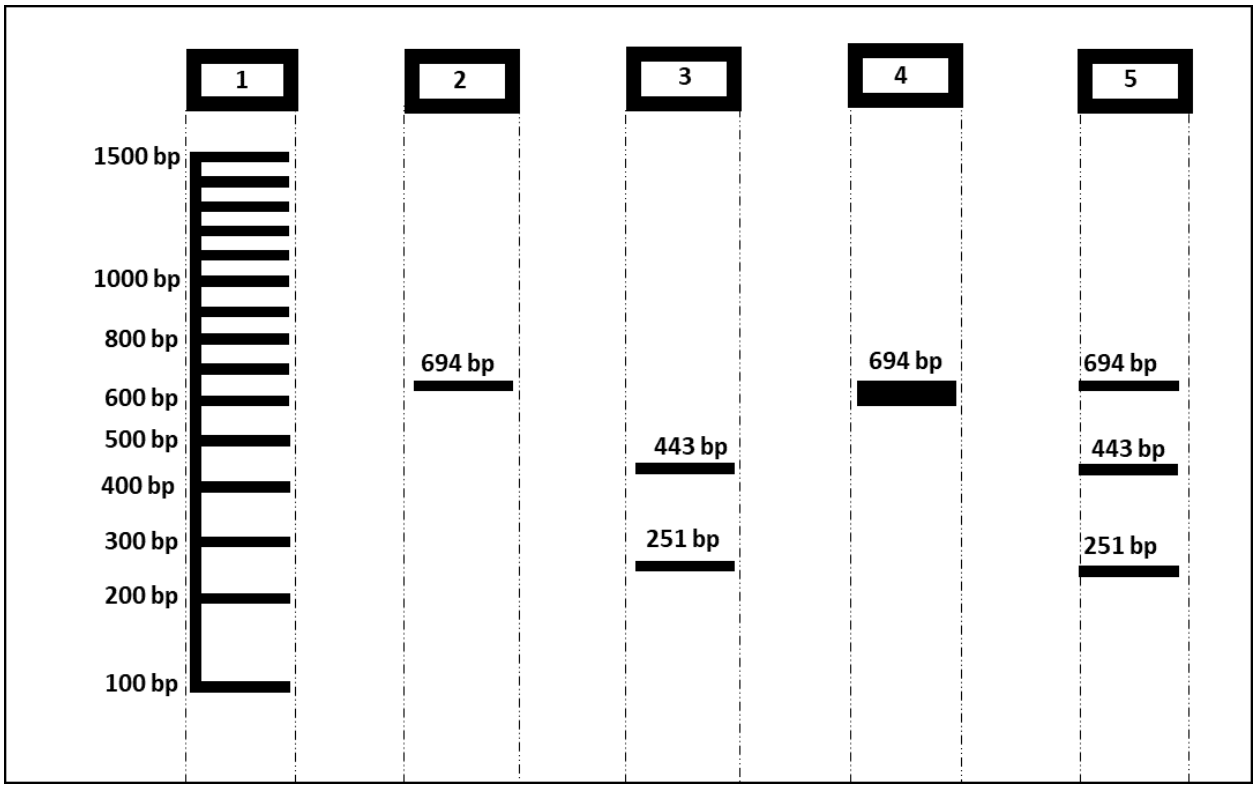

Figure 5: Schematic of gel electrophoresis patterns for *TPMT 3B on 3% agarose gel.** *The first column depicts the ladder, the second column a positive control (undigested PCR product, the third column a homozygous *3B allele, the fourth column contains a wild-type allele and the fifth column depicts a heterozygous *3B allele.*

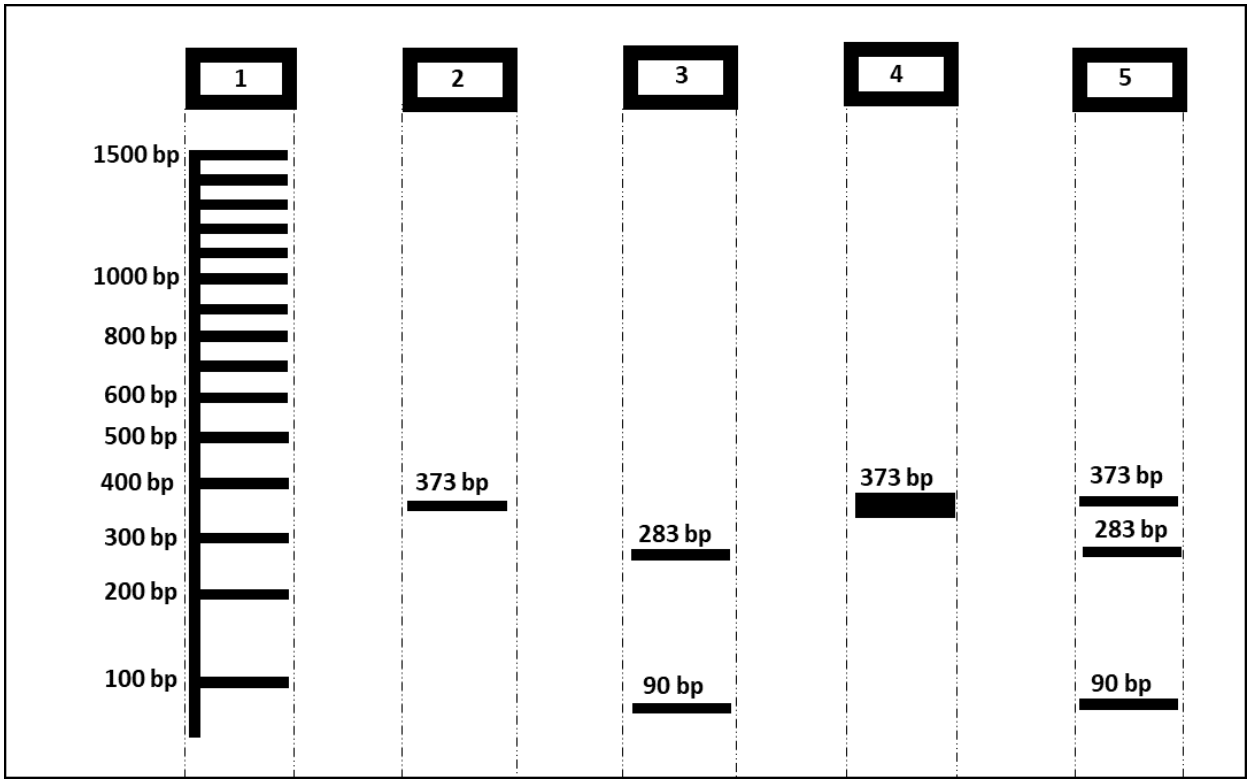

Figure 6: Schematic of gel electrophoresis patterns for *TPMT 3C on 3% agarose gel.** *The first lane depicts the ladder, the second lane a positive control (undigested PCR amplicon), the third lane a homozygous *3B allele, the fourth lane contains a wild-type allele and the fifth lane depicts a heterozygous *3B allele.*

All 40 sets of study population data as well as 40 sets of control population data were successfully analysed for the presence of *TPMT* *3A, *3B or *3C SNP and the results reported in Table 21 in the Appendix. A total of 6/40 participants presented with the *TPMT* *3A heterozygous variant, while 1/40 presented as heterozygous *3B and an unusual mutation combination of *TPMT* *3B homozygous was detected in 33/ 40 diseased patients. *TPMT* *3A is a heterozygous variant that results from a double mutation when both *TPMT* *3B and *3C is present in an individual. A wild type allele is represented with a 1. The final column refers to whether the patient reported experiencing an adverse drug reaction while on AZA therapy.

Of the 40 IBD patients; 33 patients (82.5%) indicated the presence of the *TPMT* *3B SNP on both alleles, a statistically significant finding ($p<0.001$) in comparison to the 6/40 control individuals who also presented with the homozygous *TPMT* *3B SNP as shown in Table 9. The presence of this homozygous *TPMT* *3B SNP in IBD patients was found in both diagnostic groups (UC and CD), across both genders and whether the patient reported experiencing and ADR to AZA or not. A sum of 20/40 control

participants presented with heterozygous *TPMT* *3B SNP, with the other half of the control participants presenting with heterozygous variant *TPMT* *3 (8/40), heterozygous *TPMT* *3C (1/40) and the absence of *TPMT* *3A, *3B or *3C (5/40).

Table 9: Statistical breakdown of study population (CD and UC) and control population demographics

Variable	Name / Value	Controls n (%)	Study n (%)
Gender	Male	14 (35)	13 (33)
	Female	26 (65)	27 (68)
Race	Black	19 (47.5)	4 (10)
	Caucasian	17 (42.5)	32 (80)
	Coloured	3 (7.5)	1 (2.5)
	Indian	1 (2.5)	3 (7.5)
Genotype	*3B/*3B	6 (15)	33 (82.5)
	*1/*3A	8 (20)	6 (15)
	*1/*3B	20 (50)	1 (2.5)
	*1/*3C	1 (2.5)	0
	*1/*1	5 (12.5)	0

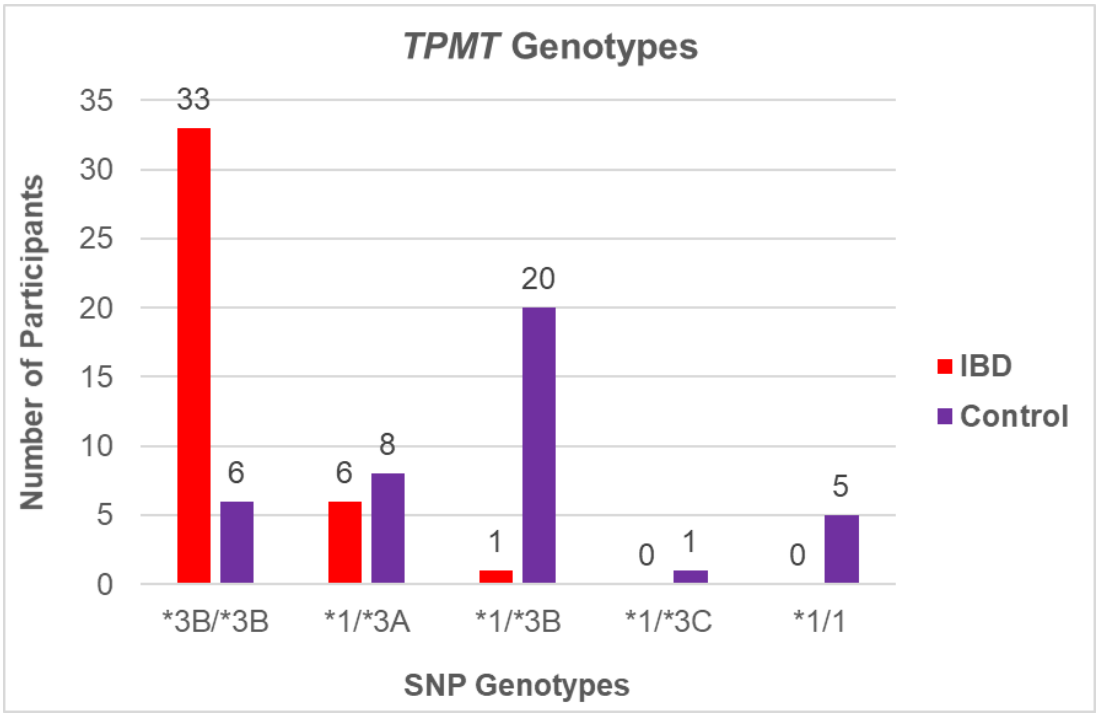

Figure 7: Graphical depiction of *TPMT* SNP's detected in the study in IBD patients and controls.

Figure 8 highlights the comparison of the number of IBD patients who reported experiencing an ADR, with the genotype of the IBD patients to comment on whether

the homozygous *TPMT* *3B phenotype can be linked to an increase in ADR's to AZA therapy.

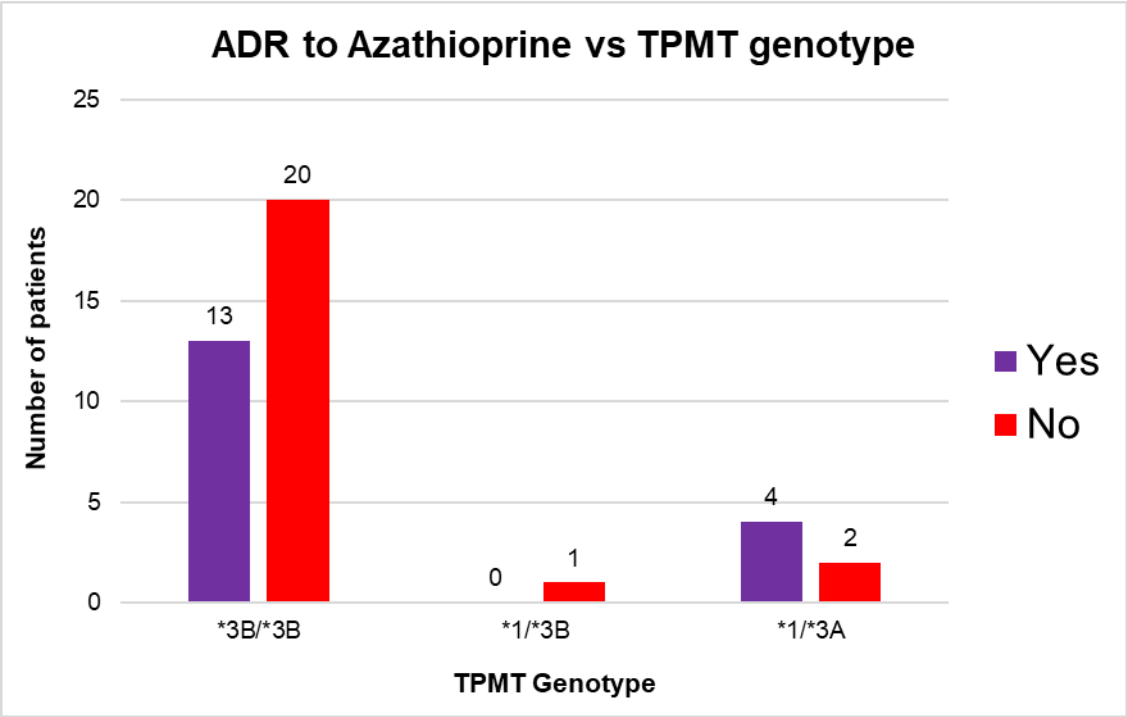

Figure 8: IBD Patients who reported experiencing an adverse drug reaction to AZA therapy and their corresponding *TPMT* genotype. *Even though 82.5% of the disease population presented with a homozygous TPMT *3B SNP, 23/33 individuals who presented with any SNP did not experience an ADR to Azathioprine. The only patient with a heterozygous TPMT *3B did not report an ADR, while 4/6 individuals with TPMT *3A did report an ADR.*

4.4.1 Sanger Sequencing

To verify the accuracy of the results via RFLP-PCR; 7 study samples were selected and sequenced via Sanger sequencing. The results are shown in the Appendix;

4.5 Discussion

Due to funding restriction for this study, only a limited number of *TPMT* SNP's could be tested for in the study population, hence, it was decided that only the most common *TPMT* SNP's would be assessed- namely *TPMT* *3A, *3B and *3C.

Literature reports that the *TPMT* *3A SNP is the most common *TPMT* polymorphism occurring in the Caucasian population [124], however, this study observed the presence of the *TPMT* *3A heterozygous variation in only 15% of the IBD patients despite the populations being 80% Caucasian. The presence of *TPMT* *3A in 20% of the control population is similar to that of other populations[8, 125, 126]. While the allelic frequency of *3C in the control population of the present study is similar to that of other populations (especially Egyptian[127] and Libyan[7] populations), the allelic frequencies of the *3A is higher than that reported for other populations, suggesting that the increase in *TPMT* *3B and *3C allelic frequencies may be unique to the South African population. This finding reiterates the fact that the majority of the research regarding *TPMT* SNPs in IBD patients has been conducted within a European Caucasian population, while a data generated regarding *TPMT* SNPs in healthy individuals has been conducted on international populations. Little is reported regarding the prevalence of *TPMT* SNP's of IBD patients of many other race, ethnic or ancestral populations globally, despite the prevalence clearly differing from that of the general healthy population.

Evidence confirms that *TPMT* *3C is the most common variant allele in the African population,[128-130] however, in this study the *TPMT* *3C allele was not detected in the IBD population although it was detected in 22.5% (allelic frequency 1.3%) of the control group and although the *TPMT* *3C allelic frequency of the control group is within the same range as other international populations, it is still far less frequent than the *TPMT* *3A and *3B alleles.

Studying the *TPMT* SNP's in this South African IBD population, produced a significant novel combination of a homozygous double mutation ($p<0.001$) of the *TPMT* *3B SNP in the South African IBD population in comparison to the healthy South African population, when analysed using Fishers Exact test of independence. This homozygous mutation was found in 82.5% of the IBD study population, but only present in 15% (allelic frequency 40%) of the control population.

The presence of *TPMT* *3B not only in the diseased population but also in the healthy control population contrasts with most published literature. A paper by Wang *et al* [115]

states that the *TPMT* *3B SNP is rare, and is usually found to have a tight linkage disequilibrium to the *TPMT* *3C SNP. This is supported by the findings from many published studies, with majority of the studies not detecting *TPMT* *3B in the healthy population at all[6, 7, 24, 131-134].

When analysing the spread of the homozygous *TPMT* *3B SNP within the IBD study population (variables listed in Table 9) it was noted that the unique SNP combination did not favour any gender, diagnosis or race despite studies proving that race does play a role in the *TPMT* phenotype [104, 112]. It must be noted that the racial demographics in this study does not accurately represent the racial demographics of the South African population, as inclusion criteria required for this specific study (i.e. IBD patients on AZA therapy) did limit the selection of patients.

More notably, there was no relationship observed between the presence of the homozygous *TPMT* *3B SNP and the patient experiencing an ADR to AZA. Calculations show that 20/33 (60%) of the IBD patients who presented with the homozygous *3B SNP did not experience an ADR. The only heterozygous *TPMT* *3B individual did not report an ADR, while 4/6 individuals with *TPMT* *3A patient in the cohort did report an ADR. A future study could use haplotyping to determine whether these *TPMT** 3A patients were in *cis* or *trans* linkage and whether the linkage plays a role in the ADR to AZA.

The ADR statistics observed in this study contrasts with what has previously been reported regarding homozygous *TPMT* SNP's – which suggests that homozygous alleles are associated with little to no *TPMT* enzyme activity and thus posing a higher risk of AZA ADRs [19, 24, 26, 27]. In 1996, Szumlanski *et al* [124] concluded that the presence of *TPMT** 3 SNP's results in decreased enzyme levels and thiopurine ADRs and toxicity [115, 124]. Hence why the FDA stance on FBC monitoring is pertinent, regular FBC testing cannot be replaced by genetic testing but should rather be used in conjunction.

A relevant limitation in this study is the reporting of ADR's. For many of the patients who had experienced an ADR to AZA at a previous physician, the physician only noted that an ADR and that the therapy was discontinued – details of the ADR were often not recorded, hence compiling data on the actual ADR's seen in this study was not a viable option. Knowledge of these ADR's could have aided in trend analysis in terms of which ADR's were noted and if there was a correlation between any of the

therapeutic markers or SNP's.

Secondly, differentiating the cause of the ADR's was also a study limitation; differentiating the cause of the ADR as being as a result of AZA therapy or due to a SNP or due to another factor altogether. Knowledge of this may have provided insight as whether the SNP detected may actually affect the enzyme activity. Patients reported having ADR's which included vomiting, nausea, itchiness and brittle nails and teeth or fatigue. These are ADR's common to AZA, however, they are experienced at extreme levels which require hospitalisation in the case of nausea or fatigue. Hence the reason why FBC's are imperative to monitoring ADR's while on AZA therapy as it is the simplest way to monitor the bodies reaction to the AZA. These ADR's that commonly occur due to AZA can be differentiated from ADR's that are due to SNP's in that SNP related ADR's usually occur within the first month of AZA therapy (steady state is not required for the symptoms to be present), they present as severe (i.e. patient may be hospitalised due to the ADR) and persist regardless of a decrease in AZA dosage. That said, determining the cause of an ADR is simple in theory but differs in practice as in most cases of IBD. The physician will make an educated decision based on the severity of the disease, the current comorbidities, concomitant medications and the presence of AE's related to these, as well as the therapeutic markers and previous experience with the patient and the drug.

The list of patients who experienced ADR's, as well as their respective SNP's, diagnosis and the change in therapeutic marker values is shown in in Table 22 and 23 in the Appendix.

Due to funding constraints, a large limitation for this objective was that only 3 SNP's were tested for. Ideally all 41 SNP's should have been tested in a population of at least 100 IBD patients and 100 matched control samples. Further enzymatic analysis of the homozygous SNP's would also have greatly benefitted the outcomes of this study.

4.6 Conclusion

When comparing the presence of *TPMT* *3A, *3B and *3C in the healthy South African population, it is evident that there is a much higher allelic frequency than other global populations; most notably in the presence of *TPMT* *3B. This study showed not only an increase in the allelic frequency of *TPMT* *3B, but also observed the statistically significant presence ($p<0.001$) of homozygous *TPMT* *3B alleles- in IBD patients when compared to controls -which has not previously been reported in published literature. Majority of the individuals with the homozygous *3B alleles did not experience an ADR contrary to known literature, this has potential for a future study.

CHAPTER 5: THERAPEUTIC MARKERS USED TO MONITOR EFFICACY OF AZATHIOPRINE

5.1 Abstract

5.1.1 Objective

To determine the efficacy of AZA in reducing the c-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) as well as to observe the change in leucocyte count from baseline AZA dosing to month 12 following initiation of AZA therapy.

5.1.2 Materials & Methods

Patients suffering from IBD who had previously or were currently using AZA for more than 12 consecutive months were recruited from Steve Biko Academic Hospital Gastroenterology Unit, as well as a private hospital in Pretoria. Once patient consent was obtained, patient files were obtained and the CRP, ESR and leucocyte counts were documented for the 12 months post initial AZA dosing.

5.1.3 Results

Over 12 months, of the 35 study participants who had CRP results on file, 74% experienced a decrease in CRP resulting in a significant difference ($p < 0,0001$) between the overall CRP at baseline versus the CRP at month 12. A total of 17 patients (42.5%) reacted to AZA, which correlated to the fact that 41% of the patients experienced a decrease in WBC. CRP counts decreased by an average of 15.67%, ESR rates increased by an average of 5.49% and the WBC count increased by an average of 4.63%.

5.1.4 Conclusion

Azathioprine therapy had the desired outcome on the IBD patients by decreasing the CRP counts in both UC and CD patients. The change in leucocyte count was linked to the occurrence of the patient having experienced an ADR on AZA therapy, with majority of patients who reported an ADR also presenting with a decrease in leukocytes.

5.2 Introduction

In healthy patients, the mucosal immune system within the digestive tract induces antigen-specific non-responsiveness when micro-organisms and antigens are administered through the diet. This lack of immune responsiveness is a learned mechanism within the intestinal mucosa termed oral tolerance where the immune cells recognise and adapt to frequently consumed micro-organisms[77, 78]. There are multiple mechanisms involved in the suppression of antigen reactive T cells that in turn suppress inflammation in the gut.

However, patients suffering from IBD lack oral tolerance and instead suffer from a hyper-responsive immune system [79]. This occurs when the parenchymal and haematopoietic cells within the intestinal mucosa react to micro-organisms, whether symbiotic or pathogenic, resulting in excessive secretion of inflammatory cytokines and an unsuppressed inflammatory cascade[77]. This inflammatory cascade is a natural response to infection, however, IBD patients lack the natural ability to inhibit this response prior to tissue damage due to the dysregulation of proinflammatory and anti-inflammatory mediators [78].

The overexpression of inflammatory cytokines in active IBD allows the use of therapeutic markers such as ESR and CRP to monitor disease activity in patients suffering from UC and CD.

There are a variety of ways to determine therapeutic efficacy of AZA therapy in patients suffering from IBD, depending on the health care system and patient standard of living.

The most common but least specific biochemical evaluations are assessments of serum CRP and ESR. Both are non-specific markers of inflammation that are used in combination to monitor and predict the outcome of certain inflammatory diseases such as rheumatoid arthritis, cardiovascular disease and IBD. Frequent measurement of CRP counts and ESR as well as full blood counts (FBC) should be mandatory in individuals on chronic AZA treatment, as suggested by the FDA and recommended by other studies[10, 27, 78].

Additional therapeutic markers include faecal calprotectin or faecal lactoferrin, which are superior to CRP in detecting active versus inactive IBD and distinguishing IBD from

irritable bowel syndrome but are not superior to endoscopic findings [39]. Calprotectin is a large protein specific to monocytes, macrophages and neutrophils, while lactoferrin is a protein found in neutrophils and mucous secretions. Both lactoferrin and calprotectin are stable in the stool for 7 days, and the presence of these faecal markers is indicative of inflammation[135]. Faecal calprotectin in the only marker that allows accurate discerning between a mild, moderate and highly active disease status[86].

Using biochemical tests to determine inflammation should be an additive tool used in combination with clinical and endoscopic findings [86].

There is no consensus as to the therapeutic endpoint that defines disease remission, as many of the clinical efficacy markers are symptom based and correlate poorly to endoscopic findings of inflammation. It has recently been suggested that mucosal healing should be considered the therapeutic endpoint defining disease remission, as opposed to achieving normal ranges of ESR and CRP counts, as emerging data is suggesting that individuals who have achieved mucosal healing have a better long-term prognosis[110]. The CD and UC patients showing mucosal healing require less hospitalization and lower requirement for systemic steroids[136].

5.2.1 Erythrocyte Sedimentation Rate

The ESR is determined by allowing uncoagulated blood to sediment for 1 hour in a narrow glass tube, with normal ESR values in males ranging from 1-17 mm/hour and in females 0-25 mm/hour but these upper limits may increase due to multiple factors e.g. age or obesity [137].

Testing the rate at which erythrocytes sediment is based on the premise that erythrocytes will settle due to gravity separating the erythrocytes from the plasma. In the presence of increased amounts of plasma proteins such as acute phase proteins, immunoglobulins or fibrinogens, the proteins bind to the erythrocytes resulting in decrease in the repulsive force between erythrocytes allowing easier aggregation of erythrocytes causing increased rates of sedimentation [138]. This test is considered to have a high sensitivity but low specificity as the ESR reflects inflammation but is influenced by factors other than inflammation [137, 139].

An accurate ESR count can be affected by factors such as anaemia, menstruation,

hypercholesterolemia, pregnancy or a sudden decrease in red blood cell numbers. Increased ESR is a common laboratory finding in infection, inflammatory disease, some cancers, nephrotic syndromes, tuberculosis and endocarditis [137, 138].

5.2.2 C-Reactive Protein

C-reactive proteins are produced in the liver in response to inflammation and forms part of the acute-phase response, increasing rapidly at the onset of inflammation, infection or cellular death. These proteins have a short half-life of 5-7 hours and fluctuate rapidly in response to inflammation and therapy in comparison to ESR, although a correlation is discernible [138, 139]. An accurate CRP count may also be influenced by pregnancy, oral contraceptives, malignant diseases or transplant rejections [140].

While CRP serum levels correlate well with disease activity in CD, CRP levels are only a modest disease activity indicator in UC, even in the presence of active inflammation and the reason for this is still unknown [78]. Normal high sensitivity-CRP (hs-CRP) is < 2 mg/L while a hs-CRP count of > 45 mg/L correlates with severe and uncontrolled inflammation in IBD and will likely result in a colectomy [139-141]. High sensitivity CRP is an immunoassay with enhanced sensitivity and a lower measurement cut-off. The common hs-CRP ranges for these diseases are listed in Table 11 below.

Studies suggest that patient CRP and ESR should be monitored weekly for the first 8 weeks of therapy, thereafter monthly for the next 6 months and then bi-annually once the patient is on long-term therapy[26]. Studies show that CRP values correlate very well to endoscopic findings in CD, but less so in UC[5, 104, 105].

Table 10: CRP Ranges for Diseases That Commonly Affect hs-CRP[89, 142, 143]

Patient Health	Hs-CRP Value (mg/dL)	Comment
Healthy	<2	
Cardiac Disease	0-1	Low Inflammation Risk
	1-3	Medium Inflammation Risk
	>3	High Inflammation Risk
Rheumatoid Arthritis	1-6	Normal
Infants	<10	
Pregnancy	1.5-2.5	Increases from 2nd trimester
Crohn's Disease[89]	<5	Disease remission
	<30	Moderate disease activity
	>30	Severe disease activity

The crucial time-lapse difference seen in CRP and ESR correlation is depicted in the adaption by Kumar *et al*[144], seen in the Figure 9.

	ESR	CRP
Usefulness for diagnosing acute inflammation	Comparatively lower	Comparatively higher
Usefulness in monitoring disease progression	High	High[a]
Accuracy in low-grade inflammation	More specific, less sensitive	More sensitive, less specific
Range of values	Limited	Wide
Affected by size/shape/ number of RBCs	Yes	No

[a]With the exception of systemic lupus erythematous.

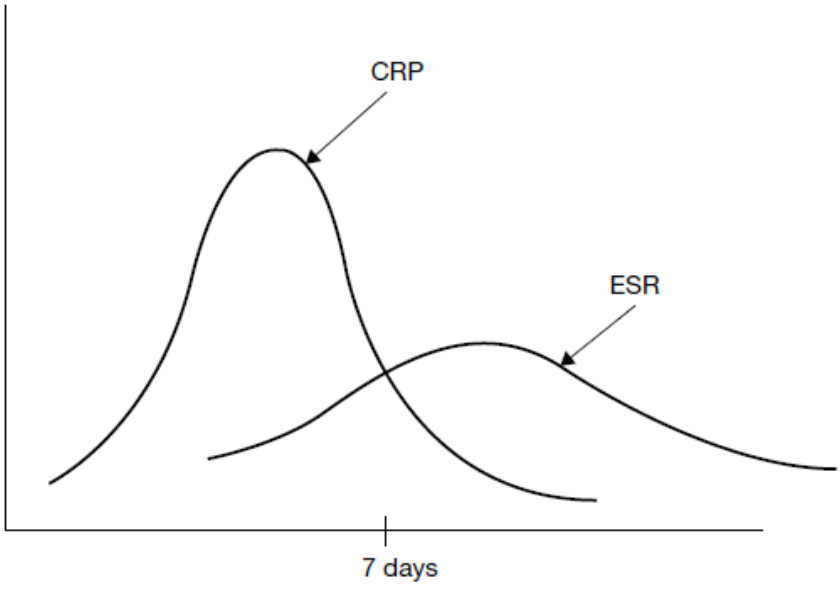

Figure 9: Time differences in the specificity of CRP and ESR[144]

5.2.3 Leucocyte counts

In severe cases of UC and CD, the patient will present with leucocytosis but it is not considered to be an accurate indicator of disease activity. However, for patients suffering from IBD undergoing AZA therapy, monitoring of leucocyte counts is vital as an early indicator of leukopenia or myelosuppression.

This is because 6-TGN, an active metabolite of AZA, has structural similarity to that of guanine resulting in frequent mis-incorporation of 6-TGN into the DNA of leukocytes. However, this mis-incorporation results in DNA strand breakage as the 6-TGN is not recognised as a base causing strand termination after incorporation. Immunosuppression occurs because of the lack of available leukocytes as they are rapidly dividing cells that are susceptible to DNA replication inhibitors [19]. Should the patient's AZA metabolism pathway be compromised by an inactive *TPMT* resulting in high concentrations of 6-TGN, myelosuppression will occur as a dose independent AE due to the over suppression of the leukocytes[145].

A review by Katsanos *et al,* notes that leukopenia cannot always be attributed to inactive or no *TPMT* and that myelosuppression may also present as a dose dependent AE in AZA therapy [146, 147]. Thus, patients on AZA therapy require regular monitoring of their leukocyte counts. This observation was made based on an article by Sandborn which stated that in a population of patients who developed leukopenia in the first 6 months of AZA therapy, 50% will have little to no *TPMT* activity while the other 50% of the population will be comprised of patients with normal *TPMT* activity [148]. Patients developing leukopenia after 6 months of AZA therapy will nearly always have normal *TPMT* activity [147].

5.3 Materials and Methods

Patient medical history files were obtained with consent from the patient. The CRP, ESR and leukocyte counts at baseline and for the first 12 months post initial AZA dosing were recorded. The concomitant medications were also recorded and are noted in Appendix. Sample analysis for ESR, CRP and leukocyte counts for all patients were conducted by pathologists at the National Health Laboratory Service at Steve Biko Academic Hospital or by Vermaak and Partners laboratory at the private hospital. ESR was determined using an Alifax Roller 20 (Alifax, Italy), the CRP

counts were determined using the Architect c4000 (Abbott, USA) with a total plasma CRP kit and the leukocyte counts were determined using a Sysmex XN (Sysmex, South Africa). These values were analysed to assess whether there was any improvement in the inflammatory parameters or if adverse side effects were evident. As the “normal” range for therapeutic markers such as ESR and CRP are considered to be relative for IBD patients, as it will differ according to diagnosis, severity and duration of disease, hence each patient served as their own baseline and instead the change in therapeutic marker value was evaluated.

5.3.1 Statistical Analysis

CRP, ESR and WBC counts were analysed to determine if an increase or decrease occurred over 12 months of AZA treatment. The number of patients who experienced an increase or decrease for each therapeutic marker was then tabulated to obtain frequency values. Frequency values were then tabulated in a contingency table according to IBD diagnosis (CD or UC) and a Fisher’s Exact test for independence was performed to determine if the IBD diagnosis played a role in whether the therapeutic marker increased or decreased. Pearson’s Correlation Coefficient test was used to calculate correlation between pairs of variables.

5.4 Results

Thirty-six sets of patient data were obtained for this study as shown in Table 12; Patient 007, Patient 012, Patient 030 and Patient 032 had previously used AZA therapy but did not have laboratory results on file as they had changed physicians. However, these patients had observed adverse reactions noted in their files and these patients were still deemed to be relevant to the study. The ESR, CRP and leukocyte counts were obtained from previous laboratory reports that were as close to the baseline and month 12 as possible. Theoretically, CRP and ESR correlate, hence many physicians choose to measure only one therapeutic parameter of choice. In this study, the one physician chose to use CRP, while the other used both therapeutic markers to track drug efficacy.

Table 11: Prevalence of measured parameters within patient cohort

Variable	Name / Value	n (%)
Gender	Male	13 (33)
	Female	27 (68)
Race	Black	4 (10)
	Caucasian	32 (80)
	Coloured	1 (2.5)
	Indian	3 (7.5)
Diagnosis	Crohn's Disease	19 (47.5)
	Ulcerative Colitis	21 (52.5)
ADR	No	23 (57.5)
	Yes	17 (42.5)
Genotype	*3B/*3B	33 (82.5)
	*1/*3A	6 (15)
	*1/*3B	1 (2.5)
	*1/*3C	0
	*1/*1	0
∆ CRP	Increase	7 (20)
	Decrease	26 (74)
	No Change	2 (5)
∆ ESR	Increase	5 (33)
	Decrease	10 (66)
	No Change	0
∆ WBC	Increase	18 (53)
	Decrease	14 (41)
	No Change	2 (5)

Over 12 months, of the 35 study participants who had CRP results on file, 74% experienced a decrease in CRP as depicted in Table 12. Patient 025, Patient 027 and Patient 038 experienced the greatest decrease from 287.5 mg/L to 2.7 mg/L, 281.7

mg/L to 38.6 mg/L and 275 mg/L to 37 mg/L respectively, over 12 months. Unfortunately, therapeutic markers such as CRP and ESR can be affected by other diseases too, and in patients who suffer from autoimmune diseases, it is often difficult to discern what the primary cause of the elevated CRP is. Thus, it must be taken into consideration that some of the CRP values in the patient files may not be directly due to IBD. In Figure 10 below, the known comorbidities of the patients are listed.

PT #	Co-morb
P002	polyarthritis, barrets esophagitis, shingles
P007	B12 deficiency, iron deficiency
P008	Nephritic syndrome
P010	Pulmonary Embolism
P013	Polyarthritis, hypothyroid, osteoporosis
P014	Chronic headache, hypertension, nodular thyroid disease
P017	PolyArthritis, osteoporosis, B12 deficiency, renal cyst
P022	B12 Deficiency
P026	anemia
P027	Fibromyalgia
P029	alopecia
P030	psoriasis
P032	Polyarthritis
P037	fibromyalgia
P039	Polyarthritis

Figure 10: Known co-morbidities for the sample IBD population *obtained from medical files*

The CRP data for Patients 025, 027 and 038 were excluded from statistical analysis and treated as outlier data as they were a lot higher than the rest of the patients, however in the case of Patients 025 and 038 there were no reported cases of comorbidities; they were having a disease flare.

Patients 008 and 014 showed normal CRP counts (1 mg/L and 2 mg/L) at baseline and no change (increase or decrease) in CRP over the 12-month period was seen as the disease was already in remission due to the use of mesalazine and prednisone as shown in Table 19. AZA therapy was prescribed as maintenance therapy to ensure the disease remained in remission.

A two-tailed paired t-test with 95% confidence revealed a significant difference ($p < 0{,}0001$) between the overall CRP at baseline versus the CRP at month 12. The line graph for each pair can be seen in Figure 11 below. Additionally, a Spearman's rho

Correlation test was conducted on the variables CRP baseline and CRP month 12, resulting in a significant pairing of r= 0,4557

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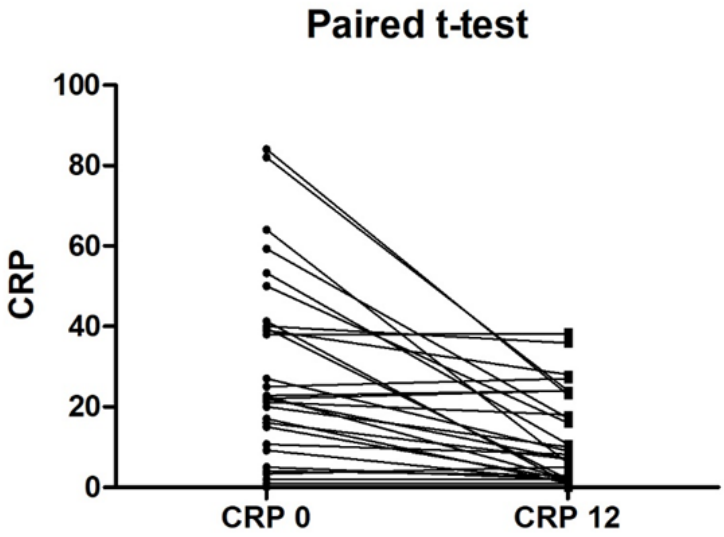

Figure 11: Line graph of the paired t-test showing CRP (mg/L) at baseline vs month 12- *outlier data for Patients 025, 027 and 038 were excluded*

In the few cases where initial CRP count was above 50 mg/L, a large decrease in CRP counts over 12 months can be observed, however, this trend is no longer observed in patients whose CRP count was below 50 mg/L at baseline, instead the change in CRP counts varied greatly irrespective of whether the initial value was higher (e.g. 38 mg/L) or lower (e.g. 5 mg/L) to begin with. CRP counts at baseline were higher than at month 12 at majority of the points, however, Patients 015, 022, 026, 031, 036 and 039 showed a slight increase in CRP counts at month 12. Patient 026 reported an ADR to AZA, while the other 5 patients that showed an increase in CRP were having a mild disease flare.

Patients diagnosed with UC and on AZA therapy showed a slightly larger decrease in CRP over 12 months (22.61% decrease) when compared to CD patients (6.76% decrease) as depicted in Figure 13. Overall there was a 15.67% decrease in CRP over 12 months between all the IBD patients on AZA therapy, with 88.8% of patients diagnosed with UC showing a decrease in CRP, compared to the 66.6% of CD patients - although when analysed using the Fisher's Exact test of independence a value of 0.2 was calculated- implying that very little non-random dependence existed between the CRP change in UC patients versus the CRP change in CD patients.

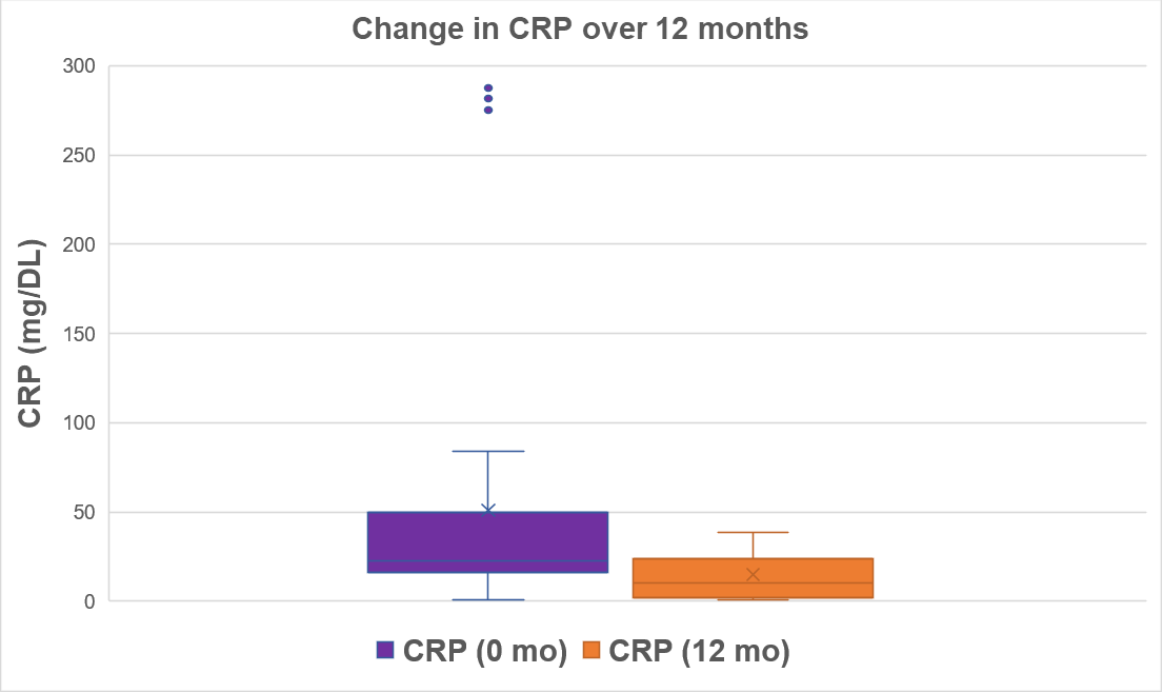

Figure 12: CRP (mg/L) counts at Baseline and Month 12 in 35 IBD patients on AZA therapy. *Including outlier data for Patients 025, 0257 and 038*

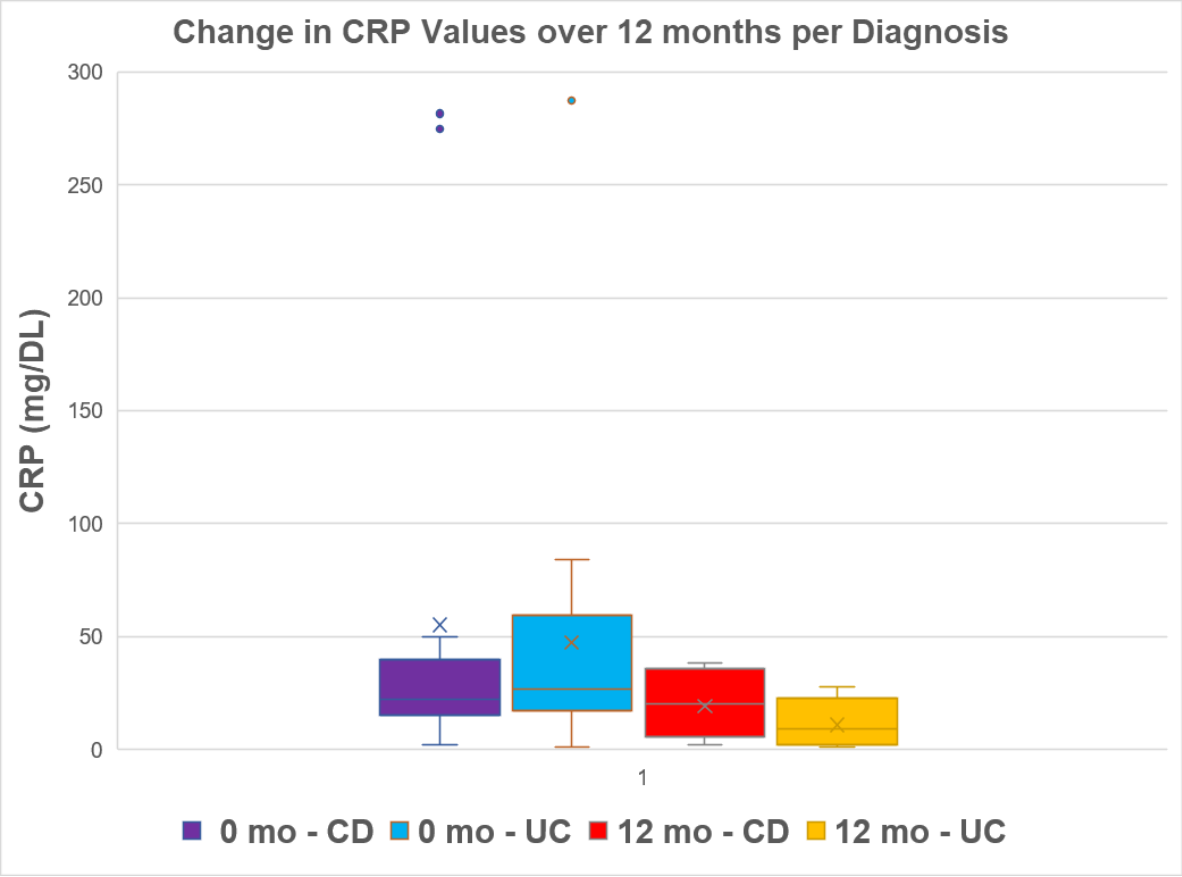

Figure 13: Comparison of CRP counts at baseline and 12 months while on AZA therapy versus patient diagnosis.

A total of 17 patients (42.5%) reported experiencing an ADR to AZA, this correlates well with the fact that 41% of the population experienced a decrease in WBC as shown in Figure 14. A two tailed paired t-test was conducted using the variables WBC baseline vs WBC month 12, with a confidence interval of 95%, which resulted in a nonsignificant finding of $p<0.9865$. However, a Spearman's rho Correlation test provided a significant pairing ($p<0.0001$) ($r=0.6201$) between WBC baseline and WBC month 12.

It must be noted that even though a patient shows symptoms and signs of an ADR such as nausea, vomiting and brittle teeth and biochemical results indicate a decrease in WBC, there may still be a decrease in inflammatory markers and thus the drug is effective at reducing the uncontrolled inflammatory reaction, but the risks of using AZA therapy can outweigh the benefit and AZA therapy should then be discontinued – this is observed in Patients 017, 018, 029 and patient 040. Or as in the case of Patients 006, 009, 025, 026, 035 and 038, the patients experienced drug efficacy shown as a decrease in both CRP and ESR and an increase or plateau in WBC, although they reported ADR to AZA. All ADR's reported in this study were obtained from the patient file and as such reported to the physician by the patient.

When comparing the change over 12 months in ESR vs the change in 12 months in CRP, no correlation was found when using the Pearson's correlation coefficient test ($r= -0,1605$). However, due to the fact that only 18 patient files contained both CRP and ESR, this finding may be skewed.

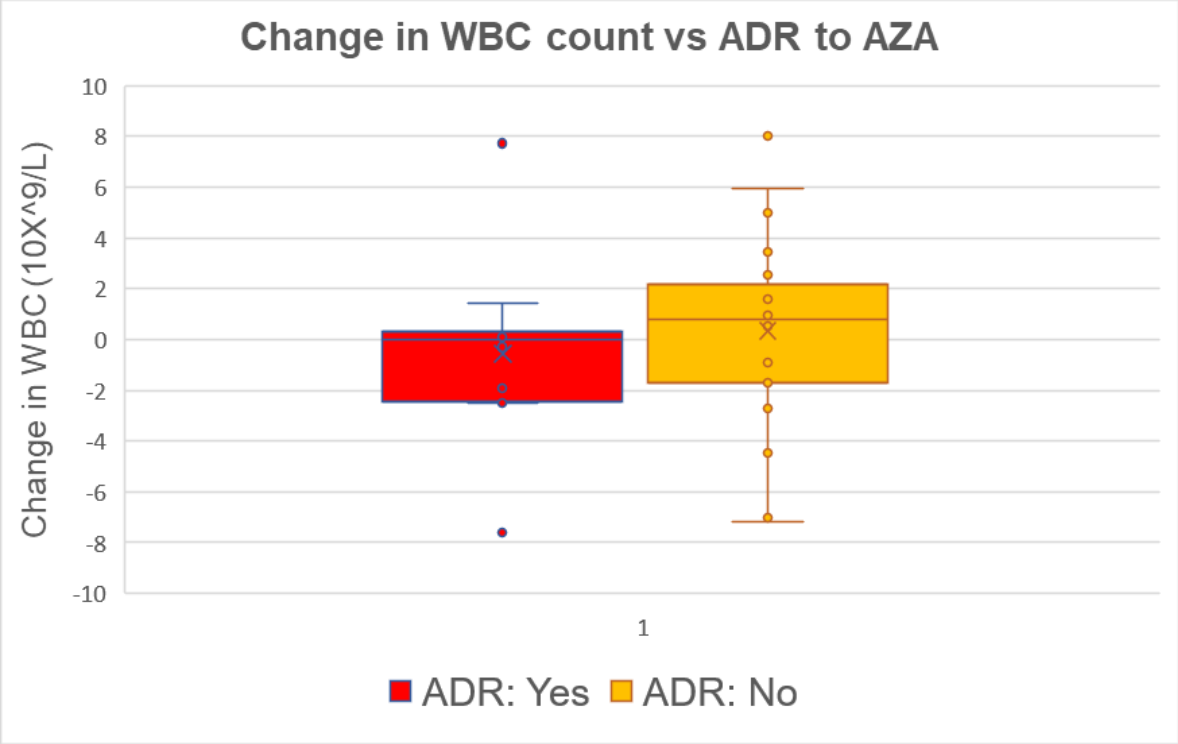

Figure 14:Change in WBC counts over 12 months in IBD patients on AZA therapy, separated according to whether an adverse drug reaction was reported. *Patients who experienced an ADR had an overall 0.459 x10^9/L decrease in WBC cell count compared to patients who did not experience an ADR while on AZA therapy who showed an average overall increase in WBC by 0.034 x10^9/L. A total of 18/40 patients reported experiencing an ADR to AZA therapy.*

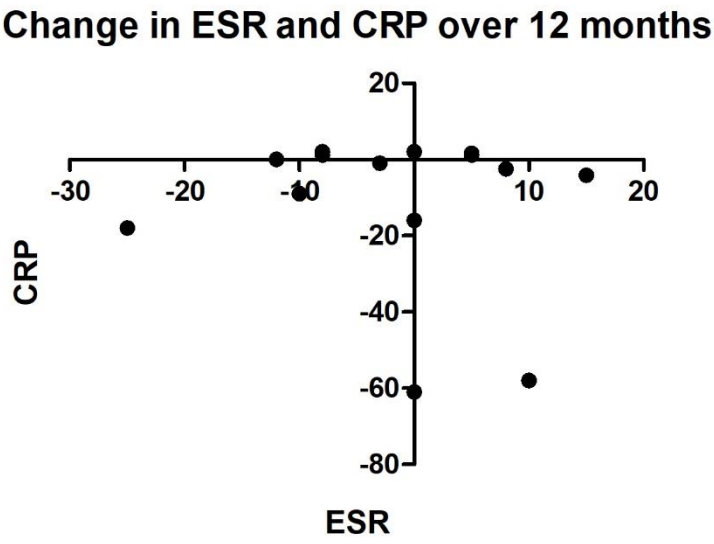

Figure 15: Spearman's rank correlation coefficient used to graph the change in CRP and ESR over 12 months in IBD patients on AZA therapy. *Contrary to literature the CRP and ESR did not have a strong association in these patients, however a weak relation can be seen at certain points. Note that this graph only details the 15 patients where both ESR and CRP counts were available on file, and excludes Patient 025, 027 and 038 who had outlier CRP counts at baseline*

5.5 Discussion

The laboratory result data obtained from this study showed a clear association – as seen in Table 22 in the Appendix- between the decrease in CRP concentrations and the increase in leucocyte (WBC) counts, even though there were only 34 sets of patient data where both CRP values and WBC counts were available for both baseline and 12 month treatment time points In practice, CRP concentrations are superior to leucocyte counts in detecting inflammation, however, WBC counts may be useful when CRP is not available as it is routinely measured in a FBC [149] as supported by the findings in this study.

As stated previously[136, 138], it has been well proven that ESR and CRP concentrations should have a strong correlation, however, findings in the study show a very weak link as seen in Figure 15. However, this may be due to lack of results as only 15 sets of both ESR and CRP results were available. CRP was shown to have a strong association with disease activity and an overall decrease of 15.67% was seen in the CRP concentration over 12 months following the AZA therapy. The ESR concentration was shown to have an overall average decrease of 9.00% however only 15 sets of data points were available. It is known that elevated CRP levels are associated with elevated ESR, anaemia and severe disease activity in IBD patients[136]. Elevated CRP cannot be correlated to histological inflammation in UC patients but shows a strong association in CD patients [136] .

Limitations to this objective were mostly due to the differences in testing procedure between the state and private health care facility. For example, state care includes ESR and CRP testing at each visit while private care mostly used CRP values and instead made use of scopes and faecal calprotectin testing on a regular basis. This resulted in data gaps for certain patients. As the samples were analysed by different labs- they would have been processed through different methodologies with different reference ranges. In this study the reference range for each patient was not included but rather an average “normal” was included from literature because ideally a reference range will differ per patient depending on factors such as gender, age or race that is calculated using an algorithm by each lab.

The exclusion criteria for this study excludes patients with rheumatoid arthritis (RA) due to the potential of RA to distort the therapeutic markers for the study. However,

patients diagnosed with spondyloarthritis (SpA) were included and this is because studies have shown that the SpA that IBD patients experience is very different from RA in that only the major joints such as the knees or ankles are affected, normally only 5 or less joints are affected and that the arthritis is not erosive to the joints and has no long term damage unlike RA[150, 151]. A paper by Orchard[151] hypothesised that while SpA and IBD may have genetic ties, the pathophysiology of IBD may actually play a larger role in that a “leaky gut” caused by the lesions and fistula common to IBD patients results in the immune system being exposed to luminal bacteria it would otherwise not be exposed which the body responds to with SpA, however, this hypothesis has yet to be proved[151]. Hence, while it seemed pertinent not to exclude the SpA patient from the study, it must be noted that a comorbidity such as this does have the potential to skew both the therapeutic marker analysis or played a role in the patients’ tolerance level of certain therapeutic drugs which could result in ADR’s.

5.6 Conclusion

An accurate representation of the association and roles of CRP, ESR and WBC with AZA therapy in IBD patients was limited by the small patient cohort and missing data, however, this study did show a change in baseline in all three therapeutic markers. CRP concentration decreased by an average of 15.67% and the WBC count increased by an average of 4.63%. Despite more than half of the subject's missing ESR values, it does appear as if AZA therapy does have the desired outcome on the IBD patients by decreasing the CRP counts in both UC and CD patients. The change in leukocyte count is associated with the presence of the patient having experienced an ADR while on AZA therapy, with majority of patients who reported an ADR also presenting with a decrease in leukocytes.

CHAPTER 6: AZA DOSING GROUP COMPARISONS

5.1 Abstract

5.1.1 Objective

To compare the frequency of *TPMT* SNP's within dose groups.

5.1.2 Materials & Methods

Patient files were obtained to determine the AZA dose concentration for each patient at baseline dosing and again at 12 months post dosing. The dosing concentration at month 12 was compared to the presence or absence of SNP's in each patient to determine if a relationship or correlation can be observed between the dose group and the frequency of the SNP.

5.1.3 Results

A total of 22/40 (55%) of IBD individuals were on ≤ 100 mg/day of AZA and 14/40 (35%) were on >100 mg/day; ≤ 150 mg/day at month 12. The calculated probability of the Fisher's Exact Test of Independence was 0.080 when comparing AZA dosage and TPMT genotype. Male individuals showed a higher average dose at baseline and patients diagnosed with UC had a lower average AZA dose at baseline.

5.1.4 Conclusion

Azathioprine therapeutic dosage was independent of the *TPMT* genotype in the IBD study population. It can however be deduced that gender and diagnosis may play a role in the dosing regimen.

5.2 Introduction

Genetic polymorphisms of the *TPMT* gene play a large role in the variability of AZA pharmacokinetics in individuals, as such, patients who possess this enzyme activity altering polymorphism needs to be treated with at least a 10% reduction of the regular AZA dose concentration and monitored for both efficacy and toxicity [152]. During the first year of AZA therapy, drug concentration may be altered due to ADR or lack of efficacy. On average it takes 17 weeks for AZA to have a therapeutic effect[10, 22, 27], implying that by 12 months post initial dose it should be apparent whether the AZA dosage needs to be adjusted, or an alternative treatment should be considered [19].

There is no consensus as to whether AZA dosing should be according to body weight or individualised factors -which includes type of disease, disease status, *TPMT* genotype, concomitant medication and other comorbidities. While current guidelines recommend weight-based dosing over individualised dosing, this can have severe consequences should the patient have an activity reducing *TPMT* SNP [153].

Product package inserts of common brand names suggest a loading dose of 1-5 mg/kg body weight/day and a maintenance dose of approximately half of that; a vague suggestion that can be adapted to an individualised situation. Other studies [113, 154, 155] found an average dose of 2-3 mg/kg body weight/day produced better therapeutic effects than the lower dosages. The dosage information adapted for a 70 kg male is listed in Table 13 below.

Table 12: Dosing Suggestions

Author	**Dose Guideline (mg/kg/day)**	**Dose for 70kg male**
Dassopoulos *et al* [153]	2.0 – 3.5	140 – 245 mg/day
Bermejo *et al* [154]	1.8 – 2.6	126 – 182 mg/day
De Boer *et al* [155]	2.0	140 mg/day
DiPiero *et al* [113]	2.0 – 3.0	140 – 210 mg/day

Another factor influencing the administered drug dose, is the route of administration; AZA is available in dosages of 50 mg tablets or 50 mg vials for injection under various brand names. Tablet form tends to be the popular choice as it can be administered by the patient at home, however, it limits the therapeutic accuracy that may be achieved by body-weight dosing as tablets cannot be accurately and precisely divided.

5.3 Materials and Methods

Upon signing consent, patients were grouped according to their AZA dosage at 12 months post first dosage. The dosage groups are listed in Table 14 below.

Table 13: AZA dosing groups

Group 1	≤ 100 mg/day
Group 2	> 100 mg/day - ≤150 mg/day
Group 3	> 150 mg/day

5.4 Results

As can be seen in Table 15, at month 12; 22/40 (55%) of the study population fell within Group 1 - ≤ 100 mg/day, 14/40 (35%) of the study population fell within Group 2 - > 100 mg/day, ≤ 150 mg/day, while only 4/40 (10%) patients fell within Group 3 - > 150 mg/day as shown in Figure 16. 33/40 patients had no change in dose over 12 months.

Table 14: Diagnosis, adverse drug reaction, genotype and dosing group numerical data of IBD patients on AZA therapy for 12 months

Variable	Name / Value	n (%)
Diagnosis	Crohn's Disease	19 (47.5)
	Ulcerative Colitis	21 (52.5)
ADR	No	23 (57.5)
	Yes	17 (42.5)
Genotype	*3B/*3B	32 (82.5)
	1/*3A	6 (15)
	1/*3B	1 (2.5)
	1/*3C	0
	1/1	0
Dosing Group	≤ 100 mg/day	22 (55)
	> 100 mg/day, ≤ 150 mg/	14 (35)
	> 150 mg/day	4 (10)

Table 15: Fisher's exact calculation derived from the tabulation of phenotype and AZA dosage in IBD study population

Genotype	Dosage (mg/day)			
	≤ 100	**> 100; ≤150**	**> 150**	**TOTAL**
Haplo *3B	2	3	1	6
Heterozyg*3B	0	0	1	1
Homozyg *3B	20	11	2	33
TOTAL	22	14	4	**40**
Fisher's exact = 0.080				

As shown in Table 16, a calculation of Fisher's exact test of independence provided a

value of 0.080 when testing for the non-random association of AZA dosage at month 12 compared to the patient genotype, proving that the *TPMT* genotype of the IBD patient does not statistically increase the chance of an individual falling within a certain dosage group. Of the 17 study participants who reported an ADR, 53% were within the lowest dosage group of ≤ 100 mg/day while the other 47% of the individuals reporting an ADR were in the middle dosage group at month 12.

Table 16: IBD patients who had a change in AZA dosage over 12 months

PT #	Dose (0 mo)	Dose (12 mo)	Dose change	Altered dose	Gender
P009	150	100	-50	Decrease in dose	M
P014	100	75	-25	Decrease in dose	F
P028	150	100	-50	Decrease in dose	M
P035	150	100	-50	Decrease in dose	M
P022	100	150	50	Increase in dose	F
P023	150	200	50	Increase in dose	M
P037	150	200	50	Increase in dose	M

Table 17 shows the 7 IBD patients that had a change from their baseline AZA dosage at some point during the initial 12 months of therapy. 4 of the patients had a decrease in dosage while 3 had an increase in dose. Patient 023 and 037 were both males who had a dose increase from 150 mg/ day – 200 mg/day and as seen in Table 23 in the Appendix both had a *TPMT* genotype of *3B/*3B, neither reported experiencing and ADR however their dosages were increased as they presented with increased disease activity. If a patient experienced dose-dependent adverse events, the dosage was decreased to see if efficacy could still be maintained.

Table 17: AZA dosage means, mode and range at baseline and month 12 according to diagnosis and gender. *All units are mg/day. StD – standard deviation, mo - months*

		Mean		StD (±)		Range at 1StD		Mode	
		0 mo	12 mo	0 mo	12 mo	0 mo	12 mo	0 mo	12 mo
	All	126.25	125.63	31.6	36.01	94.65 - 157.85	94.03 - 161.64	100	100
Gender	**Females**	126.92	126.28	31.72	36.23	95.20 - 158.64	94.56 - 162.51	100	100
	Males	127.63	126.97	31.82	36.45	95.81 - 159.45	95.15 - 163.42	150	100
Diagnosis	**UC**	124.29	125.71	30.17	34.58	94.12 - 154.46	95.54 - 160.29	100	100
	CD	126.25	125.63	31.6	36.01	94.65 - 157.85	94.03 - 161.63	100	100

Table 18 shows the numerical breakdown of AZA dosing within the study population at baseline and month 12. Note that none of the patients included in this study were dosed according to body weight. When analysed according to mean, the male group

has a slightly higher average dosing at baseline (127.63 mg/day) than the females. The male dosing average is also higher than the overall mean and at month 12 the male average normalises with the female group average, but it remains higher than the overall dosing average at month 12. This is mirrored in the modal class where the male group is the only group to have a higher frequency of individuals on 150 mg/day of AZA. The UC diagnosis group has a lower average dosing at baseline (124.29 mg/day), than the CD group (126.25 mg/day) and the overall population average (126.25 mg/day). However, at month 12 the mean dosage of the UC group normalised to that of the overall population. This lower dosage correlates to the fact that UC patients tend to be on lower doses of AZA as their symptoms are not as severe, but also that they appear to have a higher response rate to the drug compared to CD patients- this was shown in the analysis of CRP change in Chapter 5. The lower dose in UC patients is also seen at 1 standard deviation at both baseline and month 12 and is mirrored in the upper range of dosing at baseline and 12 in Table 18.

5.5 Discussion

This study showed that despite reporting ADR to AZA therapy, many of the patients still experienced therapeutic efficacy. This can be explained by the fact if a patient experienced a dose-related side effect such as nausea, vomiting or pruritis but still showed signs of clinical efficacy -case dependent- the physicians would rather lower the dose of AZA and monitor the adverse events than immediately seek alternative therapy. This is partially because finding an effective therapy for IBD is very patient specific and generally if AZA fails to elicit disease remission, the next treatment step would involve high cost biological therapy. However, should the patient not be a member of a medical aid, gaining access to biological therapy becomes very difficult and costly as South African state health care has limited access to biological therapy.

While a Fisher's Exact Test of Independence of 0.080 indicates that the study population genotype does not predict the ideal AZA dosage (i.e. the AZA dosage is independent of the *TPMT* genotype); it can be deduced as shown in Table 18 that gender may play a role in the dosing regimen. A total of 5/7 individuals who had a change in dosing regimens between baseline and month 12, were male despite the dominant female demographic of the study population. It would appear that this finding may also support previous literature studies [153] in that weight dosage play an important role in the dosing regimen of AZA and hence the weight of the patient should be

considered.

According to dosing suggestions in literature [113, 153], as shown in Table 13, the average 70kg male should receive a minimum average dose of 140 mg/day and the dosing should be increased up to maximum range of 250 mg/day if required. However, the average AZA dosing at baseline and month 12 for the overall population is lower than the dosing suggestions – this may be due to the predominantly female demographic of the study population and the fact that most of the females were on a lower dose of AZA.

In both sets of patients (from private and state health care) AZA was prescribed in tablet form, hence the physicians did not particularly make use of weight dosing and instead reverted back to a standard dosing regimen that was a “safe” starting point from previous experience – generally 100mg/day in state and 150mg/day for private care. In the cases of younger patients (e.g. under 25) the dosage was lower. If it was seen that AZA therapy was not controlling the disease activity (excessively high CRP or intestinal pain) and the patient’s WBC were still adequate, the dosage would be increased per 50mg/day until the physician felt the disease was under better control. However, in cases were the patient was showing signs of an ADR but still experiencing disease remission, the dosage would be lowered to see if the disease remission could be successfully maintained while and the ADR’s avoided. Details of the change in dosage can be seen in Table 23 in the Appendix.

In order to obtain and adequate number of patients in the study, patients who had previously been on AZA therapy were also included in the study- in these cases patient files had to be relied on for accurate patient information. As it is not common practice for doctors to prescribe AZA dosage according to body weight due to the limitations of drug in tablet form, body weight is not recorded in patient files, making dosing analysis more variable.

Lack of consistent patient data proved to be a large limitation in this study, especially as two separate doctor’s notes were used. Difference in actual dose versus what dose the patient should theoretically be receiving according to their weight played a role in accurate analysis of the data in terms of ADR’s. It was thus difficult to distinguish if the patient was really experiencing an ADR (e.g. a dose independent AE) or was it purely a dose-dependent AE that could be avoided with correct weight-dosing procedures?

5.6 Conclusion

A Fisher's Exact Test of Independence comparing dose to genotype class gave a probability of 0.080, indicates that the AZA therapeutic dosage is independent of the *TPMT* genotype in the IBD study population. It can however, be deduced that gender and diagnosis may play a role in the dosing regimen, with male individuals having on average a higher AZA dose at baseline than the overall sample population average and UC patients receiving a lower average AZA dose at both baseline and at month 12 when compared to the overall population dosing average. If the patient receives an AZA dosage that is higher than what is tolerable, they may experience an AE, if the dosage is reduced and the AE persists it will be classified as an ADR and the therapy will likely be discontinued.

CHAPTER 7: DISCUSSION & CONCLUSION SUMMARY

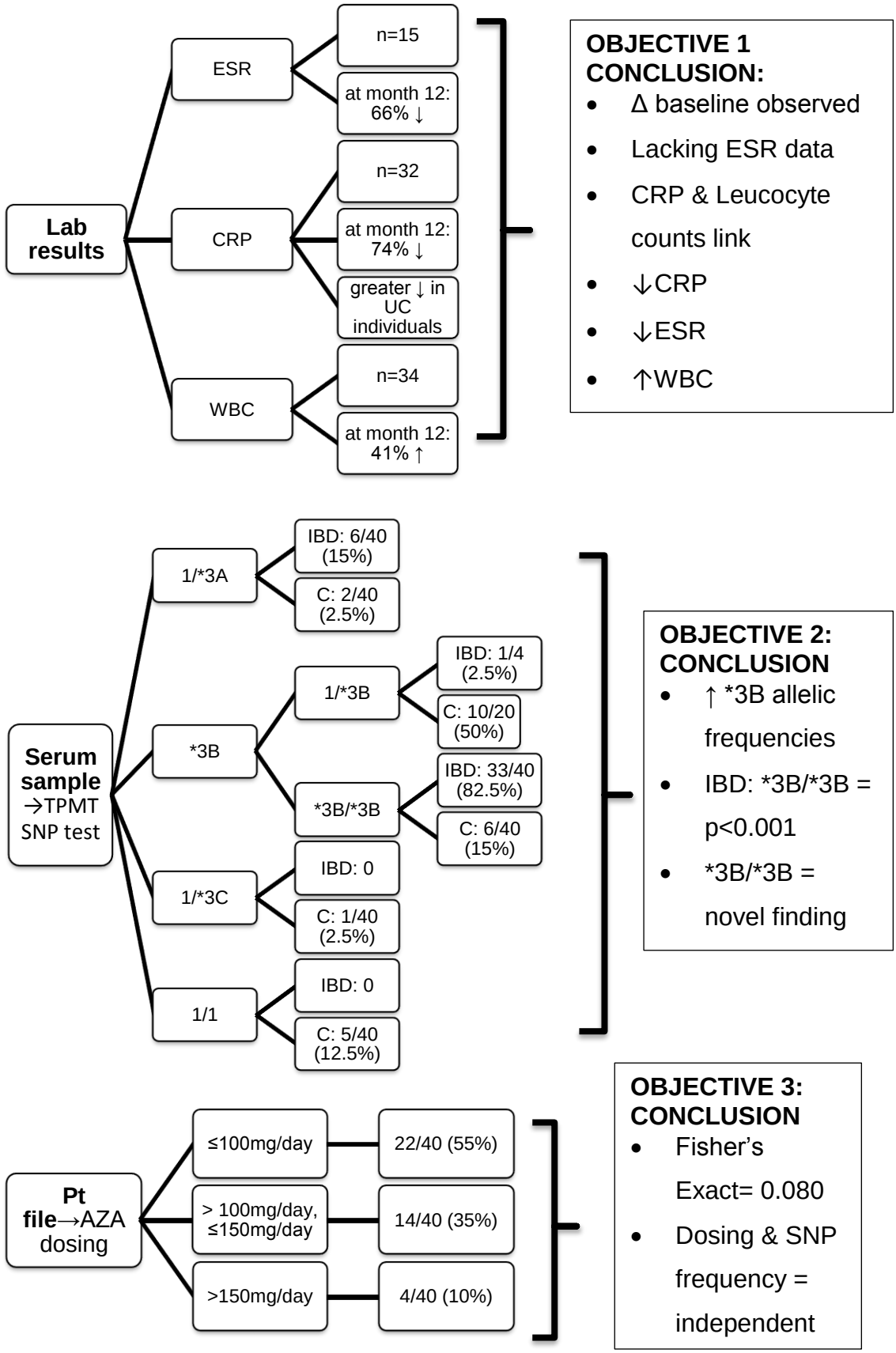

Figure 16: Summary of conclusions for each objective

As in many cases of people suffering from an autoimmune disease, many patients who had IBD also suffered from arthritis, arthralgia or fibromyalgia. The pathophysiology of these diseases, other comorbidities or even the concomitant medications used to treat these separate diseases may have construed the therapeutic marker values assessed and made the assessment of ADR's (due to drug or due to SNP) more difficult.

However, the largest limitation to this study is lack of funding and small patient numbers. Ideally the sample population should consist of at least 100 IBD patients and 100 matched control samples, and all 41 *TPMT* SNP's should be a tested. Hence these results should be regarded as a pilot study to demonstrate the potential role that genetic testing can play in the South African IBD population. Further enzymatic analysis of the homozygous SNP's would also have greatly benefitted the outcomes of this study.

When comparing the presence of *TPMT* *3A, *3B and *3C in the healthy South African population, it is evident that there is a higher allelic frequency than other global populations; most notably in the presence of *TPMT* *3B. This study showed not only an increase in the allelic frequency of *TPMT* *3B, but also observed the statistically significant presence ($p<0.001$) of homozygous *TPMT* *3B alleles in South African IBD patients when compared to healthy South African individuals- this has not previously been reported in published literature.

Thus, in future, the enzymatic effect of *TPMT* *3B/*3B should be studied in a larger sample size prior to recommending early warning SNP testing in IBD patient using AZA as these results cannot be ignored.

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APPENDIX

1. Patient Information Leaflet and Informed Consent

Protocol no: AZA-2017

Protocol title: Identification of Single Nucleotide Polymorphisms in Inflammatory Bowel Disease Patients on Azathioprine Therapy

Participant no: _____

Name of investigator: _____

Dear Participant

You are invited to join a research project involving the medication that you are currently using for the treatment of your inflammatory bowel disease. This medication is Azathioprine, also known as Imuran.

Before you agree to take part in this research project, you must please read this information sheet as it contains important information to help you decide whether or not it is in your best interests to do so.

You are encouraged to ask as many questions as needed to make sure that you understand all the study steps, including possible risks and benefits. If you have any questions that are not properly explained or answered in this form, please feel free to ask Lyla Adam to give you more information. You are welcome to take this document home with you and discuss joining the study, with your family and friends.

The study has been approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria and compliance with medical and ethical standards. In addition, the study will follow all the requirements of the declaration of Helsinki *(last updated at the* 64th WMA General Assembly, Fortaleza, Brazil, October 2013*)* and *Guidelines for good practice in the conduct of clinical trials with a human participant in South Africa,* which deal with your rights as a person joining a research study and which guide the study doctor in biomedical research involving human participants. (ICH Harmonised Tripartite Guideline for Good Clinical Practice 2015 Integrated addendum to ICH E6 (R1): Guideline for Good Clinical Practice).

Why have you been invited to take part?

You have been invited to join this research study, as your doctor has diagnosed you with inflammatory bowel disease which includes Crohn's disease or Ulcerative Colitis and has prescribed Imuran therapy. You have thus been invited to join this research study to see if this medicine is helpful in decreasing the inflammation and irritation of your disease.

Your rights as a participant

Joining this study is your own choice. You may choose not to consent to a blood sample or to have your file reviewed. If you decide not to join the study, you will still be able to see the study doctor and your medication will not be stopped.

Who may take part in this study?

☑ You must be able to answer **YES** to all of these requirements in order for you to be a good match to join this study:

1. Sign this consent form for trial participation
2. Males or females > 18years
3. Diagnosed with ulcerative colitis or Crohn's disease
4. Be on azathioprine treatment for a minimum of 12 months without stopping treatment

☒ You must be able to answer **NO** to all of these requirements in order for you to be a good match to join this study. If you have or have had any of the following, you will not be allowed to take part in the study:

1. Diagnosed with autoimmune hepatitis, rheumatoid arthritis or acute lymphoblastic leukaemia
2. Have bone marrow suppression when treatment started
3. Stop-start use of azathioprine at any point during the first 12 months
4. Individuals who are pregnant at any stage during the first 12 months of treatment
5. Unwilling to consent
6. Any other medical condition which in the opinion of the investigator could compromise the participant's ability to participate in the study

Study design

Azathioprine is normally broken down by an enzyme called Thiopurine Methyltransferase (*TPMT* for short) Sometimes this enzyme doesn't work properly because of a type of genetic change in the body called Single Nucleotide Polymorphisms (SNP's). These are different from normal genetic mutations in that the specific genetic change happens in more than 1% of all the people in the world. This is why your medicine might make you feel worse than other people's medicine will make them feel, because your body struggles to break the medicine down. Depending on the type of SNP you have, your enzyme may work less than it should. This is referred to as *TPMT* enzyme status.

This is an observational clinical study to see what the *TPMT* enzyme status is of patients on azathioprine (Imuran) medication for ulcerative colitis or Crohn's disease at the Gastroenterology Unit of Steve Biko Academic Hospital and a private hospital.

Once you have signed this form and given permission, your medical history will be collected from your hospital file. We will look at what disease you have, when you were diagnosed, what other medicine you are taking and if you have any other illnesses. We are only interested in the 12 months from when you first started taking your azathioprine (Imuran) medication. We will look at the blood results from the blood samples the doctor takes at your normal visits to see if your medicine is working to stop the swelling and irritation of the intestines. We will also look at the notes the doctors have made from any endoscopies or biopsies that may have been done in the first 12 months after you started taking the medication.

1 tube of blood (5ml) will be collected to look at the *TPMT* gene that created the enzyme by looking at a sample of your blood to see if you have the normal enzyme or a changed enzyme.

Study procedures

To determine if you me*et al*l criteria to join this study, you will be asked to do the following:

- Sign this informed consent form for joining before any study related steps
- Your date of birthdate, gender and race will be recorded
- A complete medical history will be noted
- 5 mL (1 teaspoons) of blood will be collected for the genetic testing
- Review of any medication used in the first 12 months of azathioprine treatment

What will happen to my blood and tissue samples?

Your blood will not be stored for any other testing. Blood samples will be taken to the Department of Physiology to be processed and analysed.

What are the possible risks or side-effects of being in the study?

There may be a possible risk when drawing of blood, like some discomfort that doesn't last long and sometimes bruising at the place where the needle is inserted.

What are the possible benefits of being in the study?

There will be no healing benefit to this research, but understanding what type of enzyme you have will help the doctor to be alert when looking for certain adverse events of the medicine that can make you feel sick and make sure that you are getting the best treatment for your specific disease. Once the *TPMT* gene that creates the enzyme is processed, the information will be made available to your doctor at the Gastrointestinal Unit.

The information gained during the study can benefit other people who are also using this medicine and suffering from the same disease as you by gaining useful information on the how the *TPMT* gene that creates the enzyme differ in different people, especially in the African population.

Confidentiality

All records identifying you will be kept confidential and will not be made publicly available. We will use personal information about you to conduct this research, this may include your name, medical history and information from your file. However, this personal information is not included in the study data that will be reported at the end of the research. You will be identified by a coded number in any reports of publications produced from this study.

You have the right to ask the study doctor about the data being collected on you and to see your personal health information.

Study results

You will be able to obtain information about your study results and the outcome of the study the next time you visit the Gastrointestinal Unit or by contacting the investigator:
Lyla Adam

083 2924 053

Consent Statement

By signing below, I agree that:

I have read or had read to me the information sheet and consent form, **version 1, dated 21 June 2017** for this study in a language that I understand the purpose of this study has been explained to me. I have been given time to ask questions and they have been suitably answered. I understand that choosing not to participate in this trial does not mean that my treatment will be stopped. I agree to participate in this research study.

Printed Name and Surname of participant	Signature of participant
Date (completed by participant)	Time (completed by participant)
Printed Name and Surname of Investigator	Signature of Investigator
Date	Time

I hereby verify that verbal consent was obtained from the above participant. The participant has been informed about the risks and the benefits of the research, understands such risks and benefits and is able to give consent to participate, without coercion, undue influence or inappropriate incentives.

Printed Name and Surname of Impartial Witness	Signature of Impartial Witness (IW)
Date	Time

1.1 Data Tables

Table 18: Medications used by IBD patients in study

PT #	Diag	Pentasa	Asacol	MTX	Ento	Humira	RVX	PDS	SZN
P001	CD	X	X						
P002	CD		X	X					
P003	UC		X					X	
P004	UC		X						
P005	UC	X							
P006	UC		X						
P007	CD								
P008	UC	X		X					
P009	UC		X					X	
P010	UC				X				
P011	CD								
P012	CD		X			X			
P013	UC		X	X					X
P014	CD					X		X	
P015	UC		X					X	
P016	CD							X	
P017	CD						X		
P018	UC		X				X	X	
P019	CD							X	
P020	UC	X						X	
P021	CD	X				X			
P022	CD				X				
P023	UC	X							
P024	UC	X							X
P025	CD							X	
P026	CD								
P027	UC		X			X			
P028	UC	X	X						
P029	UC		X					X	
P030	UC		X						
P031	CD							X	X
P032	CD			X				X	
P033	CD	X	X						
P034	UC	X							

P035	UC		X					X	
P036	UC		X					X	
P037	UC	X							
P038	CD							X	
P039	CD							X	X
P040	CD		X			X			

Diag; Diagnosis, Ento; Entocord, MTX; Methotrexate, RVX; Revellex, PDS; Prednisone, SZN; Salazopyrin

Table 19: Prescribed Vitamin and Mineral supplementation in IBD study patients

PT #	Gender	Diagnosis	Vit B12	Vit D	Calcium	Folic acid	Iron
P001	F	CD		X	X		
P002	F	CD					
P003	M	UC					
P004	F	UC					
P005	F	UC					
P006	F	UC				X	
P007	M	CD					
P008	F	UC					
P009	M	UC					
P010	F	UC					
P011	F	CD				X	
P012	M	CD					
P013	F	UC					
P014	F	CD					
P015	M	UC					
P016	F	CD				X	
P017	F	CD	X			X	
P018	F	UC				X	X
P019	M	CD				X	
P020	F	UC				X	
P021	F	CD				X	
P022	F	CD	X			X	
P023	M	UC					
P024	F	UC				X	
P025	F	CD				X	
P026	F	CD					

P027	F	UC					
P028	M	UC					
P029	M	UC				X	
P030	F	UC					
P031	F	CD					
P032	F	CD					
P033	F	CD		X	X		
P034	F	UC					
P035	M	UC					
P036	M	UC					
P037	M	UC					
P038	F	CD				X	
P039	F	CD					
P040	M	CD					

Table 20: Patient Demographics, *TPMT* genotype and *TPMT* phenotype

PT #	Genotype	Genotype	Gender	Race	Diagnosis	ADR
P001	*3B/*3B	Homozygous *3B	F	W	CD	No
P002	*3B/*3B	Homozygous *3B	F	W	CD	No
P003	*3B/*3B	Homozygous *3B	M	B	UC	No
P004	*3B/*3B	Homozygous *3B	F	B	UC	No
P005	*3B/*3B	Homozygous *3B	F	C	UC	No
P006	*3B/*3B	Homozygous *3B	F	B	UC	Yes
P007	*1/*3A	Heterozygous *3A	M	W	CD	Yes
P008	*3B/*3B	Homozygous *3B	F	W	UC	Yes
P009	*3B/*3B	Homozygous *3B	M	W	UC	No
P010	*3B/*3B	Homozygous *3B	F	W	UC	No
P011	*3B/*3B	Homozygous *3B	F	W	CD	Yes
P012	*3B/*3B	Homozygous *3B	M	W	CD	Yes
P013	*3B/*3B	Homozygous *3B	F	W	UC	Yes
P014	*1/*3A	Heterozygous *3A	F	W	CD	No
P015	*3B/*3B	Homozygous *3B	M	W	UC	No
P016	*1/*3A	Heterozygous *3A	F	W	CD	Yes
P017	*1/*3A	Heterozygous *3A	F	W	CD	Yes

P018	*1/*3A	Heterozygous *3A	F	W	UC	No
P019	*3B/*3B	Homozygous *3B	M	W	CD	No
P020	*3B/*3B	Homozygous *3B	F	W	UC	Yes
P021	*3B/*3B	Homozygous *3B	F	W	CD	No
P022	*3B/*3B	Homozygous *3B	F	W	CD	No
P023	*3B/*3B	Homozygous *3B	M	I	UC	No
P024	*3B/*3B	Homozygous *3B	F	W	UC	Yes
P025	*3B/*3B	Homozygous *3B	F	W	CD	Yes
P026	*3B/*3B	Homozygous *3B	F	I	CD	No
P027	*3B/*3B	Homozygous *3B	F	W	UC	No
P028	*3B/*3B	Homozygous *3B	M	W	UC	Yes
P029	*3B/*3B	Homozygous *3B	M	W	UC	Yes
P030	*3B/*3B	Homozygous *3B	F	W	UC	No
P031	*1/*3A	Heterozygous *3A	F	W	CD	Yes
P032	*3B/*3B	Homozygous *3B	F	W	CD	No
PT #	**Final Genotype**	**Phenotype**	**Gender**	**Race**	**Diagnosis**	**ADR**
P033	*3B/*3B	Homozygous *3B	F	W	CD	No
P034	*3B/*3B	Homozygous *3B	F	B	UC	Yes
P035	*3B/*3B	Homozygous *3B	M	W	UC	No
P036	*3B/*3B	Homozygous *3B	M	W	UC	No
P037	*3B/*3B	Homozygous *3B	M	I	UC	Yes
P038	*3B/*3B	Homozygous *3B	F	W	CD	No
P039	*1/*3B	Heterozygous *3B	F	W	CD	Yes
P040	*3B/*3B	Homozygous *3B	M	W	CD	No

F; female, M; male, W; white (Caucasian), B; Black, C; Coloured, I; Indian, CD; Crohn's disease, UC; Ulcerative Colitis, ADR; adverse drug reaction.

Table 21: CRP, ESR and leukocyte counts reported in medical files and the change in these parameters over 12 months in IBD patients on AZA therapy. *Patients 007, 012,030 and 032 were excluded from the Table as no laboratory results were available on file. Normal CRP is < 2 mg/L, Normal ESR 0-25 mm/hr. CRP; c-reactive proteins, ESR; erythrocyte sedimentation rate, WBC; white blood cells, mo; month*

	ESR (mm/hr)			CRP(mg/L)			WBC (x 109/L)		
PT #	0 mo	12 mo	Change	0 mo	12 mo	Change	0 mo	12 mo	Change
P001				40.00	35.90	**-4.10**	17.00	25.00	8.00
P002				10.70	8.20	**-2.50**			
P003	12.00	20.00	8.00	27.00	9.00	**-18.00**	6.58	4.92	-1.66
P004	40.00	15.00	**-25.00**				3.76	5.57	1.81
P005	100.00	10.00	**-90.00**	64.00	6.00	**-58.00**	10.26	5.79	-4.47
P006	50.00	60.00	10.00	84.00	23.00	**-61.00**	12.08	13.53	1.45
P008				1.00	1.00	0.00	8.49	8.19	-0.30
P009				39.50	2.00	**-37.50**	7.10	7.40	0.30
P010				9.20	1.60	**-7.60**	7.20	13.14	5.94
P011				15.00	2.00	**-13.00**	7.90	8.70	0.80
P013				21.20	18.00	-3.20	10.70	8.80	-1.90
P014				2.00	2.00	0.00	6.90	7.00	0.10
P015				22.80	24.00	1.20	6.30	7.90	1.60
P016	16.00	8.00	**-8.00**	16.00	7.00	**-9.00**	6.17	6.70	0.53
P017	15.00	5.00	**-10.00**	22.60	2.00	**-20.60**	11.86	9.44	-2.42
P018				53.3	10.70	**-42.60**	11.40	8.90	-2.50
P019				38	38.10	0.10	6.60	10.04	3.44
P020				39	28.00	**-11.00**	8.30	6.60	-1.70
P021				22.00	7.00	**-15.00**			
P022				3.40	5.00	1.60	5.70	8.23	2.53
P023	2.00	7.00	5.00	4.00	3.00	**-1.00**	7.18	8.15	0.97
P024	8.00	5.00	**-3.00**	20.00	10.00	**-10.00**	7.10	6.20	-0.90
P025				281.70	38.60	**-243.10**	12.00	12.00	0.00
P026	5.00	3.00	**-2.00**	25.00	27.00	2.00	7.87	15.60	7.73
P027	11.00	3.00	**-8.00**	287.50	2.70	**-284.80**	10.80	8.10	-2.70
P028				59.30	17.20	**-42.10**	12.40	5.20	-7.20
P029				17.00	1.00	**-16.00**	9.20	6.70	-2.50
P031	30.00	18.00	**-12.00**	22.00	24.00	2.00	16.21	14.83	-1.38

	ESR (mm/hr)			CRP(mg/L)			WBC (x 109/L)		
PT #	0 mo	12 mo	Change	0 mo	12 mo	Change	0 mo	12 mo	Change
P033				40.00	35.90	**-4.10**	17.00	22.00	5.00
P034	48.00	63.00	15.00	82.00	24.00	**-58.00**	11.60	12.40	0.80
P035				41.30	1.00	**-40.30**	7.10	7.40	0.30
P036				22.80	24.00	1.20	6.30	7.90	1.60
P037	2.000	7.000	5.000	5.00	2.00	**-3.00**	7.18	8.15	0.97
P038				275.00	37.00	**-238.00**	9.00	9.00	0.00
P039	29.00	17.00	**-12.00**	22.00	24.00	2.00	19.00	12.00	-7.00
P040	23.00	15.00	**-8.00**	50.00	16.00	**-34.00**	15.60	8.00	-7.60

Table 22: AZA dosing group allocations according to the AZA dosage at baseline and month 12 for IBD patients including final genotype and whether the subject reported an ADR

PT #	Dose (0 mo)	Dose (12 mo)	Dose Group	Final Genotype	ADR?
P001	100	100	1	*3B/*3B	No
P002	100	100	1	*3B/*3B	No
P003	100	100	1	*3B/*3B	No
P004	100	100	1	*3B/*3B	No
P005	100	100	1	*3B/*3B	No
P006	100	100	1	*3B/*3B	No
P007	100	100	1	*1/*3A	Yes
P008	150	150	2	*3B/*3B	Yes
P009	150	100	1	*3B/*3B	Yes
P010	100	100	1	*3B/*3B	No
P011	150	150	2	*3B/*3B	No
P012	100	100	1	*3B/*3B	Yes
P013	100	100	1	*3B/*3B	Yes
P014	100	75	1	*1/*3A	Yes
P015	150	150	2	*3B/*3B	No
P016	150	150	2	*1/*3A	No
P017	150	150	2	*1/*3A	Yes
P018	150	150	2	*1/*3A	Yes
P019	150	150	2	*3B/*3B	No
P020	100	100	1	*3B/*3B	No
P021	50	50	1	*3B/*3B	Yes
P022	100	150	2	*3B/*3B	No
P023	150	200	3	*3B/*3B	No
P024	100	100	1	*3B/*3B	No
P025	150	150	2	*3B/*3B	Yes
P026	100	100	1	*3B/*3B	Yes
P027	100	100	1	*3B/*3B	No
P028	150	100	1	*3B/*3B	No
P029	150	150	2	*3B/*3B	Yes
P030	150	150	2	*3B/*3B	Yes

PT #	Dose (0 mo)	Dose (12 mo)	Dose Group	Final Genotype	ADR?
P031	200	200	3	*1/*3A	No
P032	150	150	2	*3B/*3B	Yes
P033	100	100	1	*3B/*3B	No
P034	100	100	1	*3B/*3B	No
P035	150	100	1	*3B/*3B	Yes
P036	150	150	2	*3B/*3B	No
P037	150	200	3	*3B/*3B	No
P038	150	150	2	*3B/*3B	Yes
P039	200	200	3	*1/*3B	No
P040	100	100	1	*3B/*3B	Yes

2. Restriction Patterns

2.1 *TPMT* *3B

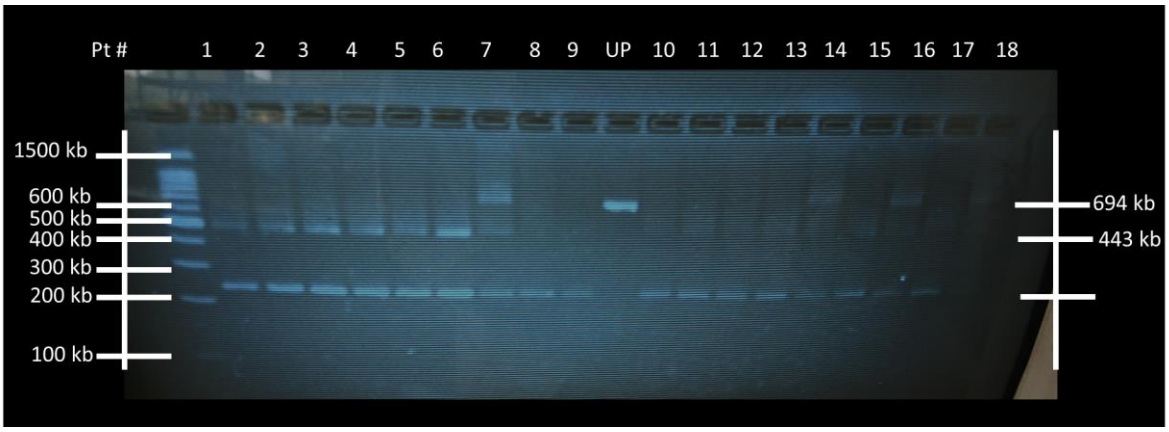

Figure 17: Gel electrophoresis patterns for IBD patients 001-018, testing for *TPMT 3B on 3% agarose gel.** *The middle column on (denoted UP) contains TPMT* 3B PCR product that has not been restricted.*

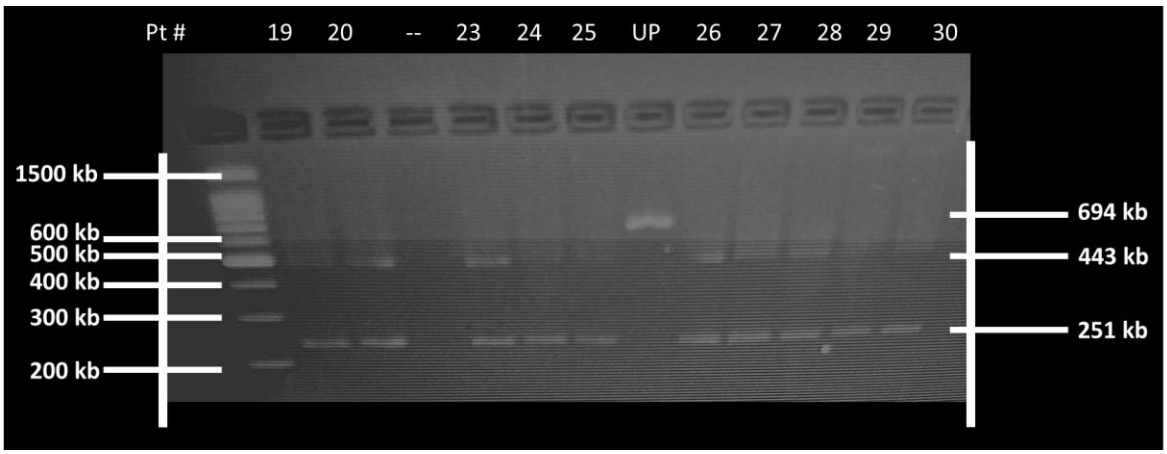

Figure 18: Gel electrophoresis patterns for IBD patients 019 & 020, 023-030, testing for *TPMT 3B on 3% agarose gel.** *The middle column on (denoted UP) contains TPMT* 3B PCR product that has not been restricted.*

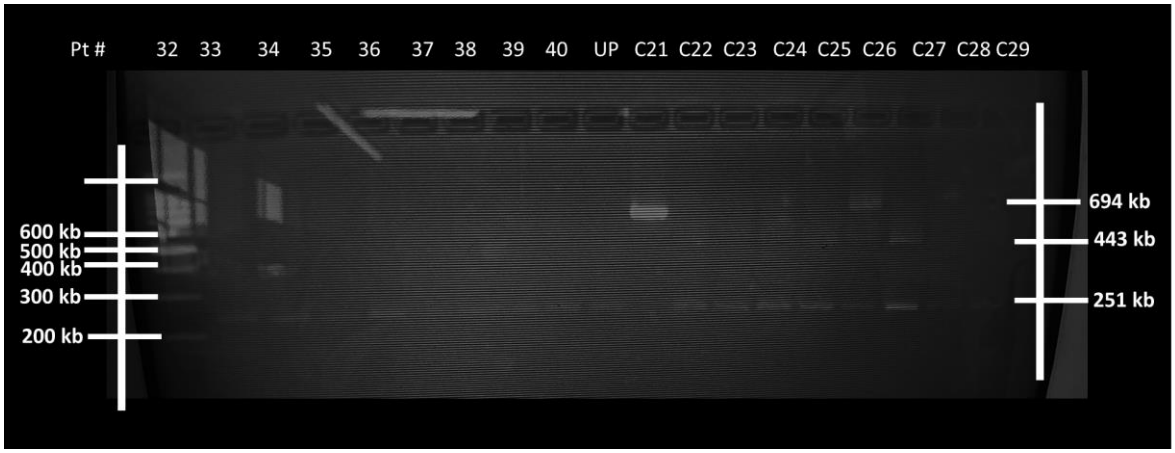

Figure 19: Gel electrophoresis patterns for IBD patients 032- 040 & control samples 021-029, testing for *TPMT 3B on 3% agarose gel.** *The middle column on (denoted UP) contains TPMT* 3B PCR product that has not been restricted.*

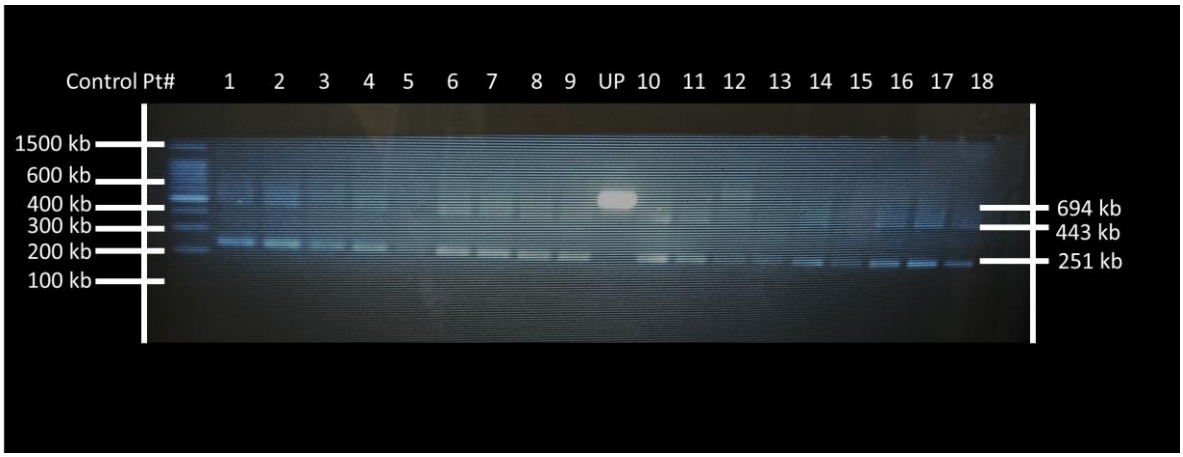

Figure 20: Gel electrophoresis patterns for control samples 001- 018, testing for *TPMT 3B on 3% agarose gel.** *The middle column on (denoted UP) contains TPMT* 3B PCR product that has not been restricted.*

2.2 TPMT *3C

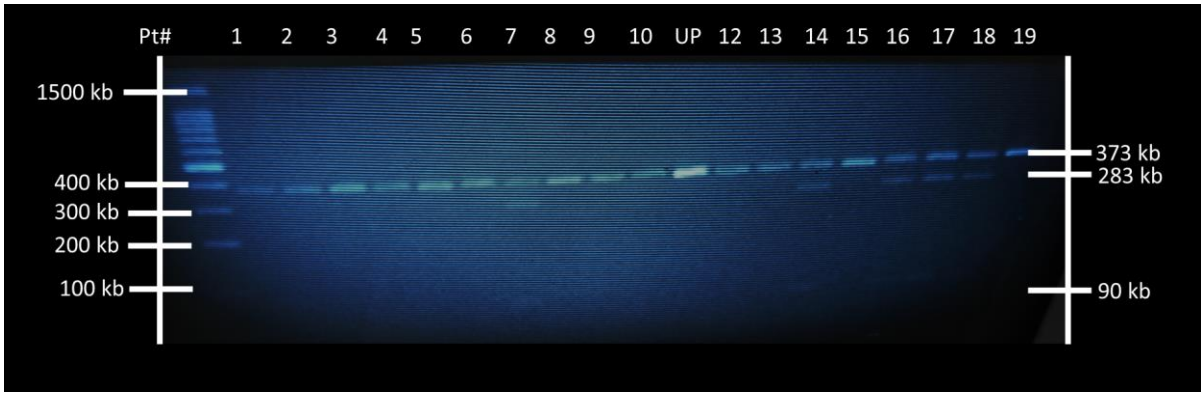

Figure 21: Gel electrophoresis patterns for *TPMT 3C in IBD patients 001-010, 012 - 019 on 3% agarose gel.** *The middle column on (denoted UP) contains TPMT* 3C PCR product that has not been restricted.*

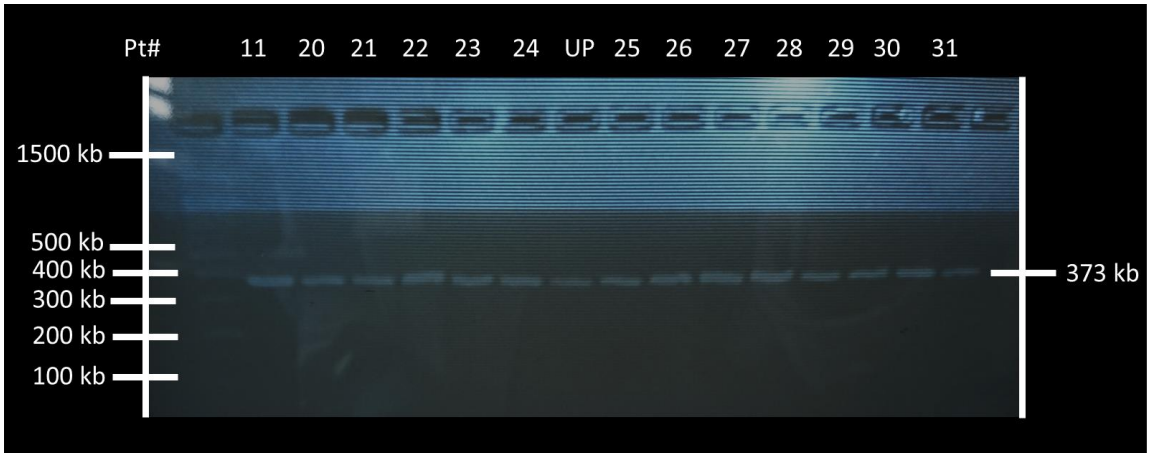

Figure 22: Gel electrophoresis patterns for *TPMT 3C in IBD patients 011, 020 - 031 on 3% agarose gel.** *The middle column on (denoted UP) contains TPMT* 3C PCR product that has not been restricted.*

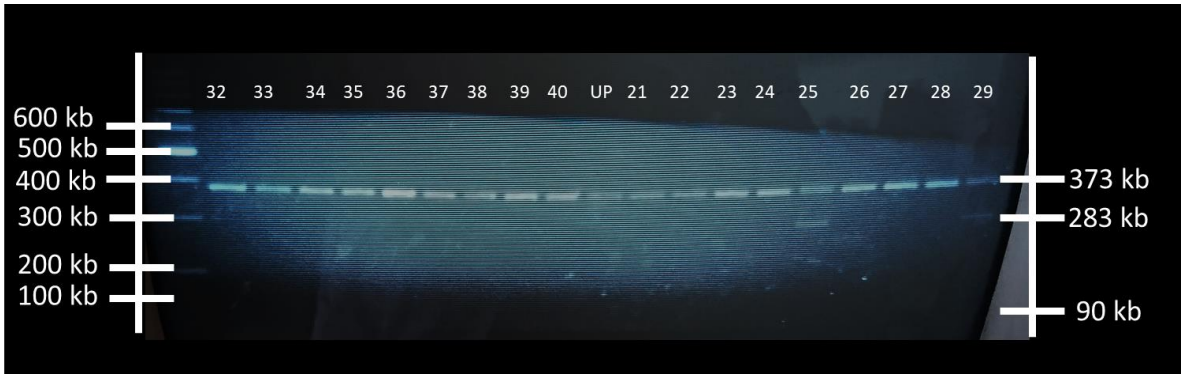

Figure 23: Gel electrophoresis patterns for *TPMT 3C in IBD patients 032- 040 & control samples 021- 029 on 3% agarose gel.** *The middle column on (denoted UP) contains TPMT* 3C PCR product that has not been restricted.*

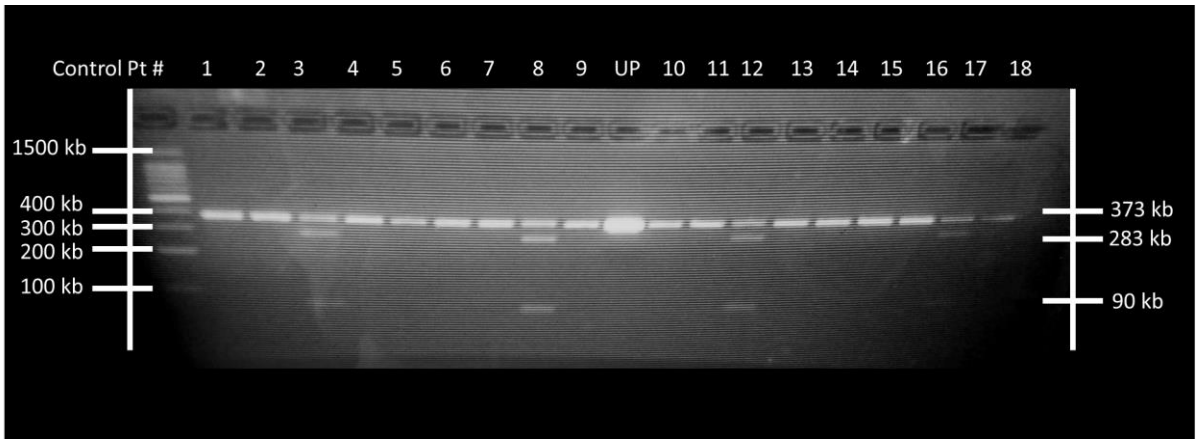

Figure 24: Gel electrophoresis patterns for *TPMT 3C in control patients 001 - 018 on 3% agarose gel.** *The middle column on (denoted UP) contains TPMT* 3C PCR product that has not been restricted.*

3. Statistics

3.1 Sample size

Letter of statistical support. FORM 3

LETTER OF STATISTICAL SUPPORT

Date: 2017/06/29

This letter is to confirm that the student, Lyla Adams, Student No: 13020740, studying at the University of Pretoria discussed the Project with the title "Identification of Single Nucleotide Polymorphisms in Inflammatory Bowel Disease Patients on Azathioprine Therapy" with me.

I hereby confirm that I am aware of the project and also undertake to assist with the statistical analysis of the data generated from the project.

The DATA ANALYIS will consist of descriptive and inferential statistics techniques for analyses. Skewness and kurtosis testing will indicate use of which formulas to apply. Tests for association of contingency tables will be performed using two-tailed Chi^2 tests. One-way ANOVA will be used (the Kruskal-Wallis test for non-parametric data) for more than 2 groups, or the Mann-Whitney test when 2 groups were compared. Correlation coefficients will be calculated using correlation matrices using applicable correlation test (non-parametric Spearman, or Pearsons test for parametric data) with p value correction for multiple testing (i.e. Bonferroni correction). Statistical significance was determined by P-value <0.05. The analyses are done using STATA 13 (Statacorp, Texas, USA).

The SAMPLE SIZE CALCULATION was made as follows:

As IBD is a rare disease effecting a small number of patients, although studies indicate and increase in the incidence of IBD. As such, all patients complying with the inclusion criteria will be drafted into the study. Expected numbers are between 30 to 40.

Name: Dr Pieter W.A. Meyer

Signature: 

Date: 2017-06-29

3.2 Data Analysis

3.2.1 t test of ESR

Table Analyzed	ESR
Column A	ESR 0
vs	vs
Column B	ESR 12
Paired t test	
P value	0.18
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.411 df=14
Number of pairs	15
How big is the difference?	
Mean of differences	9
95% confidence interval	-4.680 to 22.68
R squared	0.1245
How effective was the pairing?	
Correlation coefficient (r)	0.4232
P Value (one tailed)	0.058
P value summary	ns
Was the pairing significantly effective?	No

3.2.2 t-test of CRP

Excluding outlier CRP Value for Patient 025, Patient 027and Patient 038

Table Analyzed	CRP
Column A	CRP 0
vs	vs
Column B	CRP 12
Paired t test	
P value	0,0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.428 df=32
Number of pairs	33
How big is the difference?	
Mean of differences	15,20
95% confidence interval	8.204 to 22.19
R squared	0,3800
How effective was the pairing?	
Correlation coefficient (r)	0,4557
P Value (one tailed)	0,0038
P value summary	**
Was the pairing significantly effective?	Yes

3.2.3 t-test of WBC

Parameter	
Table Analyzed	WBC
Column A	WBC 0
vs	vs
Column B	WBC 12
Paired t test	
P value	0.9865
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t	df;t=0.01705 df=33
Number of pairs	34
How big is the difference?	
Mean of differences	0.01059
95% confidence interval	-1.254 to 1.275
R squared	0.000008809
How effective was the pairing?	
Correlation coefficient (r)	0.6201
P Value (one tailed)	P<0.0001
P value summary	***
Was the pairing significantly effective?	Yes

3.2.4 t-test: ESR and CRP correlation

Excluding outlier data from Patient 025, Patient 027 & Patient 038

Number of XY Pairs	15
Pearson r	-0,1605
95% confidence interval	-0.6217 to 0.3834
P value (two-tailed)	0,5678
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R squared	0,02574

3.2.5 Sample analysis

-> tabulation of class

class	Freq.	Percent	Cum.
Control	40	50.00	50.00
Patient	40	50.00	100.00
Total	80	100.00	

-> tabulation of pheno_clas

pheno_clas	Freq.	Percent	Cum.
Haplo *3B	14	17.50	17.50
Hetero *3B	21	26.25	43.75
Hetero *3C	1	1.25	45.00
Homo *3B	39	48.75	93.75
Homo WT	5	6.25	100.00
Total	80	100.00	

-> tabulation of dos_class

dos_class	Freq.	Percent	Cum.
<= 100 mg/day	22	55.00	55.00
>100 - <=150mg/day	14	35.00	90.00
> 150mg/day	4	10.00	100.00
Total	40	100.00	

-> tabulation of react

react	Freq.	Percent	Cum.
Did not react	22	55.00	55.00
Reacted	18	45.00	100.00
Total	40	100.00	

-> tabulation of esr_class

esr_class	Freq.	Percent	Cum.
Increased	5	33.33	33.33
Decreased	10	66.67	100.00
Total	15	100.00	

-> tabulation of crp_class

crp_class	Freq.	Percent	Cum.
Increased	7	21.21	21.21
Decreased	26	78.79	100.00
Total	33	100.00	

-> tabulation of wbc_class

wbc_class	Freq.	Percent	Cum.
Increased	18	56.25	56.25
Decreased	14	43.75	100.00
Total	32	100.00	

-> tabulation of disease

disease	Freq.	Percent	Cum.
CD	19	47.50	47.50
UC	21	52.50	100.00
Total	40	100.00	

```
. * Fishers Exact  to compare Control to Patients group TPMT SNPs *

. tab pheno_clas class, exact col r
```

Key
frequency *row percentage* *column percentage*

```
Enumerating sample-space combinations:
stage 5:  enumerations = 1
stage 4:  enumerations = 1
stage 3:  enumerations = 6
stage 2:  enumerations = 64
stage 1:  enumerations = 0
```

	class		
pheno_clas	Control	Patient	Total
Haplo *3B	8	6	14
	57.14	42.86	100.00
	20.00	15.00	17.50
Hetero *3B	20	1	21
	95.24	4.76	100.00
	50.00	2.50	26.25
Hetero *3C	1	0	1
	100.00	0.00	100.00
	2.50	0.00	1.25
Homo *3B	6	33	39
	15.38	84.62	100.00
	15.00	82.50	48.75
Homo WT	5	0	5
	100.00	0.00	100.00

	12.50	0.00	6.25
Total	40 50.00 100.00	40 50.00 100.00	80 100.00 100.00

Fisher's exact = 0.000

```
. * Thus 82.5% (n=33) of patients had the Homo *3B mutation, p=0.000 *

.
. * Frequency within dose groups *

. tab pheno_clas dos_class, exact

Enumerating sample-space combinations:
stage 3:  enumerations = 1
stage 2:  enumerations = 5
stage 1:  enumerations = 0
```

pheno_clas	dos_class <= 100 mg	>100 - <=	> 150mg/d	Total
Haplo *3B	2	3	1	6
Hetero *3B	0	0	1	1
Homo *3B	20	11	2	33
Total	22	14	4	40

Fisher's exact = 0.080

```
. tab pheno_clas dos_class,  col row exact
```

Key
frequency *row percentage* *column percentage*

```
Enumerating sample-space combinations:
stage 3:  enumerations = 1
stage 2:  enumerations = 5
stage 1:  enumerations = 0
```

pheno_clas	dos_class <= 100 mg	>100 - <=	> 150mg/d	Total
Haplo *3B	2 33.33 9.09	3 50.00 21.43	1 16.67 25.00	6 100.00 15.00
Hetero *3B	0 0.00 0.00	0 0.00 0.00	1 100.00 25.00	1 100.00 2.50
Homo *3B	20 60.61 90.91	11 33.33 78.57	2 6.06 50.00	33 100.00 82.50
Total	22 55.00 100.00	14 35.00 100.00	4 10.00 100.00	40 100.00 100.00

```
          Fisher's exact =                 0.080

. * now with frequencies, no stat difference p=0.08 *

. * efficacy *

. tabl esr_class crp_class wbc_class

-> tabulation of esr_class

  esr_class |      Freq.     Percent        Cum.
------------+-----------------------------------
  Increased |          5       33.33       33.33
  Decreased |         10       66.67      100.00
------------+-----------------------------------
      Total |         15      100.00

-> tabulation of crp_class

  crp_class |      Freq.     Percent        Cum.
------------+-----------------------------------
  Increased |          7       21.21       21.21
  Decreased |         26       78.79      100.00
------------+-----------------------------------
      Total |         33      100.00

-> tabulation of wbc_class

  wbc_class |      Freq.     Percent        Cum.
------------+-----------------------------------
  Increased |         18       56.25       56.25
  Decreased |         14       43.75      100.00
------------+-----------------------------------
      Total |         32      100.00

. * 67% of the 15 patient with ESR showed a decrease after 12 month *

. 79% of the 33 patients with CRP measurements showed a decrease after 12 month *
unrecognized command:  79 invalid command name
r(199);

. * 79% of the 33 patients with CRP measurements showed a decrease after 12 month *

. * 56% of patients of the 32 with WBC showed an increase *
```

4. Instruction Sheets

4.1 PCR

Certificate of Analysis

GoTaq® Hot Start Green Master Mix

Cat.#	Size
M5121	10 reactions
M5122	100 reactions
M5123	1,000 reactions

Includes GoTaq® Hot Start Green Master Mix, 2X, and Nuclease-Free Water.

Description: GoTaq® Hot Start Green Master Mix[a,b] is a premixed ready-to-use solution containing GoTaq® Hot Start Polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. GoTaq® Hot Start Polymerase contains the high-performance GoTaq® DNA Polymerase bound to a proprietary antibody that blocks polymerase activity. The polymerase activity is restored during the initial denaturation step when amplification reactions are heated at 94–95°C for two minutes. This allows hot-start PCR in which polymerase activity is inhibited at temperatures below 70°C, allowing convenient room-temperature reaction setup. Hot-start PCR is advantageous for some amplification targets, because it may eliminate or minimize primer-dimer and secondary products. In some cases, hot-start PCR may improve yields. GoTaq® Hot Start Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis. Reactions assembled with GoTaq® Hot Start Green Master Mix have sufficient density for direct loading onto agarose gels. GoTaq® Hot Start Polymerase exhibits 5 →3 exonuclease activity.

GoTaq® Hot Start Green Master Mix is recommended for any amplification reaction that will be visualized by agarose gel electrophoresis and ethidium bromide staining. The master mix is not recommended if any downstream applications use absorbance or fluorescence excitation, as the yellow and blue dyes in the reaction buffer may interfere with these applications. The dyes absorb between 225–300nm, making standard A_{260} readings to determine DNA concentration unreliable. The dyes have excitation peaks at 488nm and 600–700nm that correspond to the excitation wavelengths commonly used in fluorescence detection instrumentation. However, for some instrumentation, such as a fluorescent gel scanner that uses a 488nm excitation wavelength, there will be minimal interference since it is the yellow dye that absorbs at this wavelength. Gels scanned by this method will have a light grey dye front (corresponding to the yellow dye front) below the primers.

GoTaq® Hot Start Green Master Mix, 2X: GoTaq® Hot Start Polymerase is supplied in 2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 4mM $MgCl_2$. Green GoTaq® Reaction Buffer is a proprietary buffer containing a compound that increases sample density, and yellow and blue dyes, which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis. The blue dye migrates at the same rate as 3–5kb DNA fragments, and the yellow dye migrates at a rate faster than primers (<50bp), in a 1% agarose gel.

Biological Source for GoTaq® Hot Start Polymerase: The enzyme is derived from bacteria. The antibody is derived from murine cell culture.

Storage Conditions: See the Product Information Label for storage recommendations. Minimize the number of freeze-thaw cycles by storing in working aliquots. Product may be stored at 4°C for up to 12 weeks. Mix well prior to use.

Quality Control Assays

Functional Assay: GoTaq® Hot Start Green Master Mix is tested for performance in the polymerase chain reaction (PCR). GoTaq® Green Master Mix, 1X, is used to amplify a 360bp region of the α-1-antitrypsin gene and a 2.4kb region of the APC gene from 100 molecules of human genomic DNA. The resulting PCR products are visualized on an ethidium bromide-stained agarose gel.

Hot-Start Amplification Assay: GoTaq® Hot Start Green Master Mix is tested in PCR for its ability to amplify a hot-start model template to produce a single 1.5kb band, eliminating extraneous bands. In a nonhot-start PCR, this template produces an additional band at 410bp.

Nuclease Assays: No contaminating endonuclease or exonuclease activity detected.

PCR Satisfaction Guarantee

Promega's PCR Systems, enzymes and reagents are proven in PCR to ensure reliable, high performance results. Your success is important to us. Our products are backed by a worldwide team of Technical Support scientists. Please contact them for applications or technical assistance. If you are not completely satisfied with any Promega PCR product we will send a replacement or refund your account.

That's Our PCR Guarantee!

Product must be within expiration date and have been stored and used in accordance with product literature. See Promega Product Insert for specific tests performed.

[a]Use of this product for basic PCR is outside of any valid US or European patents assigned to Hoffman La-Roche or Applera. This product can be used for basic PCR in research without any license or royalty fees. This product is for research use only. [b]U.S. Pat. No. 6,242,235, Australian Pat. No. 761757, Canadian Pat. No. 2,335,153, Chinese Pat. No. ZL99808861.7, Hong Kong Pat. No. HK 1040262, Japanese Pat. No. 3673175, European Pat. No. 1088060 and other patents pending.

Signed by: R. Wheeler, Quality Assurance

GoTaq® Hot Start Green Master Mix

REF M5122 LOT 0000264022

-30°C -10°C 2020-04-26

Dispensed Lot#: 0000254673

100 reactions

For Research Use only. Not for Use in Diagnostic Procedures.
Country of Origin: USA

Promega Corporation 2800 Woods Hollow Road Madison, WI 53711-5399 USA

ADM5122 00002640222

Promega

Promega Corporation

2800 Woods Hollow Road	
Madison, WI 53711-5399	USA
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Internet	www.promega.com

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Part# 9PIM512
Printed in USA Revised 10/16.

Promega

Usage Information

1. Standard Application

Reagents to be Supplied by the User

template DNA	downstream primer
upstream primer	mineral oil (optional)

1. Thaw the GoTaq® Hot Start Green Master Mix at room temperature. Vortex the Master Mix, then centrifuge it briefly in a microcentrifuge to collect the material at the bottom of the tube.
2. Prepare one of the following reaction mixes at room temperature:

For a 25µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Hot Start Green Master Mix, 2X	12.5µl	1X
upstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
downstream primer, 10µM	0.25–2.5µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	25µl	N.A.

For a 50µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Hot Start Green Master Mix, 2X	25µl	1X
upstream primer, 10µM	0.5–5.0µl	0.1–1.0µM
downstream primer, 10µM	0.5–5.0µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	50µl	N.A.

For a 100µl reaction volume:

Component	Volume	Final Conc.
GoTaq® Hot Start Green Master Mix, 2X	50µl	1X
upstream primer, 10µM	1.0–10.0µl	0.1–1.0µM
downstream primer, 10µM	1.0–10.0µl	0.1–1.0µM
DNA template	1–5µl	<250ng
Nuclease-Free Water to	100µl	N.A.

Note: Although typically not necessary, magnesium optimization may be required to improve yield for some targets.

3. If using a thermal cycler without a heated lid, overlay the reaction mix with 1–2 drops (approximately 50µl) of mineral oil to prevent evaporation during thermal cycling. Centrifuge the reactions in a microcentrifuge for 5 seconds.
4. Place the reactions in a room-temperature thermal cycler. Perform PCR using your standard parameters. **A 2-minute initial denaturation step at 94–95°C is required to inactivate the antibody and initiate hot-start PCR.**

2. General Guidelines for Amplification by PCR

A. Denaturation

- All denaturation steps after the 2-minute initial denaturation step should be between 30 seconds and 1 minute.

B. Annealing

- Optimize the annealing conditions by performing the reaction starting approximately 5°C below the calculated melting temperature of the primers and increasing the temperature in increments of 1°C to the annealing temperature.
- The annealing step is typically 30 seconds to 1 minute.

C. Extension

- The extension reaction is typically performed at the optimal temperature for *Taq* DNA polymerase, which is 72–74°C.
- Allow approximately 1 minute for every 1kb of DNA to be amplified.
- A final extension of 5 minutes at 72–74°C is recommended.

D. Refrigeration

- If the thermal cycler has a refrigeration or "soak" cycle, the cycling reaction can be programmed to end by holding the tubes at 4°C for several hours.
- This cycle can minimize any polymerase activity that might occur at higher temperatures, although this is not usually a problem.

E. Cycle Number

- Generally, 25–30 cycles result in optimal amplification of desired products.
- Occasionally, up to 40 cycles may be performed, especially for detection of low-copy targets.

3. General Considerations

A. GoTaq® Hot Start Green Master Mix Compatibility

If both agarose gel analysis and further downstream applications involving absorbance or fluorescence will be used, the two dyes can be removed from reactions using standard PCR cleanup systems such as the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281).

B. Primer Design

PCR primers generally range in length from 15–30 bases and are designed to flank the region of interest. Primers should contain 40–60% (G + C), and care should be taken to avoid sequences that might produce internal secondary structure. The 3 -ends of the primers should not be complementary to avoid the production of primer-dimers. Primer-dimers unnecessarily deplete primers from the reaction and result in an unwanted polymerase reaction that competes with the desired reaction. Avoid three G or C nucleotides in a row near the 3 -end of the primer, as this may result in nonspecific primer annealing, increasing the synthesis of undesirable reaction products. Ideally, both primers should have nearly identical melting temperatures (T_m); in this manner, the two primers should anneal roughly at the same temperature. The annealing temperature of the reaction depends on the primer with the lowest T_m. For assistance with calculating the T_m of any primer, a T_m Calculator is provided on the BioMath page of the Promega web site at: **www.promega.com/biomath/**

C. Amplification Troubleshooting

To overcome low yield or no yield in amplifications, we recommend the following suggestions:

- Adjust annealing temperature. The reaction buffer composition affects the melting properties of DNA. See BioMath Calculator to calculate the melting temperature for primers in the GoTaq® reaction (**www.promega.com/biomath/**).
- Minimize the effect of amplification inhibitors. Some DNA isolation procedures, particularly genomic DNA isolation, can result in the copurification of amplification inhibitors. Reduce the volume of template DNA in reaction, or dilute template DNA prior to adding to reaction. Diluting samples up to 1:10,000 has been shown to be effective in improving results, depending on initial DNA concentration.
- Increase template DNA purity. Include an ethanol precipitation and wash step prior to amplification to remove inhibitors that copurify with the DNA.
- Add PCR additives. Adding PCR-enhancing agents (e.g., DMSO or betaine) may improve yields. General stabilizing agents such as BSA (Sigma Cat.# A7030; final concentration 0.16mg/ml) also may help to overcome amplification failure.

D. More Information on Amplification

More information on amplification is available online at the Promega web site:
PCR Applications: **www.promega.com/paguide/chap1.htm**
PCR Protocols and Reference: **www.promega.com/guides/pcr_guide/default.htm**

4. Summary of Changes

The following change was made to the 6/14 revision of this document:

Legal disclaimers were updated.

Part# 9PIM512
Printed in USA Revised 10/16.

4.2 Restriction Enzyme Digest

MwoI

CutSmart RR dilB 60° MW CpG

Reaction Conditions:

CutSmart Buffer, 60°C.

Time-Saver™ Protocol:

Restriction Enzyme	1 µl
DNA	1 µg
10X NEBuffer	5 µl (1X)
Total Rxn Volume	50 µl
Incubation Temperature	60°C
Incubation Time	5-15 min.*

*Can also be used overnight with no star activity.

Buffer Performance:

NEBuffer	1.1	2.1	3.1	CutSmart®
% Activity	<10	100	100	100

For detailed product information, scan the code on the front or visit www.neb.com/R0573

AccI

CutSmart RR dilA 37° HF 80° CpG

Reaction Conditions:

CutSmart Buffer, 37°C. Inactivate at 80°C for 20 min.

15 min

Time-Saver™ Protocol:

Restriction Enzyme	1 µl
DNA	1 µg
10X NEBuffer	5 µl (1X)
Total Rxn Volume	50 µl
Incubation Temperature	37°C
Incubation Time	5-15 min.*

*Can also be used overnight with no star activity.

Buffer Performance:

NEBuffer	1.1	2.1	3.1	CutSmart®
% Activity	50	50	10	100

For detailed product information, scan the code on the front or visit www.neb.com/R0161

4.3 Primer Synthesis Report

Inqaba Biotechnical Industries (Pty) Ltd
P.O. Box 14356, Hatfield 0028, South Africa
Tel: 012 343 5829
Fax: 012 343 0287
E-mail: info@inqaba.com

SYNTHESIS REPORT

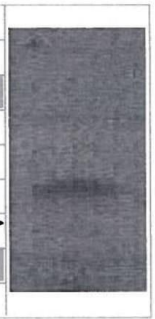
09 Mar 2018

Client Detail:

Dr Alisa Phulukdaree
University of Pretoria (UNP002) - Chemical Pathology (#1369)
Department of Physiology
Room 9-27, Basic Medical Science Blg, Prinshof Campus
Pretoria
South Africa

Name:	TPMT*3B FWD		**Barcode:** S4919	**Length:** 25 bases	
Sequence:	AGGCAGCTAGGGAAAAAGAAAGGTG				
OD	15.5952	**MW min \ max**	7860.7\7860.7	**5' Mod**	None
nmoles	48.95	**GC % min \ max**	48\48	**3' Mod**	None
Tm min \ max	64.58\64.58			**Purification**	Standard
For a 100 µM stock solution add 489.49 µl water or buffer				**PAGE QC Image >>**	
Comments					

Name:	TPMT*3B REV		**Barcode:** S491A	**Length:** 25 bases	
Sequence:	CAAGCCTTATAGCCTTACACCCAGG				
OD	18.9905	**MW min \ max**	7570\7570	**5' Mod**	None
nmoles	71.85	**GC % min \ max**	52\52	**3' Mod**	None
Tm min \ max	66.22\66.22			**Purification**	Standard
For a 100 µM stock solution add 718.52 µl water or buffer				**PAGE QC Image >>**	
Comments					



Name:	TPMT*3C FWD		**Barcode:** S491B		**Length:** 23 bases	
Sequence:	GAGACAGAGTTTCACCATCTTGG					
OD	16.2982	**MW min \ max**	7062.9\7062.9	**5' Mod**	None	
nmoles	64.7	**GC % min \ max**	47.83\47.83	**3' Mod**	None	
Tm min \ max	62.77\62.77			**Purification**	Standard	
For a 100 µM stock solution add 647.01 **µl water or buffer**					**PAGE QC Image >>**	
Comments						

Name:	TPMT*3C REV		**Barcode:** S491C		**Length:** 28 bases	
Sequence:	CAGGCTTTAGCATAATTTTCAATTCCTC					
OD	17.5408	**MW min \ max**	8487.9\8487.9	**5' Mod**	None	
nmoles	60.32	**GC % min \ max**	35.71\35.71	**3' Mod**	None	
Tm min \ max	61.69\61.69			**Purification**	Standard	
For a 100 µM stock solution add 603.19 **µl water or buffer**					**PAGE QC Image >>**	
Comments						

RECOMMENDATIONS FOR HANDLING AND STORAGE OF OLIGOS

- Lyophilized oligo pellets might become displaced from the bottom of the tube during shipment. Briefly centrifuge each tube before opening to prevent the loss of the pellet.
- Prepare stock solution of oligos (e.g. 100 uM = 100 pmole per ul) preferably with a sterile buffered solution such as TE (10 mM Tris, pH 7.5 to 8.0, 1 mM EDTA). If sterile distilled water is used, make sure that the pH is above 7.0 since acidic solutions favours oligo depurination and subsequent loss of activity.
- Working solutions might be diluted from the stock solution with sterile, nuclease-free water to prevent inhibition of enzymatic reactions (e.g. PCR) by EDTA.
- Store the oligos as concentrated stock solution or lyophilized at -20° C.
- Avoid frequent freeze-thaw cycles by dividing the stock solution into smaller aliquots for long term storage and to prevent accidental contamination.
- Dye-modified oligos are light sensitive and should always be stored in the dark.
- Re-suspend modified oligos preferably in a slightly basic solution (i.e., TE at pH 8.0). However, Cy dye modified oligos are best kept at pH 7.0 at -20° C.
- Preferably store the modified oligos as dried aliquots at -20° C.

4.4 DNA Ladder

100bp DNA Ladder

REF G2101 LOT 0000258245
-30°C -10°C
2021-10-20
Dispensed Lot#: 0000247390
250µl

For Laboratory Use
Country of Origin: CHN
CHN: G210A
USA: All others

Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711-5399 USA

Promega

ADG2101 00002582452

PEEL HERE

Certificate of Analysis

100bp DNA Ladder:

Part No.	Size
G210A	250µl

Description: The 100bp DNA Ladder is ideal for determining the size of double-stranded DNA from 100–1,500 base pairs. The ladder consists of 11 double-stranded DNA fragments with sizes of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000 and 1,500bp. The 500bp band is present at triple the intensity of the other fragments and serves as a reference indicator. All other fragments appear with equal intensity on the gel. Recommended loading volume is 5µl/lane.

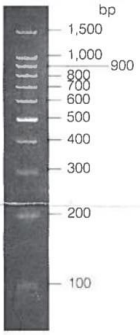
Storage Buffer: The 100bp DNA Ladder is supplied in 10mM Tris-HCl, 1mM EDTA. Final pH 7.4.

Concentration: Five microliters (650ng) of the ladder contains approximately 150ng of the 500bp DNA fragment and 50ng of each of the other ten DNA fragments.

Storage Conditions: Store at –20°C.

Usage Note: Concentration gradients may form in frozen products and should be mixed well prior to use.

Blue/Orange 6X Loading Dye (G190A): The Blue/Orange 6X Loading Dye supplied with these markers has a composition of 15% Ficoll® 400, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA. This dye is used for loading DNA samples into gel electrophoresis wells and tracking migration during electrophoresis. Recommended usage is one part loading dye for every five parts DNA solution. The xylene cyanol FF migrates at approximately 4kb, bromophenol blue at approximately 300bp and orange G at approximately 50bp in 0.5% to 1.4% agarose gels in 0.5X TBE (1).



2% agarose

Quality Control Assays

Accurate Sizing: Five microliters of the 100bp DNA Ladder are mixed with 1µl of Loading Dye and subjected to electrophoresis on a 2% agarose gel with TAE 1X buffer. The markers must show the expected pattern when compared with HaeIII-digested ΦX174 DNA Markers (Cat.# G1761).

Nuclease Assay: To test for nuclease contamination, 5µl of the 100bp DNA Ladder are incubated in restriction enzyme buffer overnight at 37°C. Following incubation, the ladder is subjected to electrophoresis and visualized on an ethidium bromide-stained agarose gel to verify the absence of visible degradation.

5′ End-Labeling: Five microliters of the 100bp DNA Ladder are added to a labeling reaction containing 1µl of T4 Polynucleotide Kinase 10X Buffer, 1µl of [γ-^{32}P]ATP (3,000Ci/mmol @ 10µCi/µl), 1µl of T4 Polynucleotide Kinase and 2µl of deionized water. This reaction is incubated at 37°C for 10 minutes, then stopped by the addition of 1µl of 0.5M EDTA. After labeling, the 100bp DNA Ladder is separated on a 4% nondenaturing polyacrylamide gel. After the gel is processed, the labeled markers must be easily visible after overnight exposure to X-ray film without an intensifying screen at –70°C.

Reference

1. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Signed by: R. Wheeler, Quality Assurance

Promega

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2800 Woods Hollow Road
Madison, WI 53711-5399 USA
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Toll Free 800-356-9526
Fax 608-277-2516
Internet www.promega.com

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Part# 9PIG210
Printed in USA. Revised 10/16.

5. *Additional Testing*

When conducting the RFLP PCR and gel electrophoresis of the IBD patient samples, the gels showed that 82% of the patient samples had the presence of the *TPMT* *3B allele. Even though this allele has never been tested for in the South African population, this statistic is still exceptionally high when compared to other international *TPMT* *3B statistics. Thus, it was decided that a set of patient samples should be Sanger sequenced to assess if the RFLP PCR was producing accurate results. 7 patient samples were sent for Sanger sequencing at Inqaba Biolabs; 3 IBD samples which showed as homozygous *3B, 3 IBD samples that showed as haplotype *3A, and 1 control group sample that showed as haplotype *3A. Each patient sample was then sequenced for *TPMT* *3C and *TPMT* *3B individually.

Two sets of sequences were obtained from the lab for each sample (forward and reverse). The forward and reverse sequences were aligned and a consensus sequence formed for each patient sample, for each SNP (*3C or *3B). Each patient consensus sequence was then compared, first to the registered SNP sequence and then to the entire *TPMT* chromosome 6 sequence in a healthy individual (containing no SNP). The alignment and comparison were conducted using the NCBI BLAST alignment application.

The full *TPMT* chromosome 6 sequence as well as the registered SNP sequences were obtained from the NCBI SNP database. The SNP sequence for *TPMT**3B is registered as *rs1800460* and the SNP sequence for *TPMT* *3C is registered as *rs1142345*

The percentage identity (PID) Expectation value was used to assess how well the two relevant sequences align. PID is calculated by multiplying the number of base matches by 100 and dividing the value by the length of the aligned region (gaps included)[156]. The PID was reported by the BLAST application.

Standard practice is to report the Expectation value, which gives an idea as to how likely it is that the database match occurred by chance- the smaller the E value, the higher the confidence that the match is non-random. However, due to the small size of the patient sample sequence and the very large size of the *TPMT* sample sequence- the E value will not be very accurate[157].

Given that the alignment is occurring between two specific sequences (as opposed to instructing BLAST to align a selected sequence to the best sequence match in the entire BLAST database) the use of the reported PID is acceptable[157].

The table below reports the PID of the patient sample vs full *TPMT* chromosome 6 sequence and the patient sample vs the registered SNP sequence for all 7 patient samples, for both SNPs.

Table 23: Percentage Identity for aligned sequences: *Sanger sequencing of pt 017 for *3C was not successful*

Pt	**SNP**	**Percent identity for *TPMT* sequence vs patient sequence (%)**	**Percent identity for rs1800460 vs patient sequence (%)**	**SNP reported per RFLP PCR**
06	*3B	100	99.08	*3B
10	*3B	98.70	99.08	*3B
14	*3B	98.70	98.17	*3B & *3C
17	*3B	98.70	99.08	*3B & *3C
23	*3B	100	99.08	*3B
31	*3B	100	99.08	*3B & *3C
C08	*3B	100	99.08	*3B & *3C
Pt	**SNP**	**Percent identity for *TPMT* sequence vs patient sequence (%)**	**Percent identity for rs1142345 vs patient sequence (%)**	**SNP reported per RFLP PCR**
06	*3C	98.36	99.08	*3B
10	*3C	98.36	99.08	*3B
14	*3C	97.54	98.17	*3B & *3C
17	*3C	NA	NA	*3B & *3C
23	*3C	98.36	99.07	*3B
31	*3C	98.36	100	*3B & *3C
C08	*3C	97.54	98.17	*3B & *3C

The PID for Patient 06, 23, 31 and control patient C08 was reported to be 100% when

aligned the *3B patient sequence to the *TPMT* chromosome 6 sequence; which indicates that the sequences are exact matches and hence no *3B SNP is present in the patient sample sequence- this is the opposite of what was detected when conducting the RFLP PCR.

When aligned using the GeneStudio application; Patient 31 shows only 1 unlike base when comparing patient sample sequence to the rs1800460 sequence- hence the 100% PID match with the *TPMT* chromosome 6 sequence. However, Patient 31 had 100% PID when aligned with rs1142345, which give an explanation for the single unlike base. Thus, out of all 7 samples sequenced, Patient 31 is the only patient to contain the true *TPMT* *3C SNP.

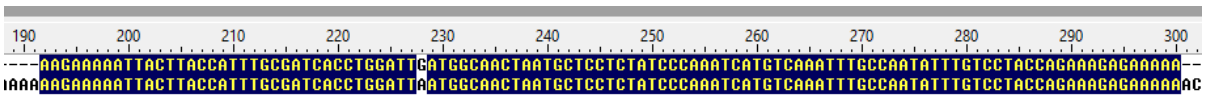

Figure 25: Patient 31 sample sequence (top) aligned to rs1800460 sequence (bottom) using GeneStudio

The PID for Patient 10, 14 and 17 were very high for the *3B sample alignment with both the *TPMT* chromosome 6 sequence and rs1800460- however neither sets of alignments had a PID of 100%. This indicates that the patient samples definitely contain a change in the base pairs, it may be that there is more than one unlike base pair or the that the unlike base is different from that which is found in the rs1800460 sequence.

An alignment assembly was performed using GeneStudio application, the figure below shows that for Patient 14 there were two different base changes, while Patient 10 only had 1 different base pair. Note that both sets of sequences show a change in similar regions of the rs1800460 sequence.

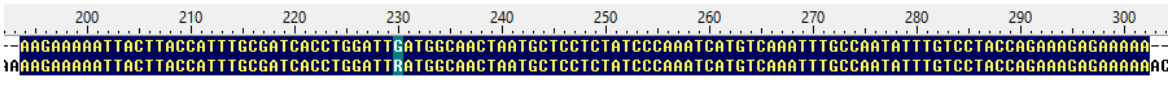

Figure 26:Patient 10 sample sequence (top) aligned to rs1800460 sequence (bottom) using GeneStudio

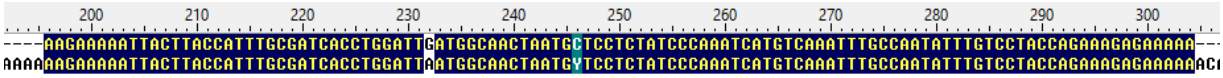

Figure 27:Patient 14 sample sequence (top) aligned to rs1800460 sequence (bottom) using GeneStudio

When comparing the *3C patient sample sequences, only Patient 31 had a 100% PID when aligning the *3C patient sample to the rs1142345 sequence. No other patient sample had 100% PID to the *TPMT* chromosome 6 sequence or rs1142345 sequence; however, PID for both sets were equally high. An indication that while the patient sample sequences were very similar to both the *TPMT* sequence and the rs1142345 sequence- it matched neither.

The fact that RFLP PCR detected the presence of *3C in these samples poses the impression that it was only detected due to the similarity of the primer sequence and that there is potentially another mutation in the same region.

This concept can be reiterated by the fact that when aligning the *3C patient sample sequences for Patient 06, 10, and 23 with rs1142345 sequence the using GeneStudio; there was only one set of unlike bases for the three different sequences, all at the same region on the rs1142345 sequence- despite the fact that Patient 06, 10 and 23 presented as negative for the presence of *TPMT* *3C when using RFLP-PCR.

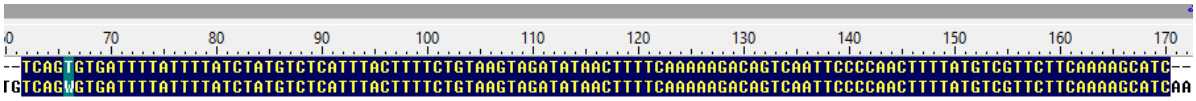

Figure 28: Patient 06 sample sequence (top) aligned to rs1142345 sequence (bottom) using GeneStudio

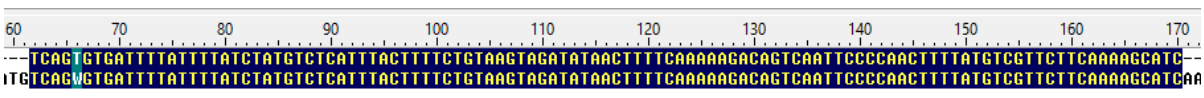

Figure 29: Patient 10 sample sequence (top) aligned to rs1142345 sequence (bottom) using GeneStudio

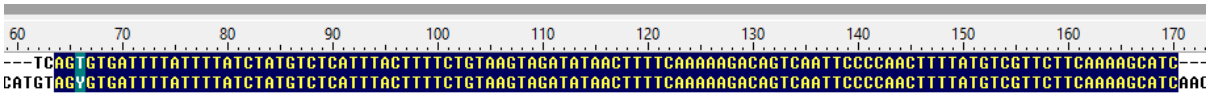

Figure 30: Patient 23 sample sequence (top) aligned to rs1142345 sequence (bottom) using GeneStudio

Thus, to conclude; the limitations of RFLP PCR became present when Sanger sequencing was conducted. Many if not most of the samples which were observed as positively showing the presence of the *TPMT* *3C or *3B SNP, may in actual fact contain a mutation different than the *TPMT* *3C or *3B SNP, but within the same region. This has potential for further studies using Sanger sequencing to determine exactly what they mutation is and if it has any effect on IBD patients.

6 Ethical Approval

6.1 University of Pretoria Ethics Committee

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

31/08/2017

Approval Certificate
New Application

Ethics Reference No: 357/2017

Title: Identification of Single Nucleotide Polymorphisms in Inflammatory Bowel Disease Patients on Azathioprine Therapy

Dear Lyla Adam

The **New Application** as supported by documents specified in your cover letter dated 22/08/2017 for your research received on the 23/08/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 30/08/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**357/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

☎ 012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria

6.2 Steve Biko Academic Hospital

Permission to access Records / Files / Data base at the Steve Biko Academic Hospital

To: Chief Executive Officer/Information Officer
SBAH Hospital

Dr AP van der Walt

From: Lyla Adam
Department of Pharmacology

Re: Permission to do research at Steve Biko AcademicHospital

I am a MSc student in the Department of Pharmacology at the University of Pretoria. I am requesting permission on behalf of all of us to conduct a study on the Steve Biko Academic Hospital grounds that involves access to patient records.

The request is lodged with you in terms of the requirements of the Promotion of Access to Information Act. No. 2 of 2000.

The title of the study is: Identification of Single Nucleotide Polymorphisms in Inflammatory Bowel Disease Patients on Azathioprine Therapy

The researchers request access to the following information:

Access to the clinical files, record book and the data base.

We intend to publish the findings of the study in a professional journal and/ or at professional meeting like symposia, congresses, or other meetings of such a nature.

We intend to protect the personal identity of the patients by assigning each patient a random code number.

We undertake not to proceed with the study until we have received approval from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria.

Yours sincerely

Signature of the Principle Investigator

Permission to do the research study at this hospital and to access the information as requested, is hereby approved.

Chief Executive Officer

Steve Biko Academic Hospital
Dr _____

DR AP VAN DER WALT
DIRECTOR CLINICAL SERVICES

Signature of the CEO

STEVE BIKO
PRIVATE BAG X169
2017 -07- 28
PRETORIA 0001
ACADEMIC HOSPITAL

Hospital Official Stamp

6.3 Private Hospital Research Committee

RESEARCH OPERATIONS COMMITTEE FINAL APPROVAL OF RESEARCH

Approval number: UNIV-2017-0040

Ms Lyla Adam

E mail: lyla.adam94@gmail.com

Dear Ms Adam

RE: IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN INFLAMMATORY BOWEL DISEASE PATIENTS ON AZATHIOPRINE THERAPY

The above-mentioned research was reviewed by the Research Operations Committee's delegated members and it is with pleasure that we inform you that your application to conduct this research at private Hospital, has been approved, subject to the following:

i) Research may now commence with this FINAL APPROVAL from the Committee.

ii) All information regarding the Company will be treated as legally privileged and confidential.

iii) The Company's name will not be mentioned without written consent from the Committee.

iv) All legal requirements regarding patient / participant's rights and confidentiality will be complied with.

v) The research will be conducted in compliance with the GUIDELINES FOR GOOD PRACTICE IN THE CONDUCT OF CLINICAL TRIALS IN HUMAN PARTICIPANTS IN SOUTH AFRICA (2006)

vi) The Company must be furnished with a STATUS REPORT on the progress of the study at least annually on 30th September irrespective of the date of approval from the Committee as well as a FINAL REPORT with reference to intention to publish and probable journals for publication, on completion of the study.

vii) A copy of the research report will be provided to the Committee once it is finally approved by the relevant primary party or tertiary institution, or once complete or if discontinued for any reason whatsoever prior to the expected completion date.

viii) The Company has the right to implement any recommendations from the research.

ix) The Company reserves the right to withdraw the approval for research at any time during the process, should the research prove to be detrimental to the subjects/ Company or should the researcher not comply with the conditions of approval.

x) APPROVAL IS VALID FOR A PERIOD OF 36 MONTHS FROM DATE OF THIS LETTER OR COMPLETION OR DISCONTINUATION OF THE TRIAL, WHICHEVER IS THE FIRST.

We wish you success in your research.

Yours faithfully

17/9/2017

Prof Dion du Plessis
Full member: Research Operations Committee & Medical Practitioner evaluating research applications as per Management and Governance Policy

Shannon Nell
Chairperson: Research Operations Committee
Date: 26/9/2017

This letter has been anonymised to ensure confidentiality in the research report. The original letter is available with author of research

7. Article 1 (Published)

Biomedicine & Pharmacotherapy 100 (2018) 8–14

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journal homepage: www.elsevier.com/locate/biopha

Review

Effective long-term solution to therapeutic remission in Inflammatory Bowel Disease: Role of Azathioprine

Lyla Adam[a], Alisa Phulukdaree[b], Prashilla Soma[b,*]

[a] University of Pretoria, Faculty of Health Sciences, School of Medicine, Department of Pharmacology, South Africa
[b] University of Pretoria, Faculty of Health Sciences, School of Medicine, Department of Physiology, South Africa

ARTICLE INFO

Keywords:
Azathioprine
Thiopurine methyltransferase
Inflammatory bowel disease

ABSTRACT

Azathioprine (AZA) is a well-known immunosuppressant used for many years for its ability to ensure long term disease remission in inflammatory bowel diseases (IBD) at an affordable cost to the public. However, the side effect profile has raised many concerns with numerous investigations into the risk, cause and prevention of these effects. Much of the side effect profile of AZA can be linked to a single nucleotide polymorphism (SNP) in the thiopurine methyltransferase (TPMT) gene which ensures the breakdown and efficacy of AZA. Mutated TPMT alleles result in low or deficient TPMT levels which directly correlate to cytotoxity. This is a review of the role of AZA in the treatment of IBD. Knowing a patient's TPMT status allows the prescribing doctor to make an informed decision about dosage and be more alert to the signs of cytotoxicity. It is essential to include "early warning" SNP testing into common practice to ensure therapeutic efficacy.

1. Introduction

Azathioprine (AZA) is an immunosuppressant drug that was first produced in 1957 and is included in the World Health Organization's (WHO) List of Essential Medicines. AZA is a purine analogue which interrupts the synthesis of purine ribonucleotides guanine and adenine, causing mis-incorporation of bases and preventing deoxyribonucleic acid (DNA) repair mechanisms [1–3]. It has a most notable effect on fast dividing cells such as T- lymphocytes; at low doses AZA works as an anti-inflammatory while at high doses it has immunosuppressant and cytotoxic characteristics [4,5]. The drug class of thiopurines is commonly used to treat dermatological conditions, malignancies, rheumatic diseases, prevention of rejection after organ transplant or for the treatment of inflammatory gastrointestinal disorders such as Inflammatory Bowel Disease (IBD). Thiopurine drugs have a very narrow therapeutic index and can cause life threatening toxicity [6].

2. Mechanism of action

As shown in Fig. 1, AZA is a prodrug which once administered, is converted to 6-mercaptopurine (6-MP). 6-MP undergoes methylation via the key enzyme thiopurine methyltransferase (TPMT) to form an inactive methylated metabolite of 6-mercaptopurine (6-Me-MP) [7]. In the absence of methylation by TPMT, 6-MP is converted into 6-thioguanine (6-TG) by xanthine oxidase, where after hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) converts 6-TG into 6-thioguanine nucleotide (6-TGN) metabolites. 6-TGN is the active metabolite that determines cytotoxicity or efficacy. TPMT competes with xanthine oxidase and HGPRT to determine how much of the 6-MP is catalyzed to 6-TGN [8,9]. TPMT enzyme activity varies greatly in patients due to the presence of polymorphic variation in the TPMT gene [3].

At normal levels of TPMT activity, 6-TGN inhibits intracellular signalling pathways and induces lymphocytic apoptosis. An increase in TPMT enzyme activity above normal results in decreased 6-TGN and hence a decrease in drug efficacy. The decrease or absence of TPMT activity (such as that seen in the TPMT polymorphisms) results in increased levels of 6-TGN which incorporates into the DNA and trigger cytotoxicity [9–11].

3. Pharmacology

Azathioprine can be administered intravenously or orally in both a delayed release oral (DRO) capsule, or tablet form. A study by Van Os *et al* (1996) compared the bioavailability of 50mg AZA when administered in the form of a DRO tablet, oral capsule, rectal hydrophilic foam (HBF), rectal hydrophobic foam (HPF) and intravenously. The oral bioavailability was 41.6%, DRO 9.6%, HBF 5.9% and HPF 1.8%, assuming the intravenous bioavailability was 100% [12]. Oral tablets are considered to be a "local" approach as it delivers a lower

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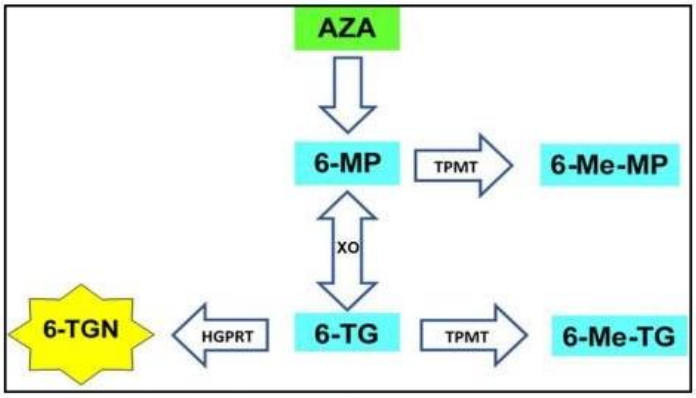

Fig. 1. Mechanism of action of thiopurine drugs and potential pathways of metabolism [8].
AZA (azathioprine), **6-MP** (6-mercaptopurine), **HGPRT** (hypoxanthine guanine phosphoribosyl transferase), **XO** (xanthine oxidase), **TPMT** (thiopurine S-methyltransferase), **6-Me-MP** (6-methyl-mercaptopurine), **6-TG** (thioguanine), **6-TGN** (6-thioguanine nucleotides).

bioavailability and thus less risk of toxicity [13].

Direct delivery to the colon is ideal as it surpasses first pass metabolism by the liver. However, direct delivery of rectal foam to the colon produces a lower bioavailability due to reduced absorption by the colon mucosa when compared to that of the gastric mucosa [12]. In a recent animal study by Helmy et al. (2017), a new therapeutic strategy was developed by loading AZA into colon-targeted chitosan beads and inserting the beads into an acid-resistant capsule [14].

The half-life of AZA is reported to be 26–80 min, or 3–5 hours if metabolites are included [15,16]. The half-life of TGN in erythrocytes is reported to be 5 days, and months may be needed to reach a steady state [17]. This may explain why a prolonged treatment period is needed before a clinical response occurs.

Therapeutic dosage concentrations range from less than 1 mg/kg bodyweight/day, to 3 mg/kg bodyweight, depending on the severity of the disease and the side effect profile. On average it takes 17 weeks for a therapeutic response to appear in most patients, but it has been suggested that the response time can be sped up by administering a loading dose of 5 mg/kg bodyweight/day to achieve a greater cumulative concentration [3,12,18]. Van Os et al (1996) observed that the patient response time can also be decreased by administering a loading dose of AZA intravenously, thus providing a portion of the cumulative dose more swiftly [12]. On average 47% of orally administered AZA reaches systemic circulation and just more than 80% of AZA is converted to 6-MP, which has a 16% bioavailability [12,13].

Although AZA has been proven to increase miscarriages by 20 fold in mice, conflicting studies and reports have been published with regards to the teratogenic nature of the drug in humans, with some studies [4,19] finding that the drug only causes low birth weight and premature birth while other studies [20,21] claim the drug is definitely teratogenic. It is difficult to relate AZA directly to foetal abnormalities as majority of the females taking the medication are on combination therapy and are advised to stay on treatment during the pregnancy to prevent disease relapse, many studies therefore conclude that any other birth abnormalities are due to underlying disease rather than the medication itself [13,19]. Yet, AZA has been listed as a class D teratogen according to the Food and Drug Administration (FDA), and is not recommended while breastfeeding [12].

AZA has been associated with a variety of side effects ranging from general nausea to myelosuppression, in very rare cases red-cell aplasia or death (in cases of TMPT polymorphism) [22,23]. Most side effects can be separated into two reaction groups, namely dose-independent (DI) and dose-dependent (DD) reactions. Drug Induced reactions tend to be hypersensitive, allergy-like reactions which tend to occur within the first few weeks after initial dosing. Symptoms such as pancreatitis, fever, joint pain, gastrointestinal disturbances and rash are common in DI reactions [17,24,25]. DD reactions tend to appear at later stages of therapy due to metabolite build up and often present as leukopenia, cholestatic jaundice, uncommon bacterial infections, hepatitis, nausea and myelosuppression. DD related side effects will generally disappear once the dosage is decreased, while DI reactions will continue until therapy is discontinued [12,13,17]. Many studies have reported patients developing toxicity long after therapy was initiated, most notably two studies totalling 1135 patients observed the onset of toxicity ranging from immediately after first dose to patients developing toxicity after 11 years of therapy [26–28]. In a more recent study by Björnsson et al (2017), it was noted that nearly three-quarters of patients who had developed thiopurine-induced hepatotoxicity, also developed cholestatic hepatitis within 3 months of starting the dose or increasing the dosage [29].

There are known associations between thiopurine treatment for rheumatoid arthritis or renal transplant and the increased risk of developing a malignancy, however there are differing opinions regarding the relationship when it comes to IBD patients [30,31]. Some studies claim there is no significant association between thiopurine therapy for IBD patients and the risk of malignancies [30], while other studies claim that there is a significant association with an increased risk of malignancies [32,33]. These malignancies include urinary tract cancer in older men, non-melanoma skin cancer (NMSC) in younger patients and lymphoma in the general population [34,35]. However, both study groups agree that there is limited data to establish causality [2].

Kandiel et al (2005) argued that a 4-fold increase in lymphoma is observed in IBD cases, but again whether this is due to the underlying disease, as a result of thiopurines or a combination of the two factors, remains unclear [2,36]. A study by Lewis et al (2000) pointed out that despite the 4-fold increase in risk of lymphoma, AZA is still a vital component of immunomodulatory therapy in IBD management and that the increased risk would have to be greater than 9.8-fold for the benefit of alternative therapies to outweigh the benefit of AZA therapy [17,37]. This statement has been reiterated recently in a study by Clowry et al (2017) on the relationship between thiopurine therapy and the risk of NMSC where, as previously concluded, due to the rising prevalence of IBD in young patients with little other alternative to immunosuppressive therapy, the benefit of this treatment outweighs the risk. Furthermore, it was noted that combination therapy for long periods of time also increased the risk factors [35].

Individuals using AZA are commonly on a combination therapy with a corticosteroid; where the steroid will slowly be weaned off once disease remission status is achieved. A study by de Jong et al (2014) has found that patients who are on a combination therapy with a higher

dosage of steroid when starting the AZA tend to have fewer and less severe adverse events [38]. It is estimated that 20–30% of all patients taking AZA monotherapy will discontinue use due to an adverse drug reaction (ADR) of some kind [9,17].

4. Pharmacogenetics

The varying degrees of therapeutic response and potential toxicity can be attributed to genetic variations of genes which encode key enzymes for the metabolism of thiopurines. These allelic variants are known as single nucleotide polymorphisms (SNP's), which are single base-pair change at a specific site, found in the human deoxyribonucleic acid (DNA) sequence. SNP's differ from normal genetic mutations in that they are only defined as a SNP when the specific allele switch is observed in more than 1% of the population [39]. The key enzymes involved in the metabolism of AZA include TPMT, HGPRT, Inosine Triphosphatase (ITPase) and Xanthine Oxidase. The polymorphisms result in the formation of variable active metabolites, of which ITPase and TPMT metabolites are the most studied [40].

4.1. Inosine Triphosphate and Azathioprine

Inosine triphosphate (ITP), a median in the purine metabolic pathway, is catalysed by ITPase to form inosine monophosphate (IMP). Deficiency of ITPase is due to a SNP on the ITP encoding gene on chromosome 20 and is characterised by irregular accumulation of ITP in erythrocytes, but it is a benign condition and under normal circumstances thought to be of no detriment to patient health [41]. However, in the presence of AZA or 6-MP, ITPase deficiency results in the accumulation of 6-thio-inosine triphosphate (6-tITP). 6-tITP is described as a "rogue nucleotide" with the potential to cause cell toxicity [42]. Currently there are five known ITPase SNP's of which three are silent and two have been linked to a decrease in ITPase activity when present in a homozygous state. It has been estimated that 6% of the population is heterozygous for a decreased activity SNP, and even less so for a homozygous SNP [17]. Despite three studies [41–43] having reported an association between ITPase deficiency and thiopurine toxicity, no consistent toxicity pattern has been reported; thus a conclusion was made by Lennard (2002) that ITP genotype testing or observation does not provide a clue as to whether a patient is at risk for cell toxicity [44,45]. This conclusion was supported by a more recent study by Citterio-Quentin et al (2017) in which it was shown that ITPase activity is rarely influenced by factors other than genetic parameters and has no influence on the concentration of AZA metabolites (6-TGN and 6-me-MP) [46].

4.2. Thiopurine methyltransferase and AZA

Thiopurine methyltransferase is encoded by the TPMT gene which is located on chromosome 6 [1]. This gene exhibits an autosomal codominant genetic polymorphism which can lead to an absent or low level of TPMT activity in individuals who are heterozygous or homozygous for this genetic polymorphism [8]. Currently there are 41 allele variants of the TPMT gene, with studies observing that allele genotype may be specific to race or ethnicity [47,48].

The most common functional allele is TPMT*1, while TPMT*2, *3A, *3B, *3C and *4 are well documented polymorphism alleles associated with heterozygous or homozygous variant genotypes as listed in Tables 1 and 2. TPMT*2, *3A and *3C account for over 90% of the inactive alleles [49].

Individuals can be homozygous normal, meaning they inherited both wild-type normal metabolizing alleles, or they can be homozygous variant meaning both inherited alleles contained SNP's and there is little to no TPMT activity. Lastly an individual can be a combination, or heterozygous, implying that they inherited both a functional and a nonfunction allele resulting in a moderate enzyme activity level.

Table 1
Nomenclature of common TPMT allele mutations, issued by the TPMT Nomenclature Committee [50].

Common Allele Name	Coding DNA	Protein
TPMT*2	238G > C	Ala80Pro
TPMT*3A	460G > A / 719A > G	Ala154Thr / Tyr240Cys
TPMT*3B	460G > A	Ala154Thr
TPMT*3C	719A > G	Tyr240Cys

There have been numerous studies on the TPMT activity in Caucasian and Asian individuals but less so in the African population. A study by Fong et al (2017) found the *3A allele to be most common in the Caucasian and Indian population and the *3C allele to be most common in the Asian population [1]. Studies in Nigeria and Libya have shown majority of the African population to have the *3C allele, a finding supported in a study on TPMT activity in African-Americans by Hon et al (1999) [8,51,52].

Updated recommendation for AZA dosing based on TPMT genotyping are frequently published by the Clinical Pharmacogenetics Implementation Consortium (CPIC), an association created to meet the need for specific guidance of pharmacogenetic testing for both laboratories and clinicians. This dosing is based on the phenotype and genotype of the individual [3].

World-wide 11% of all Caucasian individuals will have the polymorphism, of these 1/300 individuals will be homozygous for the variant allele and thus completely TPMT deficient [6]. Measuring the TPMT activity level is an effective method of determining an individual's risk of toxicity and allows for dose adjustment or drug avoidance, to prevent the risk of the individual developing bone marrow suppression, leukopenia, thrombocytopenia or neutropenia [28,55–60]. TPMT polymorphisms have only been associated with overall thiopurine-induced adverse drug reactions and not with the less severe side effects such as gastrointestinal upsets, skin reactions and pancreatitis; which are instead attributed to the underlying disease [61]. None the less, in 2003, the FDA made it compulsory to add that the status of TPMT be determined prior to dosing, to the AZA package insert [1].

TMPT status can be determined via phenotypic enzyme-level testing using red blood cells, or by creating a white blood cell genetic profile [9,55]. Determining the TPMT genotype of the individual will enable the clinician to better understand and predict variation in ADR's [62]. Abnormalities in other AZA metabolizing enzymes are extremely rare [58].

5. Gastrointestinal therapy

5.1. Inflammatory Bowel Disease

One and a half million Americans suffer from IBD- an umbrella term used to represent a group of intestinal disorders affecting all or part of the digestive tract – which are comprised of 2 major diseases: ulcerative colitis (UC) and Crohn's disease (CD). IBD is estimated to affect 0.3% of the westernised world and has a rising prevalence in newly industrialised countries such as Africa, South America and the Middle East [63,64]. Of these IBD patients at least 20% will suffer from leukopenia and eventually myelosuppression [9,41,61].

While UC and CD are very similar is symptoms, they differ vastly in location and treatment. UC only affects the inner lining of the colon whereas CD can affect any component of the gastrointestinal tract from the oesophagus to the rectum, and may also affect the skin, eyes or liver. Individuals suffering from IBD have a chronically inflamed gastrointestinal tract as the body perceives food or bacteria as a foreign substance and will respond by activating T-lymphocytes, triggering inflammation. Leukopenia is a common haematological sign of AZA

Table 2
CPIC recommended azathioprine therapy, according to TPMT phenotype [53,54].

Phenotype	TPMT Genotype	Example of Diplotypes	Recommendations: Therapeutic	Recommendations: Steady State
Homozygous wild-type - High enzyme activity	Two functioning alleles	*1/*1	2–3 mg/kg/day	Allow 2 weeks to achieve steady state between each dose adjustment
Heterozygous - intermediate enzyme activity	One functional allele + One non-functional allele	*1/*2 *1/3A *1/3B *1/3C *1/4	Start at 30–70% of target dose, increase based on tolerance	Allow 2–4 weeks to achieve steady state between each dose adjustment
Homozygous variant - low/deficient enzyme activity	Two non-functioning alleles	*2/3A *3A/3A *3A/4 *3C/2 *3C/3A	Dose 3 × weekly or reduce daily dose by 10-fold. - Consider alternative treatment	Allow 4–6 weeks to achieve steady state between each dose adjustment

Table 3
Azathioprine monotherapy randomised clinical trials [67].

Author	Year of Publication	Use of Azathioprine	Efficacy = n/N (%)
Bastida et al. [68]	2007	Induction / Maintenance	21/25 (80%)
Campbell & Ghosh [69]	2001	Maintenance	82/94 (87%)
Christodoulou et al. [70]	2003	Induction / Maintenance	15/15 (94%)
Cuffari et al. [71]	2001	Maintenance	14/19 (74%)
Falasco et al. [72]	2002	Induction / Maintenance	25/58 (43%)
Fraser et al. [73]	2002	Induction / Maintenance	201/346 (58%)
Gisbert et al. [74]	2008	Induction / Maintenance	65/156 (42%)
Hibi et al. [75]	2003	Maintenance	15/17 (88%)
Khan et al. [76]	2000	Induction / Maintenance	38/53 (72%)
Kull & Beau [77]	2002	Induction / Maintenance	23/30 (77%)
Lopez-Sanroman et al. [78]	2004	Maintenance	24/34 &71%)
Mantzaris et al. [79]	2001	Induction	24/40 (60%)
Mantzaris et al. [80]	2004	Maintenance	28/34 (82%)
Paoluzi et al. [81]	2002	Induction / Maintenance	22/32 (69%)
Sood et al. [82]	2006	Induction / Maintenance	101/111 (91%)

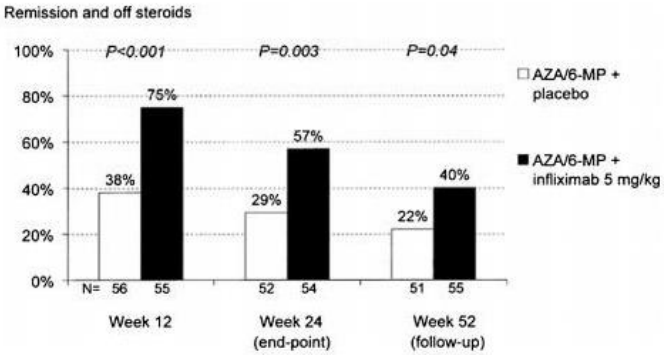

Fig. 2. Disease remission in Azathioprine and placebo vs Azathioprine and Infliximab arms from the study by Lemann et al [86].

Table 4
Details of the double-blind randomised clinical trials by Lemann et al. [86] and Colombel et al. [87].

Author	Year of Publication	Drug	Comparison	n = arm	Efficacy = n/N (%)
Lemann et al. [86]	2006	Azathioprine	Infliximab	57	32/57 (57%)
		Azathioprine	Placebo	56	16/56 (29%)
Colombel et al. [87]	2010	Azathioprine	Placebo	170	51/170 (30.0%)
		Infliximab	Placebo	169	75/169 (44.4%)
		Infliximab	Azathioprine	169	96/169 (56.8%)

toxicity, and occurs in up to 20% of IBD patients due to the TPMT polymorphism [57,65]. In 2012, 100,000 people were admitted to hospital in the United States for various complications arising from IBD resulting in an annual hospitalization cost of over $3 billion [66].

A meta-analysis was conducted by Gisbert et al (2009), to review the efficacy of AZA and mercaptopurine in the induction and maintenance of disease remission in UC. This included a selection of randomised clinical trials (RCT) comparing AZA to placebo or aminosalicylates. A total of 1065 patients were included from 15 studies, taken from the year 2000 and onward [67]. The detail of the latter studies are highlighted in Table 3. Of the 1065 patients, efficacy was observed in 697 patients (65.45%), i.e. they were found to be in disease remission at the end of the respective study period. In the majority of these studies AZA was indicated for induction and maintenance purposes.

In 2017, a 5-year review was published by Cassieri et al (2017), documented the number of IBD patients utilising AZA treatment at the IBD Outpatient Clinic at Cristo Fe Hospital in Italy. There were 260 IBD patients receiving AZA treatment over the 5-year observation period, 145 for CD and 115 for UC. Upon completion of the 5-year treatment period, 86 CD patients (59.3%) and 49 UC patients (42.6%) were still in remission [83].

Azathioprine is used in conjunction with steroids, methotrexate or biological agents such as infliximab to induce disease-remission and maintain remission, by reducing the white cell count and neutrophil count [84].

In 2017, Cholapranee et al (2017) released a systematic review of RCT's that was conducted to compare the efficacy of AZA monotherapy, Infliximab monotherapy and a combination of AZA and Infliximab in the treatment of CD. This review mentioned 12 RCT's which made use of monotherapy or combination therapy to attain disease remission induction and maintenance as well as mucosal healing [85]. Two of these studies that revealed significant results, were conducted by Lemann et al [86] (2006) and Colombel et al [87] (2010). Both studies were double-blind and made use of a placebo to maintain the blind.

The study by Lemann et al (2006) took into consideration that results may be altered in patients having previously used AZA or being treated with AZA at inclusion or commencement of the study, hence the study made use of two groups within the dosing arms; a failure stratum (previously exposed to AZA) and a naïve stratum [85]. 55 patients were part of the failure stratum and 58 of the naïve stratum. Patients were then treated with AZA and a placebo or AZA and Infliximab. The Crohn's Disease Activity Index (CDAI) was used to evaluate patients at 12 and 24 weeks respectively. A score of CDAI < 150 was used as the marker for disease remission. Of the 113 patients partaking in the study, 57% of the combination group had disease remission at 24 weeks, in comparison to the 29% experienced by the monotherapy group. These findings are depicted in Fig. 2. No significant effect was seen when comparing the stratum to the treatment group [86].

Colombel et al (2010) made use of three separate dosing arms in the double-blind study, comparing Infliximab and placebo versus AZA and placebo versus Infliximab and AZA. 508 patients were included in a 26 week study, which as seen in Table 4, showed that the combination therapy had more efficacy in reaching disease remission, than when compared to the other treatment arms (56.8% vs 44.4% vs 30.0%). This pattern was also seen in the mucosal healing within the arms [87]. This study also made use of the CDAI scoring.

There are a variety of ways to determine therapeutic efficacy, depending on the health care system and patients' standard of living. The most common mean is by biochemical evaluation where C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are assessed. CRP and ESR are both non-specific markers of inflammation that are used in combination to monitor the disease status of IBD patients. Frequent measuring of ESR and CRP counts as well as full blood counts (FBC) should be mandatory in individuals on chronic AZA treatment, as suggested by the FDA and recommended by other studies [3,18,61]. Studies suggest that patients should be monitored weekly for the first 8 weeks of therapy, thereafter monthly for the next 6 months and then bi-annually once the patient is on long-term therapy [17]. An accurate ESR count can be affected by numerous factors such as anaemia, infection or a sudden decrease in red blood cells, while CRP fluctuates rapidly and is affected by obesity or pregnancy. CRP values correlate very well to endoscopic findings in CD, but less so in UC [88–90].

Additional therapeutic markers include faecal calprotectin or faecal lactoferrin, which are superior to CRP in detecting active versus inactive IBD, but not superior to endoscopic findings. Using biochemical tests to determine inflammation should be an additive tool used in combination with clinical and endoscopic findings [91].

There is a great deal of debate as to what the exact therapeutic endpoint for disease remission should be, as many of the clinical efficacy markers are symptom based and reflect poorly on endoscopic findings of inflammation. It has recently been suggested that mucosal healing should be considered the therapeutic endpoint for disease remission, as opposed to simply achieving normal ranges of ESR and CRP counts, as emerging data is suggesting that individuals who have achieved mucosal healing have a better long-term prognosis [92,93]. These CD and UC patients will require less hospitalizations and lower the need for systemic steroids [85].

6. Conclusion

AZA therapy is an affordable, effective solution to maintain disease remission in IBD patients and while genetic testing is expensive, it still outweighs the rate of hospitalisation, and the cost of medication and treatment caused by AZA cytotoxicity. When comparing AZA to the more newer IBD therapies that are available, AZA remains a viable option for the state sector but it is essential to make "early warning" SNP testing, common practice to ensure therapeutic efficacy.

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