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Optimising the use of Azathioprine in the treatment of Inflammatory Bowel Disease

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Optimising the use of Azathioprine in the treatment of Inflammatory Bowel Disease

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MD (Res)

2013

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Communications arising from this research

Original Research Papers

NOVEL PHARMACOGENETIC MARKERS FOR TREATMENT OUTCOME IN AZATHIOPRINE-TREATED INFLAMMATORY BOWEL DISEASE

Smith, MA.; Marinaki, AM; Arenas,M; Shobowale-Bakre, M; Lewis, CM; Ansari,A; Duley,J & Sanderson,JD.

Alimentary Pharmacology and Therapeutics, 2009 Aug 15;30(4):375-84

OPTIMISING OUTCOME ON THIOPURINES IN INFLAMMATORY BOWEL DISEASE BY CO-PRESCRIPTION OF ALLOPURINOL

Melissa A. Smith¹, Paul Blaker¹, Anthony M. Marinaki², Simon Anderson¹, Peter M. Irving¹, Jeremy D. Sanderson¹

Journal of Crohn's and Colitis 2012 Oct;6(9):905-12. Epub 2012 Mar 3. Feb 2012

THE IMPACT OF INTRODUCING THIOGUANINE NUCLEOTIDE MONITORING INTO CLINICAL PRACTICE

Melissa Smith, Paul Blaker, Chenali Patel, Anthony Marinaki, Monica Arenas, Emilia Escuredo, Simon Anderson, Peter Irving, Jeremy Sanderson

Int J Clin Pract. 2013 Feb;67(2):161-9.. Epub 2012 Dec 17.

Oral Presentations:

CALCULATING THE "MISSED OPPORTUNITY" OF THIOPURINE MONOTHERAPY OVERCOME WITH THIOPURINE AND ALLOPURINOL COMBINATION THERAPY

M.A. Smith, P.Blaker, A.M. Marinaki, S. Anderson, P.M. Irving, J.D. Sanderson.

DDF (BSG) 2012

GENETIC POLYMORPHISM IN THE MULTI-DRUG RESISTANCE-5 GENE IS ASSOCIATED WITH NON-RESPONSE TO AZATHIOPRINE TREATMENT IN INFLAMMATORY BOWEL DISEASE

M.Smith; A.Marinaki; J.D.Sanderson

DDW 2010

FURTHER EXPERIENCE OF OPTIMISING TREATMENT OUTCOME ON AZATHIOPRINE BY CO-PRESCRIPTION OF ALLOPURINOL IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE

M. Smith, A. Marinaki, S. Anderson, P. Irving, J. Sanderson

BSG 2010

COMMON POLYMORPHISM IN THE ALDEHYDE OXIDASE GENE IS A MARKER OF NON-RESPONSE TO AZATHIOPRINE THERAPY IN INFLAMMATORY BOWEL DISEASE

Smith, MA; Marinaki, AM; Ansari,A; Sanderson, JD.

BSG 2008.

Poster Presentations

MYELOTOXICITY IS A LATE EVENT IN AZATHIOPRINE TREATED TPMT HETEROZYGOTES: LESSONS FOR MONITORING.

Melissa Smith, Anthony Marinaki, Monica Arenas, Simon Greenfield, Azhar Ansari, Jeremy Sanderson.

BSG 2007: Gut 56: Suppl. 11 April 2007 Abstract 371 p.A120

Poster of distinction DDW 2007

SEEKING PHARMACOGENETIC LOCI WHICH EXPLAIN NON-THIOPURINE METHYLTRANSFERASE RELATED SIDE EFFECTS IN PATIENTS TAKING AZATHIOPRINE FOR INFLAMMATORY BOWEL DISEASE

M. A. Smith, A. M. Marinaki, M. Arenas, S. Greenfield, A. Ansari, J. Sanderson.

Poster prize, United European Gastroenterology Week, 2007.

THE IMPACT OF INTRODUCING THIOGUANINE NUCLEOTIDE MONITORING INTO CLINICAL PRACTICE

Melissa Smith, Tony Marinaki, Monica Arenas, Emilia Escuredo, Jeremy Sanderson
.BSG 2008.

THIOGUANINE NUCLEOTIDE MONITORING AIDS CLINICAL DECISION-MAKING IN THE INFLAMMATORY BOWEL DISEASE CLINIC

M. A. Smith, A. M. Marinaki, M.Arenas, E. Escuredo, P.Irving, J.D.Sanderson
BSG 2010

RATIO OF METHYLATED METABOLITES TO THIOGUANINE NUCLEOTIDES PREDICTS HEPATOTOXICITY AND NON-RESPONSE TO THIOPURINE THERAPY

M. A. Smith, A. M. Marinaki, M.Arenas, E. Escuredo, P.Irving, J.D.Sanderson
BSG 2010

GENETIC POLYMORPHISM IN THE MULTI-DRUG RESISTANCE-5 GENE IS ASSOCIATED WITH NON-RESPONSE TO AZATHIOPRINE TREATMENT IN INFLAMMATORY BOWEL DISEASE

M.Smith; A.Marinaki; J.D.Sanderson
BSG 2010

FURTHER EXPERIENCE OF OPTIMISING TREATMENT OUTCOME ON AZATHIOPRINE BY CO-PRESCRIPTION OF ALLOPURINOL IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE

M. Smith, A. Marinaki, S. Anderson, P. Irving, J. Sanderson
DDW 2010

CALCULATING THE “MISSED OPPORTUNITY” OF THIOPURINE MONOTHERAPY
OVERCOME WITH THIOPURINE AND ALLOPURINOL COMBINATION THERAPY

M.A. Smith, P.Blaker, A.M. Marinaki, S. Anderson, P.M. Irving, J.D. Sanderson

DDW 2012

Review articles

PHARMACOGENOMICS IN THE TREATMENT OF INFLAMMATORY BOWEL DISEASE

Melissa A. Smith, Anthony M. Marinaki, Jeremy D. Sanderson.

Pharmacogenomics (2010) 11(3), 421–437

REVIEW ARTICLE: MALIGNANCY ON THIOPURINE TREATMENT WITH SPECIAL
REFERENCE TO INFLAMMATORY BOWEL DISEASE

Smith,MA; Irving,PM; Marinaki,AM and Sanderson,JD

Aliment Pharmacol Ther. 2010 Jul;32(2):119-30.

Book Chapters

MANAGEMENT OF THE PATIENT WITH MORE COMPLEX IBD

Dr Jeremy Sanderson and **Dr Melissa Smith**

Inflammatory Bowel Disease Nursing

Eds. K.Whayman, J.Duncan & M O'Connor

Quay Books, MA Healthcare Ltd. 2011

DO THIOPURINES WORSEN RISK AND PROGNOSIS OF CERVICAL NEOPLASIA?

Smith,M.A. and Sanderson,J.D.

Clinical Dilemmas in Inflammatory Bowel Disease: New Challenges

Eds: Peter Irving, Corey A. Siegel, David Rampton and Fergus Shanahan

Wiley-Blackwell, 2011

Abstract

Azathioprine (AZA) and mercaptopurine (MP – also known as 6-mercaptopurine or 6MP) are the first line immunomodulatory treatments for inflammatory bowel disease (IBD) with proven efficacy for multiple clinical outcomes (fistula closure, steroid withdrawal, maintenance of remission etc.). They also have a wide application in the fields of rheumatology, dermatology, haematology and transplant medicine. However these drugs also cause toxicity and may be ineffective. Both of these outcomes can have serious consequences for the individual concerned. A proportion of toxicity caused by these drugs is explained by genetic polymorphism in the enzyme thiopurine methyltransferase (TPMT), however the majority of toxicity remains unexplained and as yet there is no satisfactory explanation for the variable efficacy of these drugs.

In this thesis I explore the impact of genetic polymorphism in several novel candidate genes involved in thiopurine metabolism on the success of thiopurine treatment. Single nucleotide polymorphisms (SNPs) in xanthine oxidase/dehydrogenase (*XDH*) and the final enzymatic step which activates its essential cofactor (molybdenum cofactor sulfurase, *MOCOS*) are shown to protect against side effects to AZA therapy. Polymorphism in aldehyde oxidase (*AOX*) and multi-drug resistance protein 5 on the other hand, are shown to predict a lack of response to thiopurine treatment. Sequencing *AOX* validated the real-time PCR results and suggested that there were no other coding SNPs likely to be contributory. A pharmacogenetic index incorporating these new markers with established predictors of outcome on thiopurines is presented and the clinical utility of such an index discussed. Finally, clinical data supporting the optimisation of azathioprine therapy, both by the measurement of thioguanine metabolite profiles and through co-prescription of allopurinol are presented.

Chapter One: Literature Overview

1.1 Brief introduction to Inflammatory Bowel Disease (IBD):

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory diseases of the gastrointestinal tract, affecting primarily young adults. Both result in distressing symptoms of diarrhoea, abdominal pain, loss of weight and fatigue and are associated with a reduced quality of life and significant occupational loss¹. Both the disease and its treatments affect family planning, sexual function and fertility/fecundity². Children, particularly adolescents, are also affected, with additional problems of impaired growth, delayed sexual maturation, educational loss and adverse psychological impact³.

Despite much research and many new developments in this field, the treatment of IBD remains challenging and often fails to restore patients to their former quality of life. Treatment of all but the mildest disease consists of either immunosuppression or surgery. The majority of patients receive long-term immunomodulatory treatment and the success of these agents can determine the course of their disease.

In UC, first line treatment is the use of 5-aminosalicylates (5ASAs), with steroid treatment for acute flares that cannot be controlled on 5ASAs alone. The importance of reducing steroid exposure and the inefficacy of steroids as maintenance therapy are widely acknowledged. This means that frequent steroid courses or inability to wean from steroids should result in an early escalation of treatment to include a thiopurine. Whilst evidence for the efficacy of anti-TNF antibody therapy in UC is beginning to accumulate, the National Institute for Clinical Excellence (NICE) have still not approved their use outside acute severe UC, and evidence to support other immunomodulators is lacking. This means that for these patients optimisation of thiopurine treatment is essential. Failure to achieve remission on these agents will generally result in recourse to surgery – a devastating event in this predominantly young population.

In CD, the number needed to treat for 5ASAs to achieve clinical remission is high and they are generally not considered to be a suitable maintenance therapy. Steroids, as for UC, can be useful to induce remission in an acute severe attack, but reducing exposure to steroid therapy is

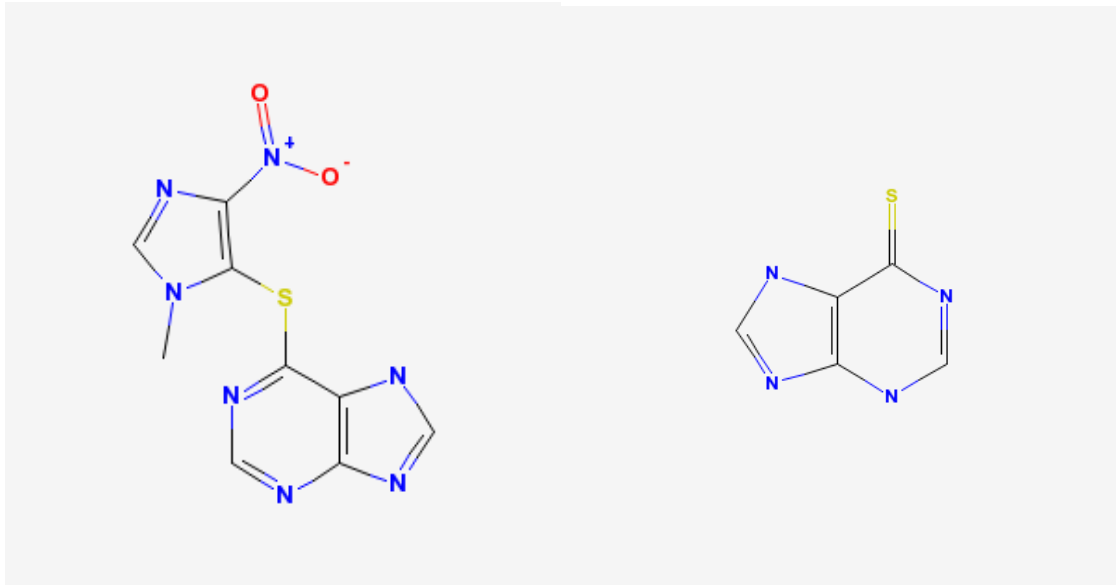
considered a key quality indicator in the treatment of CD. Whilst “top-down” (anti-TNF antibody initiated early) strategies for the treatment of CD are proposed in recent clinical trials, in practice this has translated to a “rapid step-up” regime, in which immunomodulators (in practice – thiopurines) are introduced early to limit steroid exposure and target mucosal healing and disease remission, rather than symptoms alone. Early recourse to anti-TNF therapy is implemented if these drugs are not achieving adequate disease control or the patient is considered to be at high risk from their disease due to its severity, behaviour and distribution.

Thiopurines have proven efficacy in IBD, reducing relapses, permitting steroid withdrawal, and closing fistulae^{4,5}. Indeed, as many as 60% of patients with Crohn’s disease (CD) now receive Azathioprine (AZA) or Mercaptopurine (MP)⁶, reflecting the changing goals of treatment in IBD away from symptom control alone, towards mucosal healing and altered natural history. This change has resulted in a push towards more aggressive treatment, maximising the chances of a successful clinical response. Newer biologic therapies appear to have a higher primary response rate than the classical immunomodulators, and their use is increasing. Co-prescription of azathioprine with these newer agents however, appears to provide additional benefit both for achievement and maintenance of remission⁷⁻⁹ a benefit which may be thiopurine specific⁹, meaning that the advent of newer therapies has extended rather than reduced the indications for thiopurine use in the treatment of IBD.

1.2 Thiopurine Drugs

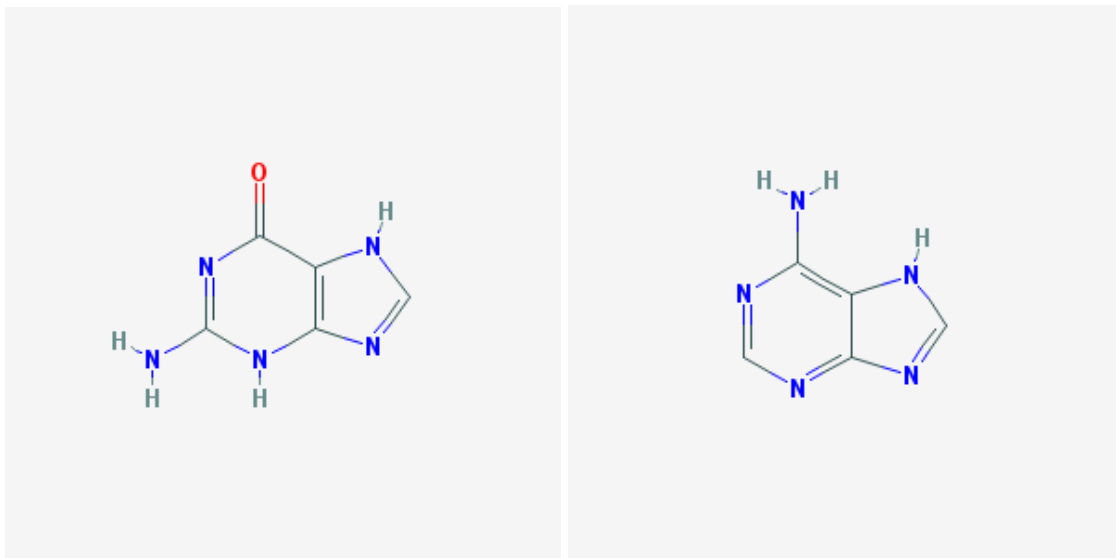
AZA and MP are therefore considered an important mainstay in the armamentarium of immunomodulators used to treat IBD, and are first line immunomodulatory treatments in the practice of most physicians. AZA is a more stable and soluble pro-drug of MP, to which it is converted by both enzymatic^{10,11} and non-enzymatic¹² cleavage of the imidazole moiety during gut absorption. Both drugs are analogues of the purine nucleotides adenine and guanine, one of the most important building blocks of life (figure 1.1).

In addition to their role as essential elements of both DNA and RNA, purine nucleotides store and transfer the energy required for intra-cellular processes and are involved in cell signalling and co-factor synthesis.



Azathioprine

Mercaptopurine



Guanine

Adenine

Figure 1.1 Chemical structure of AZA and MP, compared to the canonical nucleotides guanine and adenine. Images taken from <http://pubchem.ncbi.nlm.nih.gov/>

1.3 What is the evidence that thiopurines work in IBD?

AZA and MP have become first-line treatments for steroid-dependent or chronic relapsing IBD⁴. There are now several trials and meta-analyses which confirm the efficacy of thiopurines in IBD. The evidence is stronger and more plentiful for CD than UC, but there is now good evidence for efficacy of thiopurines in induction/maintenance of remission and steroid-sparing in both diseases and for fistula healing in CD¹³⁻¹⁷. There is also a growing body of evidence for a role in the post-operative prevention of relapse in patients with CD.¹⁸⁻²³

1.4 How do thiopurines work?

It has long been considered that incorporation of “rogue” thiopurine nucleotides into DNA was essential for the action of these drugs²⁴. However, the initial proposition that incorporated thioguanine nucleotides (TGNs) would truncate a replicating strand has been shown to be inaccurate. DNA can continue to replicate beyond an incorporated thioguanine nucleotide, but the presence of the bulky sulphur atom in thioguanine subtly disrupts DNA structure²⁵ and the base pairs formed are much less stable²⁶. In addition to these structural alterations, thioguanine codes ambiguously and could be paired with either a cytosine or a thymidine base, creating the potential for mutations to develop²⁶.

Thioguanine is also much more susceptible to methylation than standard nucleotides^{27,28}. This further disrupts DNA structure, and has been shown to encourage point mutation, silence gene expression²⁹ and increase mis-pairing with thymidine^{27,30}. Equally, incorporated TGNs are more sensitive to attack by reactive oxygen species than canonical bases and, once oxidised, are unable to form a stable pair with any base^{31,32}. The situation is complex however, and recent studies have demonstrated a reduction in global DNA methylation in response to MP and 6-thioguanine (6-TG) treatment, thought to be important in the anti-leukaemic action of thiopurines³³.

Importantly, all bases (even cytosine) are identified by mismatch repair (MMR) systems as coding faults when they pair with thioguanine (due to a combination of altered structural conformation and decreased strength of base-pairing)^{27,28}. The “faulty” daughter strand of DNA is cleaved and the cell attempts to recopy this section of DNA. However, if the thio-GTP has

been incorporated into the parent strand, the fault can never be repaired. Repeated failure to correct DNA abnormalities caused by TGN incorporation triggers cellular apoptosis^{27,28,34-36}. This selective killing of dividing cells is thought to be important in the mechanism of action of thiopurines for both their anti-inflammatory and anti-leukaemic effects.

Although this process renders cells with functioning MMR systems more sensitive to thiopurines^{28,35}, cells without functional MMR will still suffer cytotoxicity from thiopurines³⁷. A different DNA-binding protein complex is formed in these situations, which also triggers cell death in response to thiopurine incorporation in cellular DNA³⁸.

Incorporated thioguanine also inhibits cell division by interfering with the activity of a whole spectrum of enzymes responsible for DNA replication and repair, including DNA polymerase, ligase and endonuclease²⁵, topoisomerase II³⁹ and RNase H⁴⁰. Thio-GTP can also be incorporated into the telomere at the 3' region of chromosomes. The telomere is present in dividing cells and is thought to play an important role in allowing cell lines to continue to divide. When thio-GTP is incorporated into the telomere, it prevents telomerase from binding and this could also block cell division⁴¹. Reduced telomerase activity has been demonstrated in the white cells of IBD patients receiving AZA⁴².

In addition to these mechanisms, all of which depend on rogue nucleotide incorporation into DNA, there have been many other observations on the influence of thiopurines which suggest additional mechanisms of action. The best established of these is the role played by methyl-mercaptopurine ribonucleotides which interfere with purine *de novo* synthesis^{43,44}. This effect is thought to be mediated by inhibition of phosphoribosyl pyrophosphate (PRPP)-aminotransferase^{45,46}, the first enzyme in the pathway of purine synthesis. This has two important effects; firstly decreased competition for thioguanine, thereby increasing the likelihood of TGNs being incorporated into DNA, and secondly an accumulation of PRPP which is essential for TGN formation⁴⁷. It is these methylated metabolites which may in part explain the equivalent therapeutic effects of MP and 6-TG, despite the significantly higher TGN levels seen with 6-TG therapy^{48,49}.

Another explanation for the anti-proliferative effects of the thiopurines has been proposed by Dayton et al⁵⁰ who demonstrated inhibition of T-cell proliferation (in association with depletion of their adenosine triphosphate (ATP) and guanine triphosphate (GTP) pools), caused by both AZA and MP. Replenishing these depleted nucleotides blocked the anti-proliferative effect of MP but not AZA. In this same paper, further experiments showed that MP did not interfere with GTP cell receptor binding but that AZA had some ability to do this⁵⁰. The concept that thiopurines may be able to interfere with the role of GTP in cellular signalling was taken further in experiments by Tiede et al⁵¹ who demonstrated that thio-GTP (derived from AZA) binds Rac-1 receptors on T-cells in place of GTP, blocking activation and thereby down-regulating various T-cell mediators including NF-Kb, bcl-X_L and mitogen-activated protein kinase (MEK) resulting in T-cell apoptosis^{51,52}.

Another anti-inflammatory mechanism of action of thiopurines is an effect on macrophages. Several studies have demonstrated altered macrophage function in the presence of thiopurines. Effects are wide ranging and include decreased circulating monocyte numbers⁵³, attenuated recruitment⁵³⁻⁵⁵, altered phenotype and gene expression^{56,57} decreased production of nitric oxide⁵⁸ and impaired handling of antibody-antigen complexes⁵⁵. Most studies have failed to demonstrate an effect of thiopurines on phagocytosis, however a recent abstract describes enhanced phagocytosis by macrophages in the presence of 6-thioguanine⁵⁹.

Thiopurines have also been shown to down-regulate a variety of T-cell mediators such as TRAIL, TNFRS-7, α 4-integrin⁶⁰. TGN levels have been shown to correlate inversely with IFN- γ levels⁶¹, and natural killer cell activity (including specific actions against tumour cells) has been shown to be down-regulated in CD patients treated with MP⁶². Ben-Horin et al have shown that thiopurines can arrest the proliferation of activated T-cells and also have the ability to selectively reduce the CD4+ memory cell response to repeatedly encountered antigens⁶³.

1.5 Challenges to the successful use of thiopurines

Despite their central role in the treatment of IBD, immunomodulators are not without their problems. Firstly, there is considerable individual variation in clinical response to each medication⁶⁴ and secondly, but just as importantly, these drugs can cause serious side effects⁶⁵⁻⁶⁷.

As they are employed in a more aggressive fashion, predicting clinical outcome and avoiding toxicity are becoming increasingly important. Work in this area has demonstrated that pharmacogenetics has a significant role in determining an individual's outcome on thiopurine therapy. It had been hoped that this inter-individual variation could be circumvented by giving 6-t(h)ioguanine (6-TG) directly⁶⁸. However, this strategy has been shown to have its own problems, particularly with hepatotoxicity⁶⁹, and is now reserved for expert use in resistant cases. This means that the study of thiopurine metabolism has taken on a new importance.

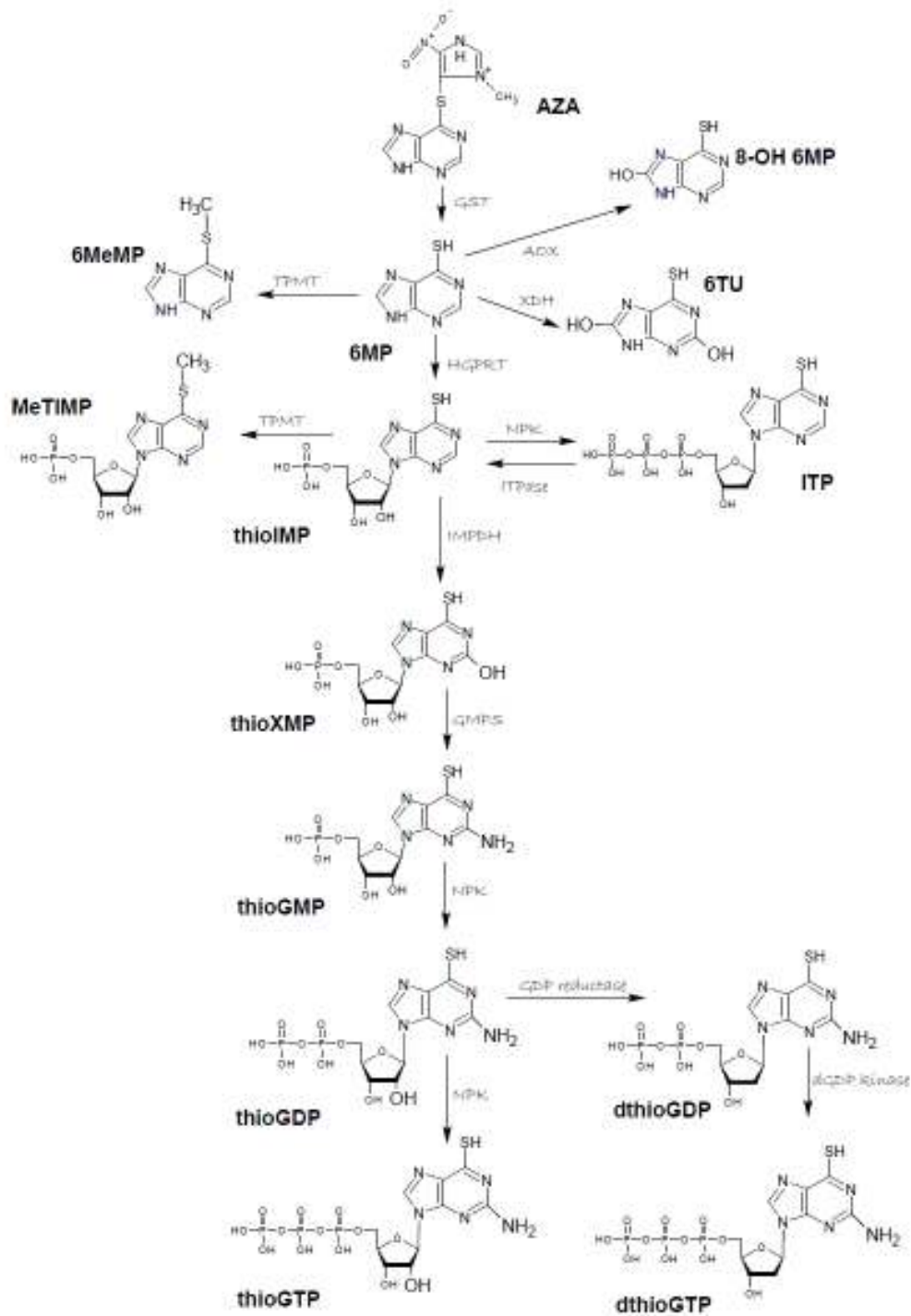


Figure 1.1: Thiopurine Metabolism. Production of the active thioguanine nucleotides from azathioprine and mercaptopurine by the endogenous purine salvage pathway.

Figure 1.2 Abbreviations:Metabolites:

AZA- azathioprine, 6MP- 6-Mercaptopurine, 8OH 6MP- 8-hydroxy 6-mercaptopurine, MeTIMP- 6-methylthioinosine monophosphate, 6MeMP- 6-methylmercaptopurine, 6TU- 6-thiouric acid, thioIMP- 6-thioinosine monophosphate, thioXMP- 6-thioxanthine monophosphate, thioGMP- 6-thioguanine monophosphate, thioGDP- 6-thioguanosine diphosphate, thioGTP- 6-thioguanosine triphosphate, dthioGDP- deoxy-6-thioguanosine diphosphate, dthioGTP- deoxy -6-thioguanosine triphosphate.

Enzymes:

GST- glutathione-s-transferase, TPMT- thiopurine methyltransferase, XDH- xanthine oxidase/dehydrogenase, HGPRT- hypoxanthine guanine phosphoribosyltransferase, ITPase- inosine triphosphatase, IMPDH- inosine monophosphate dehydrogenase, GMPS- guanine monophosphate synthetase, NPK- nucleotide phosphokinase, dGDP kinase- deoxyguanine diphosphate kinase.

1.6 What is known about thiopurine metabolism?

As a result of their molecular similarity to the purine nucleosides (figure 1.1), thiopurines are subject to the action of a large number of enzymes, primarily those from the “purine salvage” pathway, (figure 1.2). This is a complex network of enzymes which retrieve purine nucleosides produced by the breakdown of DNA and RNA and recycle them, limiting the need for energy-expensive *de novo* nucleotide synthesis (figure 1.5). Salvage of the purine bases hypoxanthine, guanine and adenine by phosphoribosyltransferases further reduces the need for expensive *de novo* synthesis. A separate parallel system exists for the salvage and inter-conversion of pyrimidines.

Thiopurine metabolism is similarly complex with clinically relevant variations in key enzymes. The best characterised of these is the enzyme thiopurine methyltransferase (TPMT). Genetically determined variation in TPMT activity is now considered a classic example of the clinical utility of the emerging field of pharmacogenetics. However, there are multiple other enzymes involved in purine and thiopurine metabolism. Many of these are also known to be subject to genetic polymorphism, the functional impact of which is as yet unknown. Since TPMT only explains a proportion of toxicity experienced on azathioprine^{70,71} it is logical to suppose that some of these other enzymes will also have a role to play.

TPMT competes with xanthine oxidase/dehydrogenase (XDH) and hypoxanthine guanine phosphoribosyltransferase (HGPRT) to act on MP, see figure 1.2. Whilst both TPMT and XDH catabolise and inactivate MP, HGPRT is the first enzyme in the anabolic pathway which will take MP on to be converted into its active metabolite – thioguanine nucleotides (TGNs) ^{72,73}. A diagrammatic representation of thiopurine metabolism is presented in figure 1.2. There follows a more detailed look at what is known about each of the enzymes involved in this system in relation to variability in thiopurine metabolism. Other key pharmacogenetic targets (from outside the purine salvage pathway) are then also reviewed.

1.7 TPMT

TPMT is just one of many enzymes involved in thiopurine metabolism, but it plays a key role both in tolerance of and response to these treatments⁷⁴. There is naturally occurring variability in TPMT activity across the population according to genetic polymorphism.

To date, 42 polymorphisms of TPMT have been reported in the literature and to the TPMT allele nomenclature committee⁷⁵ (TPMT*2 and *4-36 *3A-3E and *1, *1A & *1S) ⁷⁵⁻⁷⁹. Most compromise TPMT activity by accelerated breakdown of the TPMT protein, partly by autophagy⁸⁰, but there are other mechanisms. These include reduced efficiency of the enzyme's active site (TPMT*5), disrupted initiation of translation and therefore zero TPMT protein production (TPMT*14) and alternative splicing (TPMT*4 and *15)⁸¹. At <http://www.imh.liu.se/tpmtalleles> an up to date list of all alleles is maintained and assigned a standard nomenclature⁷⁵.

The distribution of TPMT activity in the population is tri-modal (figure 1.3), reflecting genetic co-dominance. Approximately 10% of the Caucasian population have one TPMT polymorphism resulting in half “normal” TPMT activity, whilst approximately 1 in 300 have two variant alleles resulting in negligible TPMT activity⁸². Allele frequencies in other populations vary but TPMT polymorphism remains relevant in all studied ethnic groups^{83,84}.

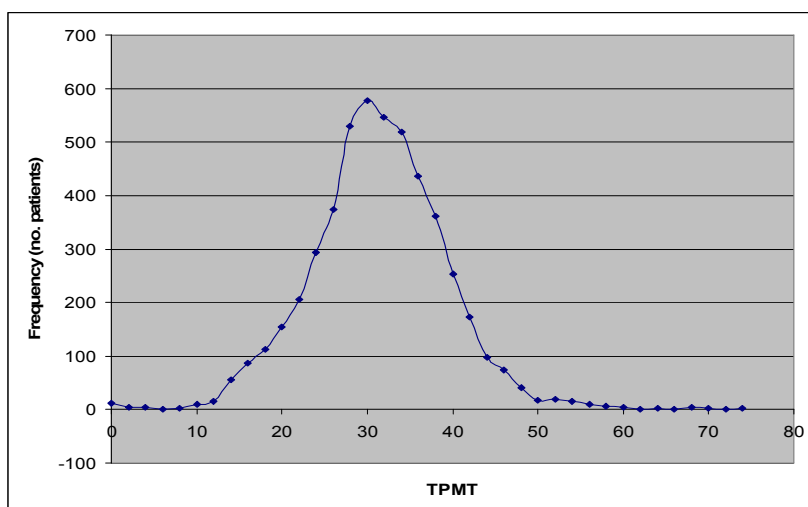


Figure 1.2 TPMT activity in the population. Data from the PRL, previously published at⁸⁵

In normal circumstances, this variation in TPMT activity has no known impact on health, but prescribing thiopurine drugs for these patients is hazardous. TPMT inactivates a large proportion of ingested thiopurine by methylation, leaving only a small proportion available to be processed by other enzymes. As a result, giving standard AZA or MP doses to those people who have no TPMT activity effectively creates a huge thiopurine overdose, with over-production of TGNs and severe life-threatening leucopenia⁸⁶. Those who have just one effective copy of the TPMT gene have intermediate (approximately 50%) activity levels and suffer milder side effects and myelotoxicity if given normal treatment doses^{71,87}. The discovery of TPMT polymorphism now means that these individuals can be identified before treatment is given. This could be life-saving in the case of those with zero TPMT, (in whom thiopurines are usually avoided,⁸⁵ although there are reports of successful treatment on 5-10% of the usual target dose).^{88,89} In those with intermediate TPMT activity, pre-treatment knowledge of their reduced methylation capacity allows dose-reduction to avoid toxicity, particularly myelotoxicity⁸⁵. At a dose of 1mg/kg⁸⁵ of AZA (equating to 0.5mg/kg MP), those with pre-treatment TPMT in the intermediate range generally tolerate thiopurines well. Table 1.1 shows the recommended use of TPMT in clinical practice.

Table 1.1 Tailoring AZA treatment on the basis of TPMT status.

Pre-treatment TPMT status	TPMT activity pmol/h/mgHb	Dosing strategy
<i>Zero</i> homozygous TPMT deficiency	<10	avoid or consider dosing at 0.1-0.2mg/kg Titrate dose by TGN at 4 weeks
<i>Intermediate</i> heterozygous TPMT deficiency	10-24	dose at 1-1.5mg/kg Titrate dose by TGN at 4 weeks
<i>Normal</i> TPMT wild type	25-50	dose at 2-2.5mg/kg Titrate dose by TGN at 4 weeks
<i>High</i> TPMT wild type	>50	dose at 2-2.5mg/kg or 0.5mg/kg with 100mg allopurinol Titrate dose by TGN at 4 weeks

Genetically determined variation in activity of TPMT is known to account for up to 30% of all adverse drug reactions (ADRs) experienced on AZA, particularly myelotoxicity, although this still leaves approximately 70% of side effects unaccounted for^{70,71,87}.

In addition to the well-documented impact of TPMT deficiency on the occurrence of side effects, TPMT status may also determine clinical response to thiopurines. Individuals with more active TPMT predominantly methylate thiopurines, leaving little to be converted to TGNs⁹⁰⁻⁹². Dose escalation in this group has been shown to be ineffective, as methylation continues to dominate. This translates to reduced rates of response to thiopurines and a high risk of hepatotoxicity, thought to be caused by an accumulation of methylated metabolites^{64,93}. Interestingly, such patients do well on a reduced dose of azathioprine (approximately 25% of standard) in combination with allopurinol, a xanthine oxidase dehydrogenase (XDH) inhibitor^{94,95}. This strategy maintains therapeutic TGN levels whilst dramatically reducing methylated metabolite production, possibly because the lower dose of AZA/MP provides less substrate for TPMT activity, but recent research suggests that increased production of thioxanthine in the presence of allopurinol blocks TPMT activity⁹⁶. The use of allopurinol co-prescription is addressed in chapter 7 of this thesis.

1.7.1 The relationship between TPMT genotype and phenotype

Although TPMT genotyping correlates well with phenotype (TPMT activity) for most patients, discrepancies remain an issue, particularly for those who are heterozygous carriers of a TPMT mutation⁹⁷⁻¹⁰⁰. The variation seen between studies could relate to differences in the methodology of the enzyme assays, however, studies consistently demonstrate a difference between the two. A proportion of this will be accounted for by rare genotypes not included in standard laboratory assays (which often test only for the *3A, *3C and *2 variants) but these are very rare and other factors must account for the majority of the genotype:phenotype discrepancy in addition to the wide variation in TPMT activity found within the wild-type population¹⁰¹. It is likely that a substantial proportion of this variability is also genetic. Indeed, family studies have shown that within the *TPMT* wild-type population, TPMT activity shows additional familial correlation¹⁰².

A HapMap analysis seeking association between genetic polymorphism and TPMT activity found that polymorphism in 96 other genes was more strongly linked to TPMT activity than polymorphism in *TPMT* itself¹⁰³, a finding which does not fit with the striking correlation between TPMT genotype and outcome on thiopurines in most studies. Amongst those genes implicated there was a significant over-representation of DNA glycosylase enzymes, which repair errors in DNA transcription. The relevance of this is not entirely clear but perhaps it could explain the minor mis-match seen between TPMT genotype and activity / outcome on thiopurines. This study also raises the possibility that, when used without target genes in mind, HapMap may be of limited value, producing a large number of “red herrings” for every true hit. Results are likely to be biased by the fact that the patients included were from 30 family trios. To date, no confirmatory studies have been done to verify any of these associations in other populations.

Another modifying influence on TPMT activity has been identified in the TPMT promoter region, which contains a GCC repeat motif. Roberts et al identified that the presence of 5 or 7 repeats (vs. 6 in most individuals) was associated with increased TPMT activity¹⁰⁴. Spire Vayron de la Moureyre et al. reported that a variable number tandem repeat element (VNTR) also in the promoter, predicted TPMT activity, with high numbers of repeats (7 or 8 vs. the usual 4-6) being associated with reduced enzyme activity¹⁰⁵. This result has been reproduced by other groups, although the size of the effect appears to be small and would only account for a small proportion of the variation seen in TPMT activity¹⁰⁶. A recent publication establishes that it is not just the number, but also the type of VNTR which determines TPMT activity, and provides a rationale for this effect by demonstrating that the VNTR region acts as a transcription factor binding site¹⁰⁷.

An additional determinant of TPMT activity is the level of available S-adenosylmethionine (SAM). SAM acts as the methyl donor for the methylating reactions catalysed by TPMT, and is additionally thought to bind to, and stabilise, the TPMT protein backbone, although the direct evidence for this comes from a SAM analogue (sinefungin) acting on the wild-type bacterial equivalent of TPMT¹⁰⁸. SAM is created by the action of ATP on methionine. Methionine is derived directly from dietary sources or can alternatively be synthesised from homocysteine by methyl exchange from either 5-methyl-tetrahydrofolate, (a B12 dependent process¹⁰⁹) or betaine.

Methionine production is therefore influenced by the folate cycle, vitamin B12 and betaine levels and folate and vitamin B12 deficiency are known to result in raised homocysteine levels¹¹⁰. Once patients are on treatment with thiopurine agents there is a further complexity to the process. Milek and co-workers have shown that Me-TIMP reduces intra-cellular ATP levels and SAM production, creating negative feedback on TPMT activity. Administration of exogenous SAM reversed these changes with increased TPMT activity resulting in higher MeMP levels but lower TGN and Me-TIMP, which lead in turn to cell salvage with reduced apoptosis¹¹¹.

Methionine production from homocysteine also competes with the conversion of homocysteine to cystathione and then on to glutathione. This pathway tends to predominate during conditions of oxidative stress, such as during a period of active disease, as glutathione is a key endogenous anti-oxidant^{109,112}. Glutathione is also required for the conversion of azathioprine to MP. Figure 1.4 shows the inter-relationship between TPMT, SAM, methionine and the folate cycle.

As a result of the importance of TPMT activity during thiopurine usage, pharmacogenetic study has extended to key enzymes in the folate cycle to establish whether, by modulating TPMT activity, they have an influence on clinical outcome.

1.8 MTHFR

5,10-methylene-tetrahydrofolate reductase (MTHFR) is the enzyme responsible for the production of 5-methyl-tetrahydrofolate (MeTHF), the methyl donor for the reaction converting homocysteine to methionine. Theoretically, deficiencies in this process would be predicted to lead to reduced SAM availability and therefore compromised TPMT activity.

MTHFR is subject to genetic polymorphism, including common variants known to have an impact on enzyme function, most importantly the *MTHFR 677C>T* polymorphism. In the presence of folate deficiency, this SNP has been shown to be associated with high homocysteine levels¹¹³. Reduced DNA methylation (also SAM dependent) has also been demonstrated where the *MTHFR 677C>T* polymorphism and folate deficiency co-exist¹¹⁴. This is an important consideration in IBD, where folate deficiency is a common complication of disease. *MTHFR 677C>T* has been associated with a wide variety of disease states, from an increased risk of

recurrent pregnancy loss¹¹⁵ and babies with neural tube defects¹¹⁶, to increased cancer risk (including oesophageal¹¹⁷ and colorectal cancers^{118,119}, acute lymphoblastic leukaemia in children¹²⁰, and from thrombophilia¹²¹ and vascular dementia¹²² to mental health problems such as schizophrenia and bipolar and unipolar depression¹²³.

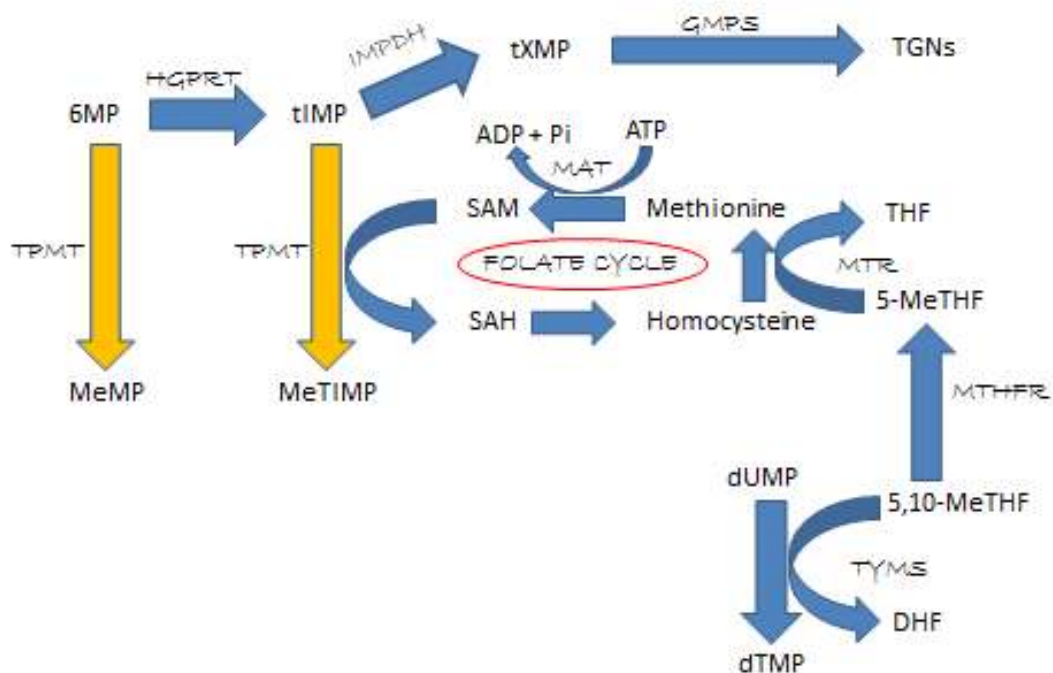


Figure 4: Interaction between the folate cycle, MTHFR, TYMS and TPMT activity

Figure 1.3: Impact of the folate cycle (and therefore MTHFR and TYMS) on TPMT activity.

Abbreviations:

Metabolites: 6MP- 6-Mercaptopurine, MeMP – 6-methylmercaptopurine, tIMP – 6-thioinosine monophosphate, tXMP – 6-thioxanthine monophosphate, TGNs – thioguanine nucleotides (6-thioguanine monophosphate, 6-thioguanosine diphosphate, & 6-thioguanosine triphosphate), Me-TIMP – methyl-thioinosine monophosphate,

Enzymes: TPMT – thiopurine methyl transferase, HGPRT – hypoxanthine guanine phosphoribosyltransferase, IMPDH – inosine monophosphate dehydrogenase, GMPS – guanine monophosphate synthetase, TYMS – thymidylate synthase, MTHFR – methyltetrahydrofolate reductase, MTR- 5-methyltetrahydrofolate homocysteine methyltransferase MAT-methionine adenosyl transferase

Substrates: SAM – S-adenosyl methionine, SAH S-adenosyl homocysteine, THF – tetrahydrofolate, 5-MeTHF – 5-methyl tetrahydrofolate, 5,10-MeTHF – 5, 10 dimethyltetrahydrofolate, DHF – dihydrofolate, dUMP – deoxyuridine

monophosphate, dTMP – deoxythymidine monophosphate, ATP – adenosine triphosphate, ADP - adenosine diphosphate, Pi – inorganic phosphate,.

Arenas et al have demonstrated that this *MTHFR* SNP is much more common than expected in those patients found to have intermediate TPMT activity, despite wild type *TPMT* genotype¹²⁴, an effect which may be dependent on gender¹²⁵ and result in reduced TPMT activity as a result of reduced SAM levels destabilising TPMT.

In patients with ALL treated with MP, there are some early suggestions that *MTHFR* genotype may be associated with an increased risk of adverse events (particularly haematological toxicity) and interruption of therapy¹²⁶⁻¹²⁸, and in one study, the occurrence of *MTHFR* and *TPMT* polymorphism together was particularly problematic¹²⁷. However, the picture is clouded by the fact that ALL patients are generally on both MP and methotrexate at the same time, during their maintenance therapy stage. Since the folate cycle is more intimately linked to the mechanism of action of methotrexate, *MTHFR* mutations are often considered more likely responsible for toxicity due to methotrexate rather than MP¹²⁶. This is despite the fact that one study clearly demonstrates that *MTHFR* polymorphism was only related to toxicity in those on the protocol using MP and not the protocol for high risk ALL which uses methotrexate alongside alternative agents¹²⁸. In other contexts, *MTHFR* polymorphism has not yet been demonstrated to be associated with toxicity^{129,130}.

1.9 TYMS

The other key enzyme which influences the availability of methyl-THF is thymidylate synthase (*TYMS*). *TYMS* competes with *MTHFR* for methyl-THF, which it uses as a cofactor during the recycling of dUMP to dTMP to maintain the pool of nucleotides required for DNA synthesis and repair. *TYMS* is also known to be subject to genetic polymorphism which has an impact on enzyme activity, in this case, due to the occurrence of a common tandem repeat in the 5' untranslated region. The more active (wild type) 3/3 variant is associated with lower folate and higher homocysteine levels¹³¹, an effect compounded both by poor folate intake and the occurrence of the *MTHFR677C>T* polymorphism¹³¹. Studies on the clinical impact of this variability are currently lacking.

Whilst TPMT is the only pharmacogenetic marker that has translated into clinical practice in IBD, there are many other potential pharmacogenetic markers and for some of these, a considerable body of evidence now supports an association with clinical outcome. This evidence is reviewed below.

1.10 ITPase

All cells require purine nucleotides and have two alternative methods by which to obtain them. Dividing cells need to make new DNA and RNA and therefore synthesize purine nucleotides *de novo*. This process requires a lot of energy however, so where possible, purine nucleotides are salvaged and recycled. It is this same pathway of enzymes which is responsible for the conversion of MP to active thioguanine nucleotides (TGNs).

Whilst the desired end-products of both *de novo* purine synthesis and purine salvage are the canonical purine nucleotides adenosine and guanine, the first nucleotide produced by both purine salvage and *de novo* synthesis is inosine monophosphate (IMP). IMP can then be converted to either adenosine monophosphate (AMP) or guanine monophosphate (GMP) as required by the cell, see figure 1.5.

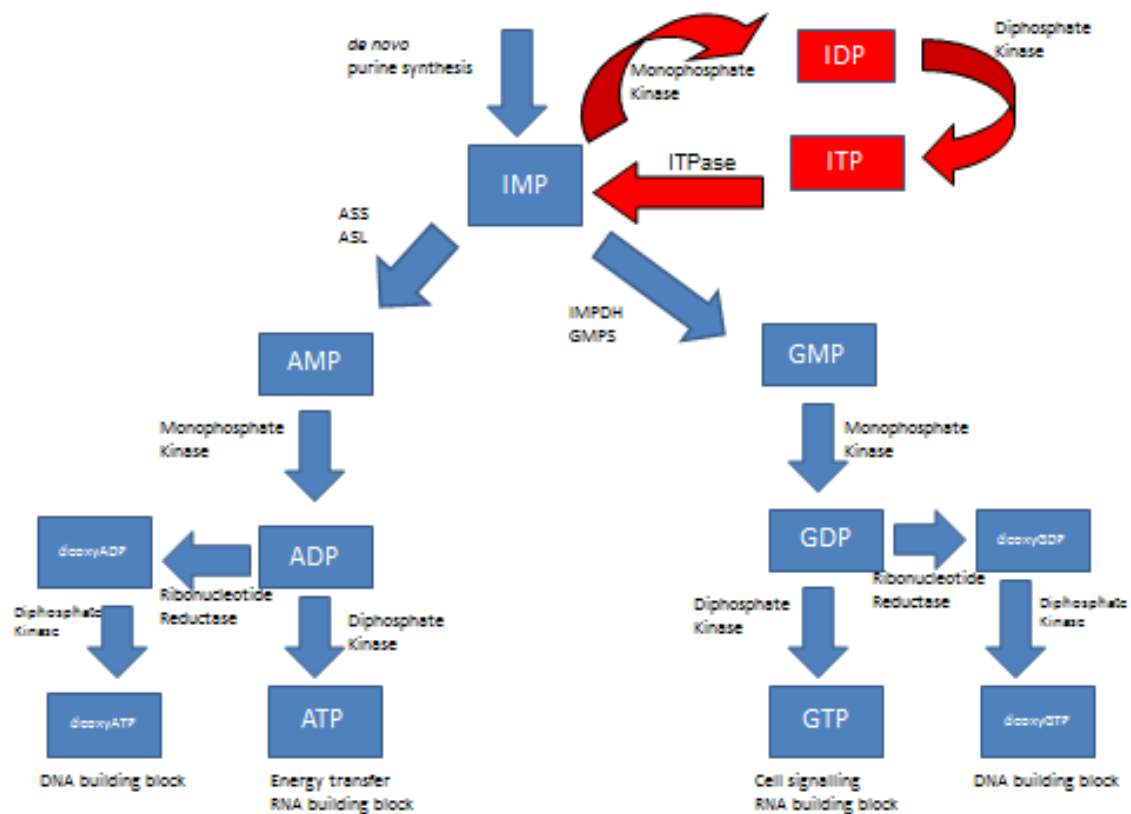


Figure 1.5 Relationship between and importance of the purine nucleotides

Nucleotides:

AMP – Adenosine monophosphate

ADP – Adenosine diphosphate

ATP – Adenosine triphosphate

GMP – Guanine monophosphate

GDP – Guanine diphosphate

GTP – Guanine triphosphate

IMP – inosine monophosphate

IDP – inosine diphosphate

ITP – inosine triphosphate

Enzymes:

IMPDH – inosine monophosphate dehydrogenase

GMPS – guanine monophosphate synthase

ASS – adenylysuccinyl synthetase

ASL – adenylysuccinyl lyase

Nucleotide kinases convert AMP and GMP into their active triphosphate forms (see figure 1.5) in which they can be incorporated into DNA and RNA, transfer energy etc. These same kinases act on IMP to create inosine diphosphate (IDP) and inosine triphosphate (ITP). However, to date, no function for IDP and ITP has been discovered and they are considered by-products of the purine metabolic pathway. To avoid wasting such valuable compounds, cells recycle ITP back to the useful building block IMP using the enzyme ITPase, see figure 1.5. The action of ITPase also prevents accumulation of ITP^{132,133}. Experimental evidence now confirms that an excess of dITP will result in its incorporation into DNA in place of canonical nucleotides¹³⁴, encouraging the occurrence of coding errors¹³⁵. Once incorporated, DNA repair enzymes struggle to uncouple and remove inosine nucleotides, especially when paired with cytosine¹³⁵. Recent work in patients with scleroderma suggested a link between high levels of inosine nucleotides and DNA damage¹³⁶ and adding inosine nucleotides to normal cells is an accepted experimental technique for encouraging mutagenesis^{136,137}. This raises an important question about the wisdom of prescribing thiopurines to patients known to be ITPase deficient, although to date no link with cancer has been demonstrated.

1.10.1 Human ITPase deficiency

ITPase deficiency in human subjects was discovered during studies on blood donors which identified that a small proportion of the healthy population have very high intracellular levels of ITP associated with low ITPase activity¹³⁸. In 2002, Sumi et al. reported the genetic basis of ITPase deficiency, describing *ITPA* c.94C>A and *ITPA* IVS2 +21A>C variants in individuals with raised ITP levels¹³⁹. The *ITPA* c.94C>A variant encodes a Pro32Thr substitution¹³⁹⁻¹⁴¹ and has the most significant impact on ITP activity. Individuals heterozygous for this variant have approximately 22.5% of expected ITPase activity and those with homozygous deficiency approximately 0%¹³⁹. Heterozygosity for the *ITPA* IVS2 +21A>C mutation in intron 2 reduces ITPase activity to 60% of expected¹³⁹.

Since these original reports, other SNPs have been shown to have an impact on ITPase activity including *ITPA* IVS2+68T>C¹⁴², *ITPA* 359_366dupTCAGCACC¹⁴³ and *ITPA* IVS2 +68T>G¹⁴⁴ and synonymous coding region SNPs have also been identified¹³⁹. However, a recent

detailed analysis of *ITPA* haplotypes suggests that the *ITPA* c.94C>A and IVS2 +21A>C are the major determinants of ITPase deficiency and that other SNP associations with reduced ITPase activity are either very rare or only likely to occur in linkage with these two causative SNPs¹⁴⁵.

The *ITPA* c.94C>A SNP has been detected in all world populations investigated with the lowest frequency in Central and Southern American populations (1-2%), intermediate in Caucasians (7%) and a relatively high incidence in Asian individuals (11-15%)¹⁴⁶. The frequency of *ITPA* IVS2+21A>C in the Caucasian population appears to be approximately 13%¹³⁹. Interestingly, it has not been detected in Japanese cohorts¹⁴⁴.

The mechanism by which these SNPs cause such a reduction in ITPase activity is of interest. The fact that individuals heterozygous for the *ITPA* c.94C>A variant had approximately 25% of wild-type ITPase activity led to suggestions that the enzyme was dimeric¹³⁹, and this has subsequently been confirmed by crystallography¹⁴⁰. The location of the Pro32Thr substitution in the crystalline structure of ITPase prompted the suggestion that the *ITPA* c.94C>A mutation would disrupt the catalytic site¹⁴⁰. More recently, substrate affinity has been shown to be unaltered, but pyrophosphohydrolysis is compromised¹⁴⁷ and protein stability reduced¹⁴⁸. However, such a mechanism would not adequately explain the 75% reduction in activity observed and in 2007, Arenas et al demonstrated that both the *ITPA* c.94C>A and the *ITPA* IVS2+21A>C variants cause mis-splicing of ITPase, resulting in deletion of exons 2 and 3, or exon 3 only, from the mRNA¹⁴⁹. This would evidently have a much greater impact on the protein structure than a single amino acid substitution. It is interesting to note that 2 of the other 3 SNPs associated with ITPase deficiency are situated in close proximity to *ITPA* IVS2+21A>C within intron 2, suggesting that they could affect ITPase function in the same manner^{142,144}.

1.10.2 Pharmacogenetic importance of ITPase deficiency

In the light of all this information it has been suspected that genetically determined ITPase deficiency must be associated with a specific clinical phenotype, but to date none has been successfully identified.

In the presence of thiopurines, ITPase has a new role, recycling 6-thio-ITP back to 6-thio-IMP¹⁴⁷ so that it can be either converted into the active end-product of thiopurine drugs, thioguanine nucleotides (TGNs) or broken down and excreted¹⁵⁰. In those with ITPase deficiency, thiopurine therapy would cause an accumulation of 6-thio-ITP which could result in toxicity^{147,151}. The only study to look at the molecular effect of an accumulation of thio-ITP showed that *in vitro*, thio-ITP inhibits the activity of RNA polymerases¹⁵². This effect could be overcome by increasing the concentration of canonical nucleotides in the reaction mixture, suggesting that the mechanism is competitive inhibition. The authors suggest that this could be an additional mechanism of azathioprine action, but in the absence of ITPase deficiency, levels of ITP and IDP (and therefore presumably thioITP and thioIDP) are kept extremely low¹⁵³. It could however explain how ITPase deficiency causes adverse events, particularly myelotoxicity – see below.

Pharmacogenetic interest in ITPA was sparked by a report from Marinaki et al. who examined a cohort of 62 patients who had experienced toxicity on exposure to thiopurine medication, seeking pharmacogenetic explanations for these adverse drug reactions (ADRs). A retrospective analysis was performed to look for ITPase genetic polymorphism. A much higher than expected incidence of ITPase mutations was detected in this group and there seemed to be a particular association between *ITPA* c.94C>A and rash, pancreatitis and flu-like symptoms¹⁵¹.

Since then several other studies have addressed this issue. Some of these have confirmed the association^{87,154} but this finding was not universal, see below. Von Ahsen et al collected data prospectively on a cohort of 71 patients with CD treated with 2.5mg/kg azathioprine. The cohort was originally recruited to assess whether the use of TGN measurements improved clinical outcomes. They reported that early drop out from the study and drop out specifically due to ADRs were both associated with *ITPA* c.94A>C¹⁵⁴. Ansari et al prospectively recruited 207 patients with IBD starting treatment with 2mg/kg of azathioprine. They detected an association between *ITPA* c.94A>C and flu-like symptoms, but no other specific ADR, and no association was seen between *ITPA* c.94A>C and overall withdrawal from the study due to ADRs⁸⁷. Zabala-Fernandez confirmed this in their retrospective cohort of 232 IBD patients on thiopurine monotherapy¹⁵⁵. In the post-transplant context, flu-like symptoms and GI disturbance have also

been associated with *ITPA* c.94A>C. This study contained three patients homozygous for *ITPA* c.94A>C, none of whom could tolerate thiopurine therapy¹⁵⁶.

Several other studies have, however, failed to find a link between ADRs and *ITPA* c.94A>C¹⁵⁷⁻¹⁶⁰. These negative studies are mostly retrospective and contain small numbers in the relevant subgroups (2 contained no patients homozygous for the *ITPA* c.94C>A variant). Others have reported atypical genotype frequencies which raises the suspicion of either genotyping errors [frequently reported for restriction fragment length polymorphism TPMT genotyping¹⁶¹] or a bias in recruitment^{157,159,162}.

Gearry et al retrospectively recruited 147 patients with IBD of whom 73 had stopped azathioprine within 6 months due to ADRs and 74 had tolerated 6 months of treatment. They found no significant over-representation of *ITPA* c.94A>C in the group that had experienced ADRs and no association between *ITPA* c.94A>C and specific ADRs, including rash, pancreatitis and flu-like symptoms¹⁵⁸. Van Dieren et al reported that in 109 patients beginning azathioprine treatment for IBD, no association between *ITPA* c.94A>C and ADRs could be demonstrated¹⁵⁹, whilst de Ridder et al, working with the same group, failed to detect an association in 72 paediatric patients¹⁵⁷. Hindorf et al prospectively recruited 60 patients starting 2.5mg AZA or 1.25mg MP for IBD. 27 patients completed the study and 27 were withdrawn due to ADRs. Again, there was no association detected between *ITPA* c.94A>C and either specific or overall ADRs¹⁶⁰.

The original Marinaki cohort did not show an association between ITPase deficiency and myelotoxicity but this association has been examined in other groups. Again, results have been conflicting, some groups showing a significant association^{163,164} but others finding none^{157,165,166}.

Zelinkova et al. published the results of a Dutch retrospective study of 262 patients treated with AZA doses between 2 and 2.5mg/kg for IBD. They found a significant association between *ITPA* c.94A>C and leucopenia with an odds ratio of 3.5 (95%CI 1.1-11.0). No other side effects were studied¹⁶³. Hawwa et al. studied a mixed group of patients, 19 children being treated for ALL and 35 IBD patients, genotyping them for both the *ITPA* c.94C>A and *ITPA* IVS2+21A>C. In

their cohort, occurrence of the *ITPA* IVS2+21A>C variant was associated with a decrease in platelet count, but not a change in white blood cells¹⁶⁴.

Meanwhile, a multi-centre retrospective study from Italy reported the outcome of 422 patients treated with AZA or MP for IBD. Whilst finding a trend towards lower white cells in those with the *ITPA* c.94C>A variant, this failed to reach significance (p=0.2)¹⁶⁷. Allorge et al reported a cohort of patients experiencing myelotoxicity on thiopurines, sequencing for the *ITPA* c.94C>A and *ITPA* IVS2+21A>C variants. In this group, the allele frequency of the *ITPA* variants was similar to that seen in a control population¹⁶⁵. Similarly, a study in 157 renal transplant recipients could not identify any link between *ITPA* variants (*ITPA* c.94C>A or *ITPA* IVS2+21A>C) and myelotoxic events¹⁶⁶.

Two recent studies have re-awakened interest in *ITPA* polymorphism and myelotoxicity. The first showed a high prevalence of the *ITPA* c.94C>A polymorphism in the Japanese population where it was associated with an increased risk of myelotoxicity¹⁶⁸. The second, a study in childhood ALL, demonstrated that if thiopurine doses are adjusted for an individual's TPMT activity, *ITPase* polymorphism emerges as a predictor of myelotoxicity, apparently in association with a higher level of methylated metabolites, which they postulate could be caused by an accumulation of methyl-thioITP¹⁶⁹.

An accumulation of thio-ITP could theoretically reduce the amount of thiopurine drug available to be converted to the active metabolite, thioguanine nucleotides (TGNs) and therefore be associated with lack of clinical response to azathioprine. Palmieri et al did report this in the Italian cohort of 422 IBD patients described above¹⁶⁷ and a retrospective cohort of 232 Spanish patients with IBD and also found an association between *ITPA* 94A>C and non-response¹⁵⁵. However, in another study, TGNs were found to be increased in those with the *ITPA* IVS2+21A variant (although unchanged in those with the *ITPA* 94A>C variant)¹⁶⁴. In a cohort treated with azathioprine for SLE, those with the *ITPA* 94A>C variant had a higher chance of achieving clinical response¹⁷⁰. This could be due to the effect of thio-ITP on RNA production described above and raises the possibility that thio-ITP has therapeutic, as well as adverse effects.

A meta-analysis performed in 2007 failed to find a consistent association between the *ITPA* c.94A>C variant and all ADRs, myelotoxicity, hepatotoxicity or pancreatitis¹⁷¹. However, the studies that did find a significant association between azathioprine ADRs and ITPase deficiency were relatively robust prospective cohorts. This means that the role of ITPase deficiency in adverse ADRs to thiopurines remains uncertain.

1.10.3 Testing for ITPase deficiency

Due to the lack of consistency in these results, ITPase testing has not been widely adopted in clinical practice and is currently only performed in one clinical laboratory¹⁵⁰. ITPase activity can be measured directly in red cells^{142,172} and inferred from the relative concentrations of ITP and IMP¹⁵³, making pre-treatment testing alongside TPMT feasible. Although activity does decrease if the cells are kept in storage, this seems to be a slow and consistent process which would allow such measurements within a realistic laboratory timeframe¹⁷³. The only problem with this approach is that heterozygous carriers of those SNPs with only a minor impact on enzyme activity (such as *ITPA* IVS2+21A>C) could be overlooked¹⁴³. An alternative would be to look for the two important ITPase mutations directly¹⁷⁴. In view of the recent haplotype analysis¹⁴⁵ this would be a reasonable approach to screening, but as with genetic TPMT testing, carries a risk of missing novel and rare variants.

In isolation, knowledge of an individual's ITPase status cannot yet aid prescribing decisions. However, with modern thiopurine treatment mainly individualised to TPMT status, it is anticipated that *ITPA* polymorphism will emerge more strongly as a predictor of both toxicity and outcome¹⁷⁵. Additionally, as increasing numbers of pharmacogenetic predictors of both toxicity and lack of response to thiopurines are discovered, it is likely that ITPase will form an important part of a panel of markers which would give the physician a personalised thiopurine risk:benefit assessment for each patient. *ITPA* variants may be particularly important in Asian populations where *ITPA* c.94C>A is more common and has been linked to more serious side effects such as myelotoxicity. Further studies are warranted to elucidate the exact risk of prescribing thiopurines in individuals with ITPase deficiency.

1.11 Other enzymes

TPMT and ITPase have been the subject of the vast majority of the work undertaken on azathioprine pharmacogenetics. However, TPMT accounts for only 10-25% of azathioprine intolerance and less than 30% of myelotoxicity.^{70,71} The role of ITPase is still unclear. This leaves a large amount of azathioprine-related toxicity and non-response unexplained, a proportion of which is highly likely to be due to polymorphism at additional loci. The following section reviews the evidence for the involvement of additional enzymes in the pharmacogenetics of azathioprine.

1.11.1 Xanthine Oxidase/Dehydrogenase

Xanthine oxidase/dehydrogenase (XDH) competes with TPMT to inactivate MP^{74,176} (see figure 1.2). The competition is indirect as XDH is mainly responsible for the significant first pass metabolism of orally administered thiopurines in the small intestine and liver¹⁷⁷, but is absent from the target tissue itself⁷². Low XDH activity would be predicted to cause high TGN levels with dose-related toxicity, a supposition backed by the effects of blocking XDH activity with allopurinol whilst on thiopurine treatment, and the 70-80% thiopurine dose-reduction required in this situation¹⁷⁸. High XDH activity would be predicted to be associated with non-response to thiopurine agents.

XDH activity is influenced by genetic polymorphism associated with both increased and decreased activity¹⁷⁹. Most of these polymorphic variants alter XDH activity against thioxanthine¹⁸⁰ and appear to alter azathioprine metabolite levels¹⁶⁴. Although true deficiency (Type 1 Xanthinuria) is rare¹⁸¹, there is a case report of a renal transplant patient experiencing profound and early neutropenia after administration of thiopurines due to a combination of congenital XDH deficiency and *TPMT* heterozygosity¹⁸².

Variations in XDH activity would also be predicted to be associated with hepatic and gastrointestinal toxicity to orally administered thiopurines, due to its concentration in these tissues and the effect of first pass metabolism. In keeping with this, orally administered thiopurines cause predominantly gastrointestinal side effects^{71,87}, whilst when given intravenously (as in early ALL studies) the most commonly occurring side effect was

myelosuppression¹⁸³. An *in vitro* study found that rat hepatocytes cultured in the presence of therapeutic concentrations of thiopurines deteriorated much more quickly than those cultured without them. This effect was blocked by the addition of allopurinol, strongly suggesting that the action of XDH on the thiopurines was responsible¹⁸⁴. In a further attempt to confirm that the cell damage was being caused by XDH, the group postulated that the damage could also be blocked by a strong anti-oxidant such as Trolox. This was borne out in further experiments and a combination of Trolox and allopurinol completely inhibited the damaging effects seen in previous cultures with thiopurines.¹⁸⁴ The authors also postulated that the relative preponderance of thiopurine-associated liver injury in male patients could result from the higher XDH activity seen in males.

Not all studies have, however, found that reactive oxygen species (ROS) are the cause of thiopurine liver injury. In a study on human hepatocytes and hepatoma cell lines, Petit et al demonstrated that the damage appears to relate instead to ATP depletion and by implication mitochondrial damage¹⁸⁵ rather than damage by ROS. This process could still however be related to the actions of XDH as allopurinol has been shown to preserve ATP pools and mitochondrial function in hepatic ischaemia/reperfusion injury¹⁸⁶. Allopurinol could also contribute to reduced liver toxicity by raising hypoxanthine levels which could lead to improved cell salvage/repair¹⁸⁷.

This provides a rationale for using reduced dose AZA with allopurinol for treatment of patients experiencing thiopurine hepatotoxicity in the presence of normal methylated metabolite levels. The use of allopurinol co-treatment is addressed in chapter 7.

1.11.1.1 Interaction between XDH and TPMT

Co-prescription of allopurinol with AZA (effectively obliterating XDH metabolism of AZA¹⁸⁸), requires a dose-reduction to 25% of the usual target AZA dose. This demonstrates that XDH is a major contributor to metabolism of thiopurine drugs, although complete TPMT deficiency requires an azathioprine dose reduction to 2.5-5%^{85,189} of the standard dose, suggesting that methylation by TPMT is dominant. Why deficiency of either one of these enzymes should require such large dose reductions is of interest. Ansari et al demonstrated that in patients on standard dose AZA, approximately 10% of the daily dose could be retrieved in urine as 6-thiouric acid (6-

TU). However, in a TPMT deficient patient receiving 5mg/day of AZA, 89% of the daily AZA dose was retrieved in the urine as 6TU, whilst in a patient co-prescribed AZA and allopurinol urinary 6TU was undetectable¹⁸⁸. Additionally, patients co-prescribed allopurinol and AZA demonstrate very low methylated metabolite levels¹⁹⁰. The reason why blocking XDH should have such a profound effect on methylated metabolite levels is not clear. Allopurinol (or the active metabolite oxypurinol) are not thought to have a direct impact on TPMT activity⁹⁴, although published data is lacking in this area. A novel explanation is that increased production of thioxanthine in the presence of allopurinol inhibits TPMT^{96,191}. An alternative explanation is that the pharmacokinetics of TPMT are such that, at the lower drug doses permitted by co-administration of allopurinol, TPMT metabolism of thiopurines and thiopurine metabolites is much less efficient. The shift in balance between TGN and methylated products may also be partly explained by decreased breakdown of the TGNs, for which XDH and aldehyde oxidase are responsible.

The relationship between genetic variability in *XDH* and clinical outcome of patients with IBD is addressed in the research presented in this thesis and can be found in chapters 3 & 5.

1.11.2 Molybdenum cofactor sulfurase

XDH and AOX require an essential cofactor, molybdenum cofactor (MOCO), in order to be active¹⁹². MOCO is also required for the action of sulphite oxidase, and MOCO deficiency results in severe neuro-degeneration and early infant death due to inactivity of this enzyme¹⁹³. The final step in MOCO adaptation for compatibility with XDH and AOX is performed by the enzyme molybdenum cofactor sulfurase (MOCOS). MOCOS deficiency, in which sulphite oxidase activity is unaffected, is associated with Type 2 XDH deficiency, a relatively benign condition, with a minor predisposition to renal stones¹⁹⁴ being the only described phenotype. The *MOCOS* gene is subject to genetic polymorphism but no studies have addressed the impact of this on thiopurine pharmacogenetics.

The relationship between genetic variability in *MOCOS* and clinical outcome of patients with IBD is addressed in the research presented in this thesis and can be found in chapters 3 & 5.

1.11.3 Aldehyde Oxidase

It has also been demonstrated that aldehyde oxidase (AOX) acts on azathioprine, MP and their metabolites, contributing to catabolism of thiopurines^{176,195-197}. However, the contribution of this enzyme tends to be neglected in reviews on the subject and little additional work has been done in this area. Despite significant quantities of AOX products being found in the above studies, it seems to have been assumed that the role of AOX is relatively minor. The only functional study of the thiopurine metabolites produced by AOX showed that 8-hydroxy-6-MP did not slow the growth of rat sarcoma¹⁹⁸.

AOX is a close relation of XDH and also has an essential requirement for MOCO¹⁹². The role of AOX in human physiology remains unclear. It is much more widely distributed than XDH and has a broad range of substrates^{195,199}. It is therefore thought to have additional functions over and above its contribution to purine catabolism²⁰⁰. There is significant inter-individual variability in AOX activity^{201,202}, whether this is genetic or attributable to other factors²⁰³ remains unknown. However, rat studies have demonstrated a SNP which prevents dimerisation, and therefore activity of AOX²⁰⁴ and studies on methotrexate suggest that there are 2 phenotypes in the human population^{205,206} with implications for clinical outcome.

Interestingly AOX has been shown to interact with and alter the activity of the membrane-bound pump ABCA1²⁰⁷. Whilst this specific pump is not thought to be involved in thiopurine metabolism, nothing is known about whether AOX interacts with other ABC pumps, some of which do transport thiopurine metabolites (see below). No study has addressed the implications of *AOX* genetic variability in thiopurine treatment to date.

Work addressing the relationship between genetic variability in *AOX* and clinical outcome of patients with IBD is presented in chapters 3 & 5.

1.11.4 Hypoxanthine Guanine Phosphoribosyltransferase (HGPRT)

As the first enzyme in the metabolic pathway converting MP to its active metabolite TGNs, variation in HGPRT would be predicted to have a profound effect on MP metabolism. Although significant systemic loss of activity of HGPRT has serious results for the affected person,

resulting in Lesch-Nyhan syndrome²⁰⁸, HGPRT activity in PBMCs has been shown to be highly variable both within and between patients with IBD. Administration of thiopurines appears to induce HGPRT activity in white cells, although higher and much more stable levels were documented in red cells^{209,210}. Activity also appears to be enhanced on azathioprine/allopurinol co-treatment when compared to monotherapy²¹¹.

Studies on leukemic cell lines demonstrate that lower HGPRT activity is associated with thiopurine resistance²¹². The clinical significance of this for thiopurine therapy in IBD is not yet clear.

1.11.5 Inosine monophosphate dehydrogenase (IMPDH)

IMPDH I & II constitute the enzymatic step subsequent to HGPRT on the pathway to TGN production. Acting in direct competition with TPMT, the balance of activity of these enzymes partly determines how much active end-product is produced from the administered thiopurine²¹³. It would be logical to suppose that reduced IMPDH activity would create a bottleneck in the creation of TGNs, allowing TPMT the opportunity to inactivate a greater proportion of the ingested drug. This would manifest clinically as a predominant methylation phenotype, known to be associated with non-response to thiopurine therapy and an increased risk of hepatotoxicity⁶⁴.

Mycophenolate mofetil (MMF), an alternative immunosuppressive agent, acts by inhibiting IMPDH and this has allowed the effect of IMPDH activity on nucleotide pools to be studied. Blocking IMPDH has the predicted effect (decrease) on GTP levels in white cells²¹⁴, but in red cells, the opposite effect is seen²¹⁵. The authors propose that this could be due to MMF stabilising IMPDH in red cells, where the lack of a nucleus means that no new enzymes can be made. The only study to date which has looked at IMPDH activity and nucleotide pools during thiopurine therapy did not demonstrate the expected relationship to TGN levels, suggesting that the proposed model is over-simplistic or that TGN measurements in red cells are not a suitable surrogate marker²¹⁶.

Roberts et al studied the IMPDH gene in a small group of patients who were azathioprine resistant, with predominant methylation despite TPMT activities in the normal range. Whilst they did find one patient with an IMPDH promoter mutation, all the other subjects had wild type

IMPDH. They concluded that this mutation, even if it was demonstrated to affect IMPDH activity, could only be a very rare cause of azathioprine resistance²¹³.

1.11.6 5'-nucleotidase

5'-nucleotidase cleaves a phosphate from the 5' end of sugar moieties, converting monophosphorylated nucleotides to their respective nucleosides. It exists as membrane-bound ecto-5'-nucleotidase (where it appears to form an important part of a cellular circulation of nucleotides²¹⁷) and in soluble form, see figure 1.6. Regulation of 5'-nucleotidase is not fully understood. Activity levels have been shown to decrease with age and be lower in men in a population of patients with arthritis and normal controls²¹⁸. Multiple SNPs have been demonstrated in the 5'-nucleotidase gene and its 5' flanking region, many of which have been demonstrated to have an impact on function²¹⁷.

Early work in ALL demonstrated that patients in whom blasts expressed 5'-nucleotidase had a lower chance of complete remission²¹⁹. More recently 194 ALL cell lines have been analysed with gene-expression profiling. 5'-nucleotidase expression was positively correlated with TGN levels and was one of only 3 genes for which expression correlated with toxicity experienced on thiopurine therapy (the other 2 were TPMT and ABCC4)²¹⁷.

A small study in rheumatoid arthritis showed that higher 5'-nucleotidase activity was associated with good response, whilst those with lower activity developed side effects during AZA and MP treatment²²⁰. Other work by the same group, (again in patients with RA) failed to confirm this association²²¹ although they have since reported a series of patients developing myelotoxicity, despite normal TPMT activity, who were all discovered to have low 5'- nucleotidase activity^{222,223}. No work has been done to date on the effect of 5'-nucleotidase activity in patients with IBD.

1.11.7 Nucleoside phosphokinase

Nucleoside phosphokinases are responsible for conversion of thioguanine monophosphate to diphosphate, and diphosphate to triphosphate²²⁴. As it has become clear that the triphosphated

form acts differently to the other forms of thioguanine⁵¹ and that the ratio of di- to triphosphate is associated with response²²⁵, phosphokinase has become of pharmacogenetic interest. However, to date no work has been done on its clinical significance in thiopurine therapy. Inter-individual variation in nucleoside diphosphate kinase (NDPK) has been demonstrated²²⁴ but whether this is genetically determined is not known. Additionally, the activity of NDPK is not related to the concentration of the triphosphated nucleotide²²⁴. The reason for this is not clear but reflects the complexity of the system.

The same enzyme is responsible for the conversion of methyl-mercaptopurine riboside to methyl thioIMP²²⁶ which is the metabolite responsible for the inhibition of *de novo* purine synthesis²²⁷. This provides an alternative mechanism by which nucleoside kinases might be responsible for pharmacogenetic variability in thiopurine response.

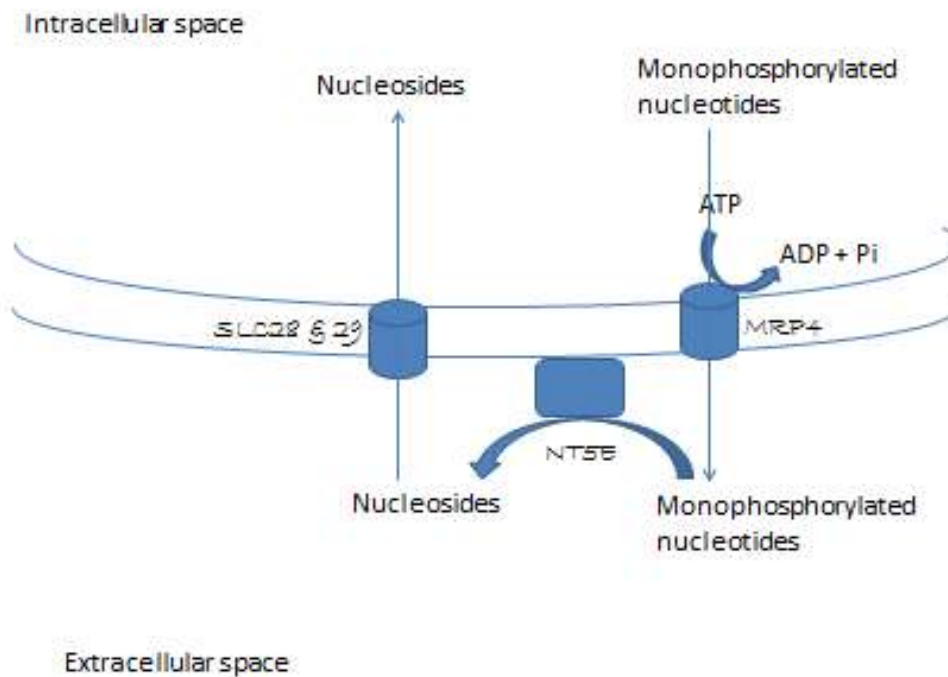


Figure 1.6: Nucleotide and nucleoside transport across cellular membranes

Abbreviations: SLC28 & 29 are the genes for 2 families of transporters – the concentrative nucleoside transporters and the equilibrative nucleoside transporters respectively, NT5E – Ecto-5'-nucleotidase, MRP4 – multi-drug resistance protein 4, ATP – adenosine triphosphate, ADP – adenosine diphosphate, Pi – inorganic phosphate.

1.11.8 MRP4 & MRP5

Multi-drug resistance proteins are ATP-binding cassette (ABC) transporters which are located on the cell surface and are responsible for energy-dependent efflux from cells, particularly the removal of xenobiotics, including drugs. There are a large number of different ABC transporters which are all thought to derive from a common ancestor. The sequence is highly conserved across pro and eukaryotes, suggesting an essential function. Their location (renal tubule, bile canaliculae, placenta, gut epithelium) suggests a role in physiological efflux. Work to date has centred on p-glycoprotein (Multi-drug resistance protein-1, MDR-1) in which common polymorphism has been shown to influence outcome on chemotherapy, conferring resistance to a wide variety of chemotherapeutic agents. There is also evidence that polymorphism in MDR-1 is associated with steroid-refractoriness in IBD²²⁸ and may possibly be a factor in the development of IBD^{229,230} although this finding has not been universal^{231,232}.

However, although most studies have focussed on MDR-1 (p-glycoprotein), thiopurines cannot be transported by this pump^{233,234}, but have instead been shown to be exported from cells by MRP-4 and MRP-5 (otherwise known as ABCC4 & ABCC5). Efflux is so efficient that additional pumps are postulated²³⁵, early evidence suggesting that ABCG2 could also be involved²³⁶. AZA and MP cannot themselves be transported²³⁷, but their metabolites, essential for these pro-drugs to work, are effluxed in an ATP-consuming process. More detailed studies have shown that Me-tIMP and other methylated metabolites²³⁸ are exported by both transporters but more efficiently by MRP-4, which also preferentially transports tGMP²³⁵. tIMP is exported more efficiently by MRP-5 whilst tXMP can only be exported by MRP-5²³⁵. MRP5 can also transport unphosphorylated nucleotides²³⁸. Cells expressing high numbers of these pumps efflux thiopurine metabolites so rapidly that lower intra-cellular TGN levels have been documented²³⁹ and several authors postulate that they could have a role in non-response to thiopurine drugs^{235,240,241}.

The question of whether over-expression of MRP-4 & 5 leads to MP resistance has been addressed in a number of ways. Peng et al²⁴² grew ALL cell lines in medium containing MP to select out resistant cells. These were then characterised and found to have higher MRP-4, (but lower hENT1, hCNT2 and hCNT3 levels and higher TPMT activity). The cells accumulated less

MP and metabolites suggesting that a transporter was responsible and this process was ATP-dependent, implying a role for MRP-4. Meanwhile, Wijnholds et al²⁴¹ transfected cells with MRP-5 and discovered that this also conferred resistance to MP by rapid efflux of tIMP. Reid et al²⁴³ found that HEK293 cell lines which over-express MRP-4 & 5 demonstrate resistance to thiopurines which is proportional to the level of MRP expression. However, in these experiments the kinetics of the transport suggest that MRPs only transport thiopurines when they reach high intracellular levels and may therefore only be a minor factor in *in vivo* resistance. This conclusion would however be challenged by emerging evidence from the clinical context that MRPs do indeed affect an individual's response to drug therapy.

Plasschaert et al²⁴⁴ took blasts from 105 ALL patients at diagnosis and characterised their level of expression of MRP 1-6 before patients received their standard chemotherapy regimens, which included MP in the maintenance phase. Levels of all 6 MRPs correlated with risk of relapse and this reached significance for all except MRP-4 ($p=0.208$), for MRP-5 $p=0.016$. The authors noted that patients found to over-express one MRP often expressed high levels of several other MRPs. Further experiments have however shown that blasts taken at the point of relapse do not necessarily have higher MRP levels than paired blasts from the time of original diagnosis²⁴⁵.

The importance of MRP-4 has been further explored by the development of an MRP knock-out mouse model which under normal circumstances has no phenotype²⁴⁶. When exposed to nucleotide analogues, serum levels of MRP were the same as those seen in wild type animals but several tissues, particularly bone marrow, thymus, spleen & gastrointestinal tract, experienced severe toxicity and PMEA accumulated in the brain²⁴⁶. Additional experiments demonstrated that MRP-4 KO mice accumulated higher levels of TGNs in the bone marrow after exposure to thiopurines (despite normal serum levels) which translated to dose-dependent toxicity²³⁹. This effect could be mimicked in wild type animals by blocking MRP-4 activity with drugs.²³⁹

Thus, as would be predicted, over-expression of MRP-4 & 5 has been linked to drug resistance and deficiency to drug-induced toxicity. Cells over-expressing MRPs seem to have an increased sensitivity to methotrexate^{241,242} which is difficult to account for, but makes the MRPs extremely

interesting pharmacogenetic targets, as information on MRP expression could direct personalised choice of immunomodulatory agent.

MRPs are subject to genetic polymorphism, which could be an important predictor of MRP function²¹⁷, see below. However, regulation of MRP expression is complex and many other factors are involved, for example, availability of other substrates (e.g. folate²⁴⁷) and other transporters (e.g. NHERF1²⁴⁸). Other substrates can also be co-excreted or compete for transport.²⁴⁹ A number of drugs act as inhibitors to MRPs e.g. ibuprofen, sildenafil, probenecid, dipyridamole, celecoxib and indomethacin^{250,251}. Evidence from KO mice suggests that reducing levels of one MRP results in up-regulation of others with a similar spectrum of substrates. In the case of MRP-4 KO mice this has been demonstrated with ABCG2²³⁶. Pharmacogenetics does, however, still play a role, and several groups have now produced evidence that SNPs in MRP relate to measurable changes in drug transport and even in clinical outcome.

Abla et al²⁵² transfected cells with naturally occurring variants of the MRP-4 gene which they found by sequencing an ethnically diverse population. They demonstrated that the polymorphism *MRP4 G559T* correlates with decreased expression of MRP-4 protein and resulted in a 69% decrease in 9-(2-phosphonmethoxyethyl)adenine (PMEA) transport and a 43% decrease in zidovudine (AZT). *MRP4 G1460A* also significantly decreased transport of both drugs, whilst *MRP4 C232G* and *MRP4 C1208T* both caused a significant decrease in AZT and a trend to decrease PMEA transport. The *MRP4 G2867C* polymorphism resulted in increased efficiency of drug extrusion. *MRP4 G559T*, *A934C* and *A2230G* are all of particular interest as they have a frequency of >5% in their population. *In vitro* studies by Janke et al²⁵³ also showed that in *MRP4 Y556C*, *E757K*, *V776I* & *T1142M* all resulted in decreased expression of MRP-4 and *V776I* was associated with a significant reduction in the transport of MP.

In patient studies, Anderson et al.²⁵⁴ demonstrated that *MRP4* SNPs *T4131G* and *G3724A* were associated with increased levels of nucleotide analogues (lamivudine and zidovudine respectively).

In a Japanese cohort *MRP4 G2269A* caused decreased membrane localisation of MRP-4 and increase sensitivity to thiopurines²³⁹. When this was looked at specifically in IBD, *MRP4 G2269A* was associated with higher TGNs and lower WBCs, although it was not associated with the 3 cases of severe low WBC²⁵⁵. Ansari et al²⁵⁶ analysed all *MRP4* SNPs and found 8 SNPs which tag the most frequent haplotypes, some of which have a frequency of >5%: C-1015T & A4131C. They then analysed this in childhood ALL where the tag SNPs T-1393C & A934C affected both toxicity and outcome. However, another group failed to replicate this in their cohort²⁵⁷. A study of 194 ALL cell lines demonstrated that *MRP4* gene expression was one of only 3 candidates found to be associated with the experience of toxicity on thiopurine therapy²¹⁷.

A large number of the ABC transporters have been characterised and they have been divided into subfamilies, see Figure 1.7. *MDR-1* is in the B-subfamily and otherwise known as *ABCB1*. Other key members of ABCB subfamily are: *ABCB4* which encodes MDR-3 p-glycoprotein and *ABCB5* which encodes MDR-5 p-glycoprotein, both of which are thought to be closely related to *ABCB1*²⁵⁸. These transporters have been the subject of much less study than *ABCB1*. However, current knowledge about their function is presented below.

ABCB4 has been implicated in resistance to paclitaxel in ovarian cancer²⁵⁹ and is up-regulated in chemo-resistant cell lines²⁶⁰. It has recently been shown to predict a shorter disease-free period after chemotherapy for colorectal cancer with 5-fluouracil²⁶¹. Deficiency due to genetic polymorphism creates a wide spectrum of problems, particularly biliary / lipid abnormalities²⁶².

ABCB5 has been the subject of detailed studies in doxorubicin resistant melanoma cell lines²⁶³, and shown to predict resistance to chemotherapy in hepatic tumours²⁶⁴, bowel cancer²⁶⁵ and in haematological malignancy²⁶⁶. Expression level has been shown to correlate with resistance to a wide variety of chemotherapeutic agents²⁶⁷ and differing variants of MDR-5 confer different levels of resistance to chemotherapy regimens²⁶⁸. Additional roles are postulated for *ABCB5*, which has been demonstrated to be responsible for cell fusion and differentiation²⁶⁹. *ABCB5* is subject to multiple potentially deleterious single nucleotide polymorphisms²⁵⁸.

The relationship between polymorphism in *MDR/MRP* genes and clinical outcome of patients with IBD is part of the research presented in this thesis and can be found in chapters 4 and 5.

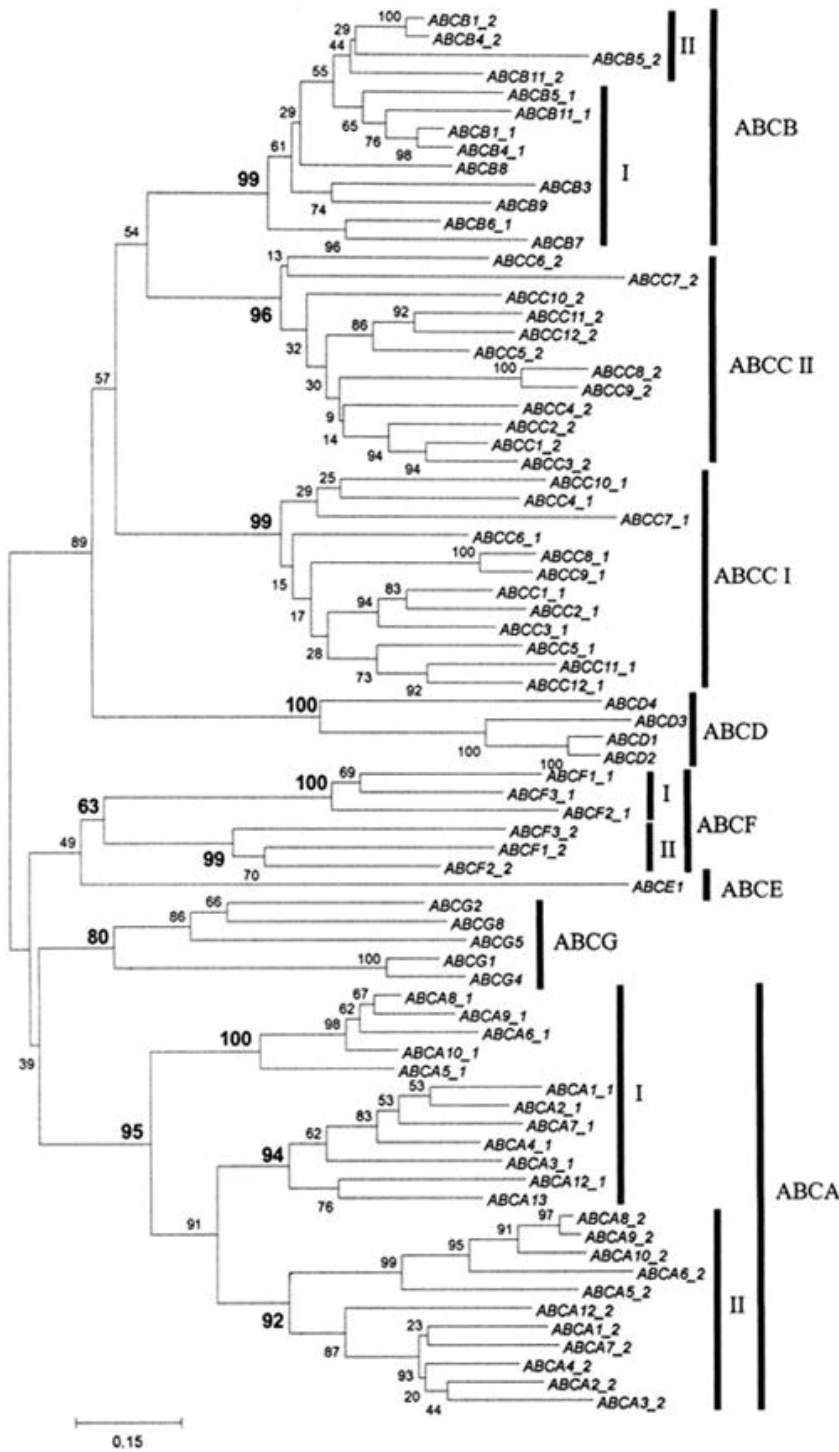


Figure 1.7: The ABC transporters divided into their subfamilies, Taken from: The human ABC Transporter Superfamily M. Dean, 2002 <http://www.ncbi.nlm.nih.gov/books/NBK31/> accessed on 24.5.12

Table 1.2: SNPs in MRP genes and reported associations with altered function (Gene freq. for Caucasians)

SNP	αα substitution	rs number	gene freq	functional data
G559T	Gly187Trp	rs11568658	0.04	reduced expression of MRP4 and significantly reduced transport of PMEA & AZT ²⁵²
G1460A	Gly487Glu	rs11568668	0.000	significantly reduced transport of PMEA & AZT ²⁵²
C232G	Pro78Ala	rs11568689	0.000	sig. reduced transport of AZT, trend for PMEA ²⁵²
C1208T	Pro403Leu	rs11568705	0.018	sig. reduced transport of AZT, trend for PMEA ²⁵²
G2867C	Cys956Ser	rs11568707	0.002	increased efficiency of drug extrusion ²⁵²
G2326A	Val776Ile	rs146708960	0.000	decreased expression of MRP4 & significant reduction in MP transport ²⁵³ .
C3425T	Thr1142Met	rs11568644	0.018	decreased expression of MRP4 ²⁵³
	Y556C	not in dbSNP		decreased expression of MRP4 but increased PMEA transport ²⁵³
T4131G		not in dbSNP		increased drug level zidovudine, lamivudine ²⁵⁴
G3724A		not in dbSNP		increased drug level zidovudine, lamivudine ²⁵⁴
G2269A	Glu757Lys	rs3765534	0.027	decreased expression of MRP4 ²⁵³ , decreased membrane localisation of MRP4 & increased sensitivity to thiopurines. ²³⁹ higher TGNs and lower WBCs ²⁵⁵
T-1393C				higher promoter activity, better event free survival and lower methotrexate plasma levels ²⁵⁶ Not replicated ²⁵⁷
A934C	Lys304Asn	rs2274407	0.05	high incidence of severe thrombocytopenia & lower event free survival ²⁵⁶ Not replicated ²⁵⁷
2000C>T	Pro667Leu	rs11568697	0.002	None
C52A	Leu18Ile	rs11568681	0.06	None
G2459T	Arg820Ile	rs11568659	0.009	None
G3211A	Val1071Ile	rs11568653	0.009	None
C3425T	Thr1142Met	rs11568644	0.018	None
G3659A	Arg1220Gln	rs11568639	0.018	None
A2230G	Met744Val	rs9282570	0.000-0.05	did not correlate with PMEA or AZT transport or membrane levels of MRP4 ²⁵²

1.11.9 Glutathione S-transferase

The conversion of AZA to MP is usually reported as being non-enzymatic. However, there is quite a body of evidence supporting an enzymatic component to this conversion, due to the action of the glutathione S-transferases, (GST)^{10,11,270-272}. The action of these enzymes, particularly through glutathione depletion, and therefore organ specific oxidative stress, is implicated in adverse events on azathioprine therapy.²⁷²⁻²⁷⁷ Patients with IBD have been shown to have low glutathione levels in gut mucosa, especially at sites of active disease²⁷⁸. It is possible that this could contribute to the high incidence of GI side effects experienced on azathioprine therapy and the success in these cases of switching to MP therapy²⁷⁹, although there is no evidence to support this theory at present.

Recently, it has been established that the subtypes of GST involved in azathioprine metabolism are GST M1-1, A1-1 and A2-2²⁷². Genetic variation in these enzymes has been shown to result in variability in their activity²⁸⁰ which is now known to specifically relate to azathioprine metabolism²⁸¹. A more active version, particularly if found in association with low TPMT activity, could cause side effects²⁷². Stocco et al published the only clinical data to date and reported that, in their cohort of 70 patients on azathioprine for IBD, those with wild type GST M1 were at increased risk of lymphopenia and adverse events. The authors provide rationales for supposing that GST might be particularly involved in the development of pancreatitis and liver damage, but unfortunately their numbers were inadequate to show a significant association with other subtypes of ADR²⁷⁷. The confirmation of an association between GST wild-type status and the development of pancreatitis in other situations^{273,282} would strengthen this hypothesis.

1.11.10 Soluble -HLA-G

HLA-G is not involved in the transport or metabolism of thiopurines. However, the gene encoding HLA-G contains a 14 base-pair insertion deletion polymorphism which has been associated with response to methotrexate in the context of rheumatological disorders²⁸³. HLA-G is a non-classical HLA protein, which has much less genetic variability than other HLA subtypes and exists at immunologically privileged sites, such as in the placenta, cornea and thymus^{284,285}. It inhibits both adaptive and innate immunity by inhibition of natural killer cells²⁸⁶, lymphocytes²⁸⁷ and dendritic cells²⁸⁸. HLA-G has been found to be up-regulated in a variety of

pathological states such as cancer²⁸⁹ and expression appears to be enhanced by some viruses as part of their strategy for evasion of host immunity²⁸⁸. Induction of HLA-G is considered to be an important part of successful tolerance of transplanted organs²⁹⁰. It was therefore considered that polymorphism in *HLA-G* could contribute to the variable response to thiopurines in IBD. Work by my colleague Dr Bijay Baburajan in the PRL was directed at investigating the effect of this polymorphism on response to both methotrexate and azathioprine in IBD and this work is included in the pharmacogenetic index presented later in the thesis.

1.12 Thiopurine Metabolite Monitoring

As discussed in the sections above regarding thiopurine metabolism, the active end product of azathioprine metabolism (figure 1.2) is considered to be the thioguanine nucleotides, (TGNs). These can be measured, alongside methylated metabolites, in the clinical setting as a guide to treatment decisions, and are increasingly recognised as a useful adjunct to therapy in clinical guidelines²⁹¹⁻²⁹³. A lack of correlation between the dose/kg of thiopurine given and the level of TGNs achieved has been extensively reported²⁹⁴⁻²⁹⁶. Some of this will represent inter-individual variability in metabolism of thiopurines and therefore, metabolite monitoring is considered by some groups to be extremely valuable in the assessment of cases of non-response to thiopurine therapy²⁹⁷. TGN / MeMP measurements confirm adherence to therapy²⁹⁸, detect preferential methylation¹⁹⁰ (associated with a low probability of clinical response and high risk of hepatotoxicity) and identify over-dosing and under-dosing²⁹⁹. TGN levels reach a plateau within 4 weeks of initiating therapy^{87,300}, facilitating early treatment optimisation which could save these patients months of poorly-controlled disease and decrease the likelihood of developing disease complications. TGNs can also establish the cause of non-response in those established on therapy and allow individualised dosing in treatment responders, maintaining effective therapy whilst minimising the risks of toxicity.

Measurement of MeMP is also important. Some studies relate MeMP and Me-TIMP to therapeutic effects^{44-46,227} by a potent inhibition of de novo purine synthesis, whilst others implicate these methylated metabolites in toxicity⁶⁴. Dervieux et al⁴⁴ showed that in those cells with active TPMT, MP was more effective at suppressing cell division than TG, whilst in those without TPMT the opposite was true. The authors attributed this differential effect to the creation of methylated metabolites which appear to work synergistically with TGNs³⁶. This could explain

why in patients with *TPMT* mutations, despite 50% dosing, equivalent response rates and toxicity are seen with TGN levels twice that expected in the wild-type population³⁰¹. Several authors have instead suggested that the ratio of different metabolites would better predict thiopurine efficacy. An example of this is the work of Derijks et al. who showed that a ratio of MeMP to TGN of <11 predicted efficacy more accurately than the levels of either metabolite in isolation.³⁰⁰ This ratio has been associated with both non-response and toxicity in other studies⁶⁴ and patients with a ratio greater than 11 do well on combination azathioprine and allopurinol treatment (see chapter 7).

Despite mounting evidence for their usefulness and the publication of guidelines recommending their use however, many physicians do not use TGN measurements in routine clinical practice.

1.12.1 Problems with the current systems of TGN monitoring

There are a few issues with current metabolite measurements which are easily surmountable. These include:

1) TGN levels have been measured by two separate methods which provide very different results from the same samples and are not directly comparable.³⁰² Care must therefore be taken that the methods used are standardised and particularly that the normal range adopted is relevant to the measurement method. TGNs degrade steadily if samples are stored at room temperature but are stable for a week in the fridge or 6 months in a freezer at -80°C. Methylated metabolites, on the other hand, decrease steadily and significantly by day 7 despite refrigeration and by 6 months despite freezing at -80 °C³⁰³

2) Even those studies which use the same analysis method have produced conflicting results, some showing a significant relationship between TGN level and response to thiopurine treatment and others showing no relationship. Meta-analysis²⁹⁷ confirms that the relationship is significant and that the study by Lowry et al³⁰⁴, which seemed to provide the strongest evidence against the usefulness of TGNs, is a statistical outlier. Exclusion of these results strengthens the association further. Those studies which took more than one measurement of TGN level showed that this also strengthened the relationship with clinical outcome^{305,306}.

3) TGN levels in most studies have been measured in erythrocytes (RBC) for practical ease^{91,295,305,307,308}. This is problematic both because erythrocytes are not the target cells for AZA, but also because, lacking a nucleus, they do not have all the enzymes requisite for thiopurine metabolism and production of TGNs^{183,309}. Such levels are therefore a distant reflection of the situation in the target white blood cells (WBCs) and bone marrow¹⁸³ and may not be fit for purpose²¹⁶. TGN levels in white cells are, however, difficult to measure as WBCs are a heterogeneous cell population and are difficult to extract without RBC contamination^{183,310}.

Some work has been done however on WBC metabolite levels,^{60,61,310-312} although most is from other disease backgrounds such as leukaemia and renal transplantation,^{310,312} where MP is given differently (intravenously or in the context of multiple other immunomodulatory treatments), making interpretation for our purposes difficult. This work does however highlight the difference between red and white cell TGN levels which appear to be most dramatic when using 6-thioguanine³¹³. In IBD, a small pilot study showed a correlation between white cell TGN levels and those measured in RBCs³¹¹, confirmed in a later study by the same group⁶¹.

4) TGN measurements have traditionally been performed using a method which cannot distinguish the mono, di and triphosphate subgroups from each other, neither can they tell the difference between these activated nucleotides and exogenously administered 6TG¹⁸³. However, since Thio-GTP is likely to be primarily responsible for bioactivity of AZA⁵¹ measuring all nucleotides together could loosen the association between metabolite measurements and response to treatment. As a result, the three subgroups of nucleotides have been measured separately and the levels analysed with treatment response²²⁵. This shows that a threshold level of 100pmol/8x10⁸RBC of the triphosphate nucleotide predicts response to treatment, and patients with an elevated fraction of thio-GDP show an attenuated response. Conversion from thio-GDP to the active thio-GTP is the responsibility of the enzyme nucleotide diphosphate kinase, and inter-individual variability in this enzyme might account for some of the variability seen in response to thiopurines²²⁵ (see above). High variability in thio-GDP:thioGTP ratios has also been seen in treatment with 6-thioguanine³¹⁴.

1.12.2 Alternatives to TGNs

Mean cell volume (MCV), change in MCV, WBC and lymphocyte counts have all be shown to change during thiopurine treatment and have been proposed as suitable alternatives to TGN measurement for treatment monitoring. However, whilst these markers correlate overall with TGN levels^{5,307,315-318}, their value in clinical practice is limited by a high degree of inter-patient variability^{294,299,318-320}. Likewise, deliberately pushing white cell counts down to ensure adequate thiopurine dose has been shown to cause toxicity^{299,321-323}.

One group has described the use of an algorithm based on standard blood monitoring results which apparently out-performs TGNs in terms of correlation with clinical response³²⁴. Perhaps the ability of this algorithm to measure response so accurately is not surprising, since changes in MCV and white cell counts are, in a way, directly measuring the body's response to thiopurines. Within the group of non-responders in this study however, the algorithm could not tell whether TGNs were sub-therapeutic, therapeutic or high, although non-adherence and predominant methylation could be predicted. There are several problems with using this however. The first is that interpretation is difficult, secondly the usefulness of such an algorithm is justified mainly on how expensive it is to request TGNs in the USA whilst in the UK TGN testing is considerably cheaper and thirdly, and perhaps most importantly, it is not clear how this measure guides therapy³²⁵. It cannot prevent toxicity from early detection of high TGN levels or optimise dosing at 4 weeks. Even at 3-4 months, it does not give an explanation for non-response unless due to non-adherence or predominant methylation. A study to determine the usefulness of TGNs in IBD clinical practice is presented in chapter 6 of this thesis.

Aims of the thesis:

The work presented in this thesis aimed to explore different strategies to optimise and personalise the way in which thiopurines are prescribed for our patients. In chapters 3 & 4 work to identify new, clinically relevant pharmacogenetic markers of outcome on thiopurines is presented. This work sought association between frequently occurring SNPs in key genes involved in thiopurine metabolism with clinical outcome (toxicity and non-response) on thiopurine therapy. Chapter 5 explores how this new information could be collated with established predictors of toxicity and non-response to create a pharmacogenetic index which would guide selection of immunomodulators and aid dosing and monitoring decisions in clinical practice. Chapter 6 presents the result of introducing thiopurine metabolite monitoring into clinical practice in an IBD clinic and chapter 7 presents the clinical outcome of introducing co-prescription of azathioprine and allopurinol.

Chapter Two: Materials and Methods

2.1 Patient Recruitment

2.1.1 Genotyping studies (chapters 3 & 4):

Samples originated from a prospective study of the pharmacogenetics of AZA in the treatment of IBD⁸⁷. 208 patients were included in the analysis of the original study, but only 192 were included in these genotyping studies, due to availability of adequate DNA for analysis. Patients were all adults, gave written informed consent, had IBD diagnosed by standard criteria and received 2mg/kg of AZA. Complete TPMT deficiency was an exclusion criterion, however none of these patients were encountered. Ethical approval was obtained for both the original study (Guy's Hospital Research Ethics Committee, MREC 00/1/33) and the additional pharmacogenetic work (Bexley and Greenwich LREC, 06/Q0707/84).

2.1.2 AOX sequencing (chapter 3)

Patients were selected from the cohort described above for more complete assessment of polymorphism in *AOX* by sequencing of the entire exomic sequence of this gene. This was partly to ensure that the real-time markers were accurate, but also to check that the highlighted polymorphism was not linked to a second polymorphism responsible for the observed problems with non-response to thiopurines. Five patients heterozygous for the SNP *AOX* 3404A>G by real-time testing and five who were wild-type for this gene were selected. All were non-responders to azathioprine despite therapeutic TGN levels and were wild-type for *TPMT**3C and *3A and *ITPA* c.94C>A variants.

2.1.3 TGN study (chapter 6)

Patients who had TGNs measured to monitor either AZA or MP treatment were identified from clinical records and from the Purine Research Laboratory (PRL) database. Only those patients attending the specialist IBD clinic at Guy's and St Thomas' NHS Foundation Trust were eligible for inclusion. In a proportion of patients, multiple TGN measurements had been taken. Patients receiving tioguanine or thiopurine/allopurinol co-treatment at the index TGN were excluded. In

each case, clinical records and laboratory results were reviewed retrospectively to record data on demographics, type/extent of IBD, indication for treatment, thiopurine dose and toxicity

2.1.4 Azathioprine and allopurinol co-treatment

Patients prescribed combination treatment with allopurinol and AZA or MP were identified from pharmacy and clinic records of co-prescription, and via TGN monitoring requests received by the PRL, GSTS Pathology, Guy's and St Thomas' NHS Foundation Trust (GSTFT), London, UK. Patient demographics, disease characteristics, indication for allopurinol co-treatment and clinical outcome were established retrospectively from electronic and paper case notes.

2.1.4.1 Eligibility for combination therapy with azathioprine and allopurinol

Combination treatment with thiopurine and allopurinol was considered in the following circumstances:

Hepatotoxicity on thiopurine monotherapy – new abnormality of liver function tests (LFTs) occurring on thiopurine therapy which the treating physician attributed to the thiopurine drug. All patients developing hepatotoxicity were investigated for other causes of abnormal LFTs.

Other side effects on thiopurine monotherapy. These ADRs had to coincide with thiopurine therapy and resolve on ceasing the drug, and the treating physician must both attribute the ADR to the thiopurine and consider it severe enough to stop the thiopurine. In cases where low doses of thiopurine were tolerated but side effects limited dose-escalation (and therefore clinical response), patients were included as sub-optimal/non-responders.

Sub-optimal response – inability to achieve clinical remission on thiopurine monotherapy. Additionally, the TGN profile had to suggest either hyper-methylation (MeMP:TGN>11) or under-dosing which could not be corrected due to side effects on dose-escalation. This group also included patients with a historical label of non-response alongside a TPMT activity >35 pmol/h/mgHb suggesting that they were at high risk of hyper-methylation.

Predominant methylation only – patients with a ratio of MeMP:TGN>11 but not currently experiencing a loss of response or toxicity.

High pre-treatment TPMT activity – patients with a TPMT greater than 35 pmol/h/mgHb have previously been demonstrated to be at high risk of non-response⁸⁷ and were considered for combination treatment with allopurinol as first line thiopurine therapy.

2.2 Determination of response

In all of the studies, determination of response to thiopurines was a key measure of outcome. The methods used for this in each section of the study are detailed below.

2.2.1 Genotyping studies (chapters 3 & 4)

Response was defined according to each patient's stated treatment goal. This was most commonly steroid withdrawal, which was defined as withdrawal of steroid treatment by 3 months and maintenance, steroid-free, for a further 3 months. If AZA was started for maintenance of remission, then remission had to be maintained for at least 6 months for treatment to be considered successful. Remission of active disease was recorded prospectively and defined by Harvey-Bradshaw index or Truelove and Witts criteria. Any patient who required escalation of treatment, either to surgery or a biologic agent, and any who required addition of, or switch to, an alternative immunomodulator was considered to have experienced treatment failure. Adverse drug reactions were included only if they resulted in cessation of the AZA or MP treatment.

2.2.2 TGN study (chapter 6)

Clinical response was established retrospectively from review of the notes and electronic patient records. In addition to the assessment of the treating physician, (considered a soft measure, particularly when determined in retrospect from the clinical records) steroid-free remission rates at 6 months from index TGN were recorded for all those patients in whom the TGN result dictated optimisation of thiopurine therapy (rather than recourse to surgery or biologic).

Any documentation of a change in management based on the TGN level was noted and outcomes were analysed according to whether the management decisions made in clinic were appropriate (according to the TGN result) or at odds with what the TGN result would suggest.

Assessment of non-response to thiopurines is a key indication for TGN measurement. Therefore, a separate analysis of the impact of the TGN results on the management of non-responders was made. Patients were only included in this analysis if it was clear from the documentation at the time of the initial TGN test that it had been ordered to establish the cause for clinical non-response.

2.2.3 Azathioprine / allopurinol co-treatment

The primary outcome measure for this part of the work was successful circumvention of the problem encountered on thiopurine monotherapy. In some cases this was a failure to achieve clinical remission, but in the majority was drug intolerance, normalisation of LFTs or normalisation of the thiopurine metabolite profile. Our primary outcomes could therefore be obtained objectively from biochemistry results for the majority of patients.

In order to confirm that combination therapy was effective in achieving good clinical outcomes in all patients, clinical remission rates for the whole group were also calculated as a secondary endpoint. Due to the nature of the study, this was a retrospective analysis and formal clinical disease activity measures (Harvey-Bradshaw Index [HBI], Simple Colitis Activity Index [SCAI] etc.) were not generally available. Clinical response was therefore assessed at one year of combination treatment on the basis of the treating physician's global assessment, as determined retrospectively from clinic records. Whilst this measure could be rather subjective, the use of steroids, biologics or surgery (much harder measures) was interpreted as an indication of treatment failure. Patients who had been on treatment for long enough to assess outcome (greater than 4 months) but not yet for one year were included in a secondary analysis.

Adverse Drug Reactions (ADRs) were defined as any adverse event occurring on combination therapy which was both severe enough to require the cessation of combination treatment and which was considered to relate to combination therapy by the treating physician.

2.3 Genotyping Studies: laboratory methods

2.3.1. SNP selection

Known coding region SNPs in *XDH*, *MOCOS* and *AOXI* with a Caucasian minor allele frequency of at least 0.02 were selected for genotyping. SNPs that encoded a non-conservative change in amino-acid were preferred.

Table 2.1: SNPs selected for analysis in AOX, XDH &MOCOS including predicted minor allele frequencies in the Caucasians population

rs number	Gene	Exon	cDNA base change	Amino-acid substitution	Frequency
rs2295475	XDH 2p23.1a	21	2211C>T	Ile737Ile	0.31
rs1884725	XDH 2p23.1a	27	3030C>T	Phe1010Phe	0.23
rs4407290	XDH 2p23.1a	10	837C>T	Val279Val	0.02
rs207440	XDH 2p23.1a	34	3717G>A	Glu1239Glu	0.06
rs17011368	XDH 2p23.1a	20	2107A>G	Ile703Val	0.05
rs17323225	XDH 2p23.1a	18	1936A>G	Ile646Val	0.05
rs59445	MOCOS 18q12.2a	11	2107A>C	Asn703His	0.34
rs623053	MOCOS 18q12.2a	4	509T>C	Ile170Thr	0.03
rs678560	MOCOS 18q12.2a	6	1072A>G	Met358Val	0.03
rs1057251	MOCOS 18q12.2a	15	2600T>G	Val867Ala	0.10
rs3744900	MOCOS 18q12.2a	4	359G>A	Ser120Asn	0.03
rs55754655	AOX 2q33.1e	30	3404A>G	Asn1135Ser	0.16

C – cytosine, T – thymine, G – guanine, A – adenine. Ile – isoleucine, Phe – phenylalanine, Val – valine, Glu – glutamate, Asn – asparagine, His – histidine, Thr – threonine, Met – methionine, Ala – alanine.

In *ABCB4* there was one reported common non-synonymous coding SNP and 2 common synonymous SNPs with proposed functionality in other contexts³²⁶ which we selected for analysis. In *ABCB5* modelling has proposed 4 key polymorphism sites for drug resistance²⁵⁸, of these only 3 are common in the Caucasian population, and were selected for analysis.

Table 2.2 Details of the SNPs in *ABCB4* and *ABCB5* which were selected for pharmacogenetic analysis including minor allele frequencies in the Caucasian population.

rs number	Gene	Exon	Base change	Amino acid change	Minor allele frequency	Frequency in our population
rs2302387	<i>ABCB4</i>	4	175C>T	Leu59Leu	0.11-0.21	0.15
rs2109505	<i>ABCB4</i>	8	711A>T	Ile237Ile	0.14-0.27	0.18
rs8187799	<i>ABCB4</i>	16	1954A>G	Arg652Gly	0.04-0.14	0.08
rs34603556	<i>ABCB5</i>	4	2T>C	Met1Thr	0.2-0.34	0.2
rs2301641	<i>ABCB5</i>	5	343A>G	Lys115Glu	0.27-0.43	0.26
rs6461515	<i>ABCB5</i>	15	1573G>A	Glu525Lys	0.12-0.17	0.16

C – cytosine, T – thymine, G – guanine, A – adenine. Leu- leucine, Ile – isoleucine, Arg- arginine, Gly – glycine, Glu – glutamate, Thr – threonine, Met – methionine, Lys – lysine.

2.3.2 DNA extraction:

DNA extraction had already been performed as part of the original study⁸⁷. However, additional DNA extractions were necessary from some patient samples, to obtain sufficient DNA for study. DNA was extracted from EDTA whole blood samples using the QIAmp DNA Mini Kit (Qiagen Ltd. Crawley, UK). Briefly, protease and buffer AL were added to 200 µl whole blood. This mixture was vortexed and incubated at 56°C for 10 min. 200µl of 100% ethanol was added, the mixture was vortexed again and transferred to a spin column from the above DNA extraction kit, within a 2ml collection tube and centrifuged at 8000g for 1 min. The column was then washed to remove contaminants and transferred to a clean collection tube in which the DNA was eluted using 200µl of buffer AE from the QIAmp kit and a further centrifuge at 8000g for 1 min. In the experience of the laboratory, this method produces 6µg of total DNA in 20-30kb lengths from

200µl of whole blood. The extracted DNA was mixed with 50µl of tris-EDTA (x1 mixture) to inhibit DNAases and stored in a freezer at -20°C.

2.3.3 Real-time PCR genotyping

Patients were genotyped by real-time PCR using a Biorad Miniopticon (Biorad, Hemel Hempstead, UK). 1.8µl of DNA was mixed with Absolute QPCR Mix (Abgene, Epsom, UK) and the selected TaqMan® SNP genotyping assays (Applied Biosystems, Warrington, UK). The reaction mixture was made up to a volume of 10µl with DNA-free water. SNP probe details are shown in Table 3.1. PCR conditions were 15 min enzyme activation at 95°C, then 42 cycles of: denaturation (15 s at 95°C) and anneal/extension (1 min at 60°C).

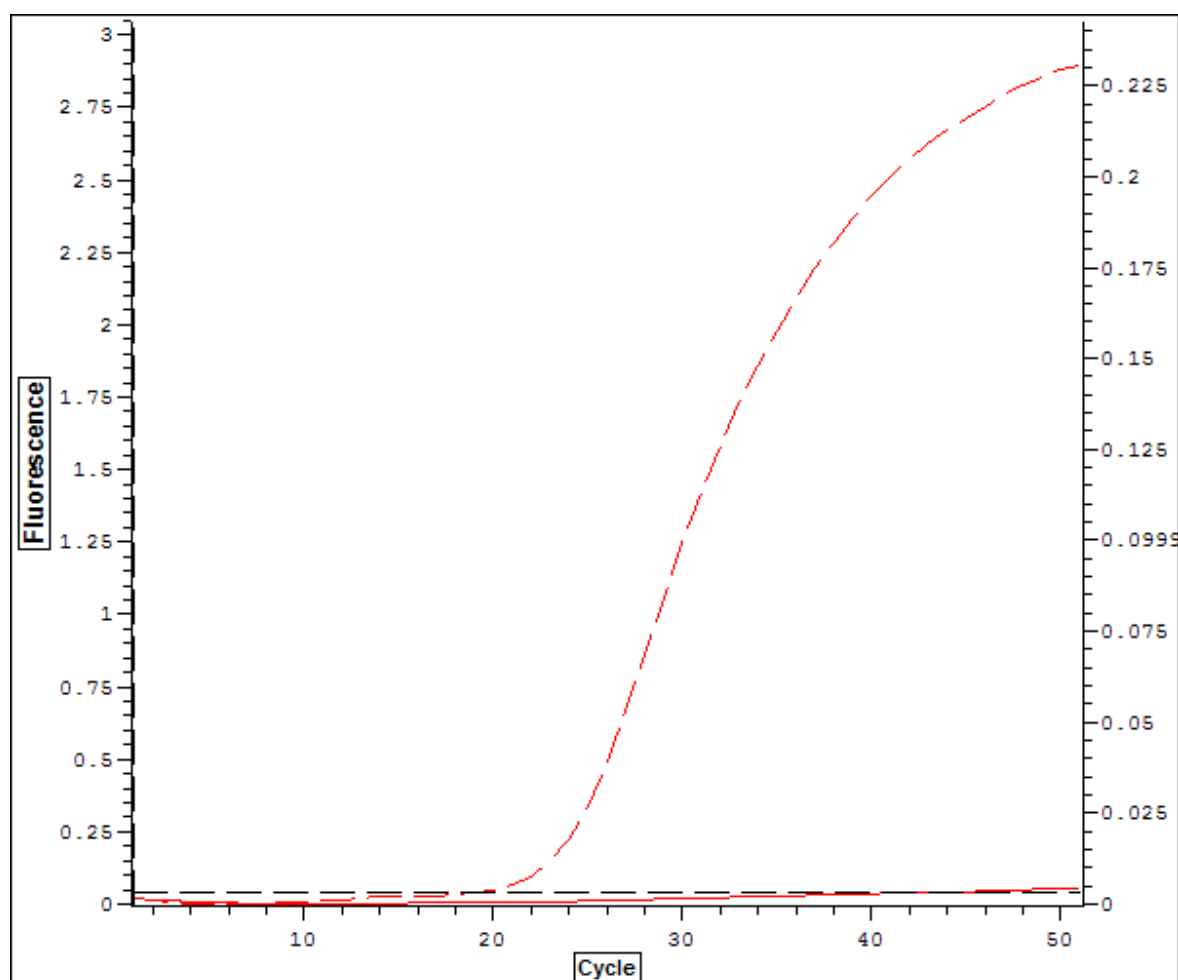


Figure 2.1 Example of a real-time trace from a wild-type individual

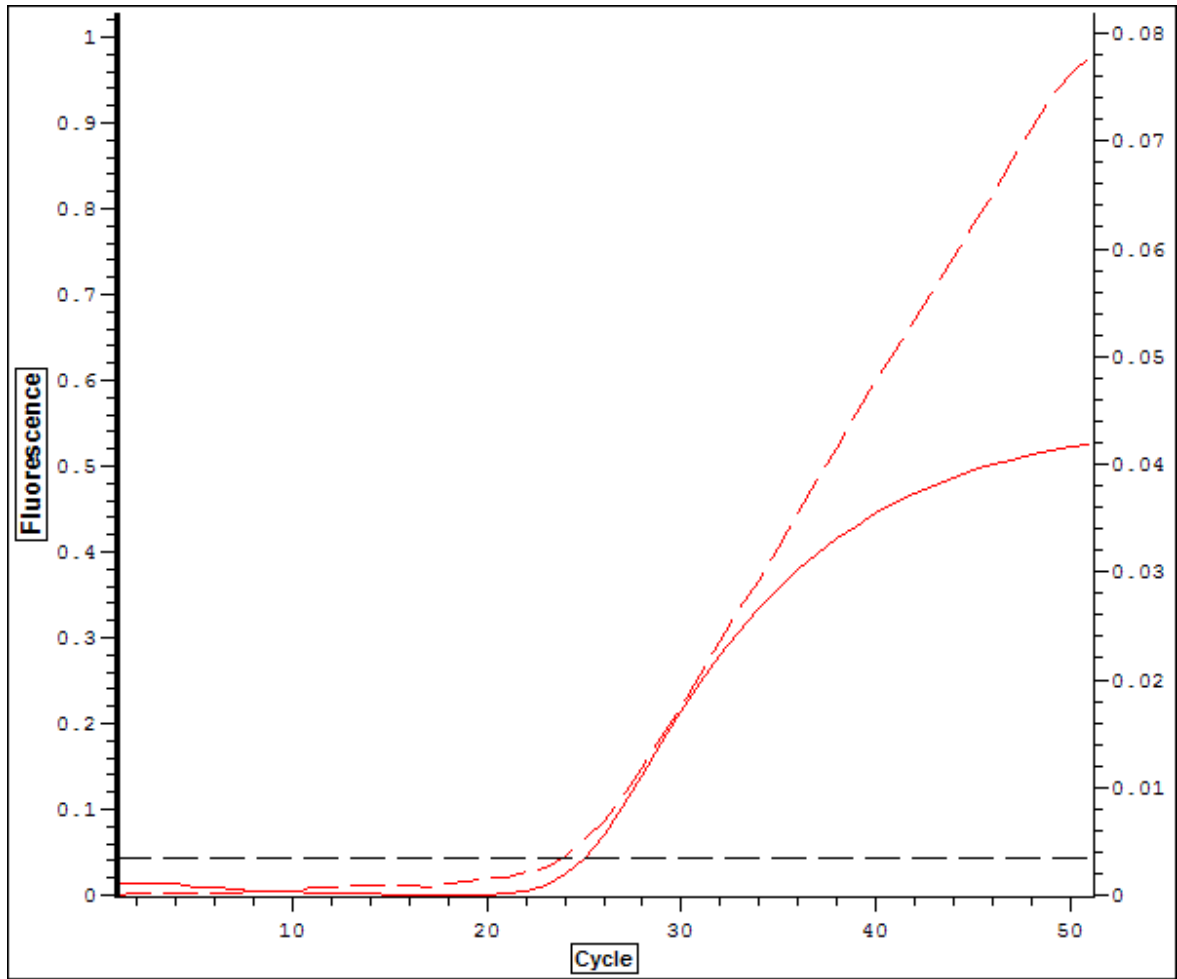


Figure 2.2 Example of a real time trace from a heterozygous carrier

2.3.4 Statistical Analysis

Genotypes for each SNP were tested for departure from Hardy-Weinberg equilibrium and the frequency in the study population compared to those published for Caucasian individuals in dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). Dominant and recessive models were used to test for association between the SNP minor allele and clinical outcome (adverse drug reaction or clinical response). Chi square tests or Fisher's Exact tests were used to test for differences between groups in a 2x2 contingency table using InStat version 3.0a for Macintosh (Graphpad Software, San Diego, CA, USA www.graphpad.com). Chi square test for trend was used for 2x2x2 contingency tables. An unpaired Student's t-test was used to assess difference in means. No correction for multiple testing was applied. Haplotype analysis was performed in XDH and MOCOS using UNPHASED³²⁷.

2.4 Sequencing the Aldehyde Oxidase Gene in non-responders to azathioprine

2.4.1 Primers:

For *AOX* sequencing, primers for each exon were designed using the web-based tool primer3 (<http://frodo.wi.mit.edu/>) and synthesised by MWG Biotech, (London, UK). The primer sequences are shown in Table 2.3.

Table 2.3: Primers used to amplify and sequence *AOX* exons.

Exon	forward primer	reverse primer
1	5'-AGCTCAGGAACGTTGGATCTTA-3'	5'-AGGAAAGGGGACATAAGGAAG-3'
2	5'-GGTGGGGCTCAAGATTACAC-3'	5'-GTTCTTCTGACGGCCACATT-3'
3	5'-GTTGTTTTCAAAGGGAGAATGC-3'	5'AGGACCATGTCTGTCTTGTTCA-3'
4	5'-TCACTCTGATCCTCAGATTCCA-3'	5'-ATATGCCAGGCTTCACTCTTGT-3'
5	5'-GCCTTAAGTGAAGCTCAAAAGC-3'	5'-GCACAAGAATTGCTTGAACCT-3'
6	5'-TGTCACAAGCAGAAGGCATAAC-3'	5'-GCCCTGCTCAAGATCAAATAAC-3'
7	5'-TAATGCTATGTGCCACGCTTAG-3'	5'-AGATGGAGTGAGAATGGGAGAG-3'
8	5'-TTTGTGTTAGAGCCCTTGGTTT-3'	5'-TCCTCCTCCTCTTCCTTAGTCC-3'
9	5'-TCTTTGAGACAAGAGCACCAAA-3'	5'-GACTGCTTTTCCTGAGAACACA-3'
10	5'-CCAGCCATTCTCTCTGTATGTG-3'	5'-AATGGGCAAAGGATATGAACAG-3'
11/12	5'-TGGACATTTTCAGATCAGCAGT-3'	5'-TTCCAGTATCCAGAAAAGCAGA-3'
13	5'-GAAGCATAGGAAGAGCACCTGT-3'	5'-ATAGGGGCATGCGTAATAACAAG-3'
14	5'-ACATACACATACCGTCCAGCAC-3'	5'-TCCCAGCCATTAAATAGAAAGGT-3'
15	5'-TGCCTGATCCAGAGCTTGTACT-3'	5'-AAACTCATACCCACCCACAGAC-3'
16	5'-CTGGTGGAAGCCAGTTACCTAC-3'	5'-CCCATAAAGAGGCTGTGAGTTT-3'
17/18	5'CAGCCCAATAGTGCTTTTTTCATA-3'	5'-TGAAGCACCGTTAACTGAGAAA-3'
19	5'-GAGGCCAGCTGTGGTTATAGTC-3'	5'-AGCAGAGAAGAGTGGCAAAAAG-3'
20	5'-TTACATTTCCCTCACTGGGGAGT-3'	5'-AGGGAAACACATTTGTGAATGA-3'
21	5'-GGCAGACACAAAAGATTCTCCT-3'	5'-GAGTGCAGTGGTGTGATCATAGA-3'
22	5'-GGTTGTTGGACATTATCCATT-3'	5'-GCAGCTTGTTTGTGTGTTTGTA-3'
23	5'-TGGCATTATCCCTGACCTAAC-3'	5'-CTAAGCAGAAAAGCCTCCATTG-3'
24	5'-TGGTTACACAGCTTGCCTAAGA-3'	5'-TGTGTGCATCTAGGAAAAGAGC-3'
25	5'-TGGTTTTGGTCTGAGGTCGTAG-3'	5'-CAGTCCTCAGGATCTTCTGCTT-3'
26	5'-TGAATGGATGAGCCTATGTCTG-3'	5'-GCTCTGGTCTCCTCTGGAGTAA-3'
27	5'-CACCCCAAAAAGAAACCTAAAA-3'	5'-CTGGTCTCGAACTCCTTCACTT-3'
28	5'-AAGAACTTTTCATGGCTGGAG-3'	5'-GGAGTGAGGGAGAAAAGAGGT-3'
29	5'-AGGCCAGTTCTCTCTCACAC-3'	5'-ACAAGAGTTCCTCCACCACACT-3'
30	5'-AGGTGCTGAACAAACAGCTACA-3'	5'-CCACAACCATGGATTAAGTAGC-3'
31	5'-GAGGCACCAGTCAGGAGTAGTT-3'	5'-TCTGGTAATCCAGGGAAATCAC-3'
32	5'-TCTGTTGCATCATAACAGCTTGA-3'	5'-ATTTGAGCTCTGCAGTTGGTCT-3'
33	5'-AAAATTCAAGCAAACAGCATGA-3'	5'-CAATCCTGGTAAAAATCCCAAA-3'
34	5'-GAGGTTGCAGTGAGCTGAGATT-3'	5'-AGTGCGTTCACGAAAACCTACC-3'
35	5'-AGATGATAACCCAGGACCTCT-3'	5'-GACGGATATCACCTAGAGGAA-3'

2.4.2 PCR

The primers were diluted to a concentration of 50pmol/ μ l and then 1 μ l of each primer for the exon under investigation was included in a 100 μ l per reaction master mix. This master mix contained (per 100 μ l reaction) 10 μ l of 10x PCR buffer, 6 μ l of 25mM MgCl₂, 4 μ l of 5mM deoxyNTP mixture and 0.5 μ l of 5U/ μ l Taq DNA polymerase, the volume was made up to 100 μ l with sterile deionised water. Finally, 2 μ l of the selected patients' DNA was added. PCR conditions were 1 minute denaturation at 94°C then 35 cycles of: 45 s denaturation at 94°C, then 30 s annealing at 54°C and 50 s extension at 72°C. When 35 cycles were complete an extension phase of 5 min was added to ensure all strands had reached the full required length. A template control was included in each PCR run for each set of primers to check for contamination.

Where initial attempts at polymerisation failed, various strategies were attempted to optimise the PCR reaction. These included altering the annealing temperature of the PCR reaction, the addition of 5% dimethylsulfoxide (DMSO) to the master mix and the use of a hot-start Taq polymerase.

Once the PCR reaction was complete, 10 μ l of the PCR product was mixed with loading buffer (bromophenol blue, xylene cyanol and sucrose) and PCR fragments separated on a 2% agarose gel containing ethidium bromide. Fragments were sized relative to a 100 bp DNA ladder. The gels were then visualised under ultraviolet light to ensure that PCR had been successful, before any further work was done on the reaction products.

2.4.3 PCR Product Purification:

Where the checking gels confirmed a successful PCR reaction, the remaining 90µl of PCR product was then purified to remove unincorporated nucleotides and primers using QIAquick PCR clean-up kit according to the manufacturer's instructions (Qiagen Ltd. Crawley, UK). Briefly the 90µl of remaining PCR product was added to a QIAgen spin column with 500µl buffer PBI. This mixture was then centrifuged at 12000 g for 30 s to adsorb the DNA onto the column matrix. After two washing steps using 750µl buffer PE, the columns were spun for one minute to dry them before being transferred to clean collection tubes. 30µl of elution buffer (10mM Tris-Cl, pH 8.5) was added to each column and these were then re-centrifuged for 1 min at 12000 g. The resultant purified DNA was transferred to a clean tube sealed and frozen at -20°C until required.

2.4.4 Sequencing:

The purified PCR products were then sequenced using Beckman Coulter Dye Terminator Sequencing Kit, (Fullerton, CA, USA), according to the manufacturer's instructions. Briefly, 4µl of Dye Terminator Cycle Sequencing (DCTS) Quick Start Master Mix was combined with 4µl of sequencing dilution buffer and 8µl of DNA-free de-ionised water per reaction. Each exon was sequenced in both forward and reverse directions by adding 3µl of purified PCR product and 1µl (5 pmol) of either the forward or the reverse primer to the above mixture. The sequencing protocol was: 1 minute at 94°C, then 35 cycles of (1 min at 94°C, 30 s at 48°C and 3 min at 68°C) and finally an extension step of 10 min at 68°C.

Agencourt CleanSEQ (Beckman-Coulter) was used to remove unincorporated dye terminators from the sequencing reaction. Briefly, the products of the sequencing reaction were bound to paramagnetic particles in the presence of 62µl of 80% ethanol. The tubes were then transferred to a ring magnet plate which pulls the particles with attached DNA to the edge of the tube. The beads were then repeatedly washed with 80% ethanol and then left to dry. Finally, 30µl of formamide was added to each tube and the tubes removed from the magnetic plate and frozen at -40°C until run on a Beckman capillary sequencer.

The sequences were analysed using Chromas (<http://technelysium.com.au>) and Mutation Surveyor (Biogen, Kimbolton, UK)

These sequences were examined for any polymorphism and compared to the reference sequences obtained from the NCBI website (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide,cDNA:BC117179,genomicDNA:AC007163>).

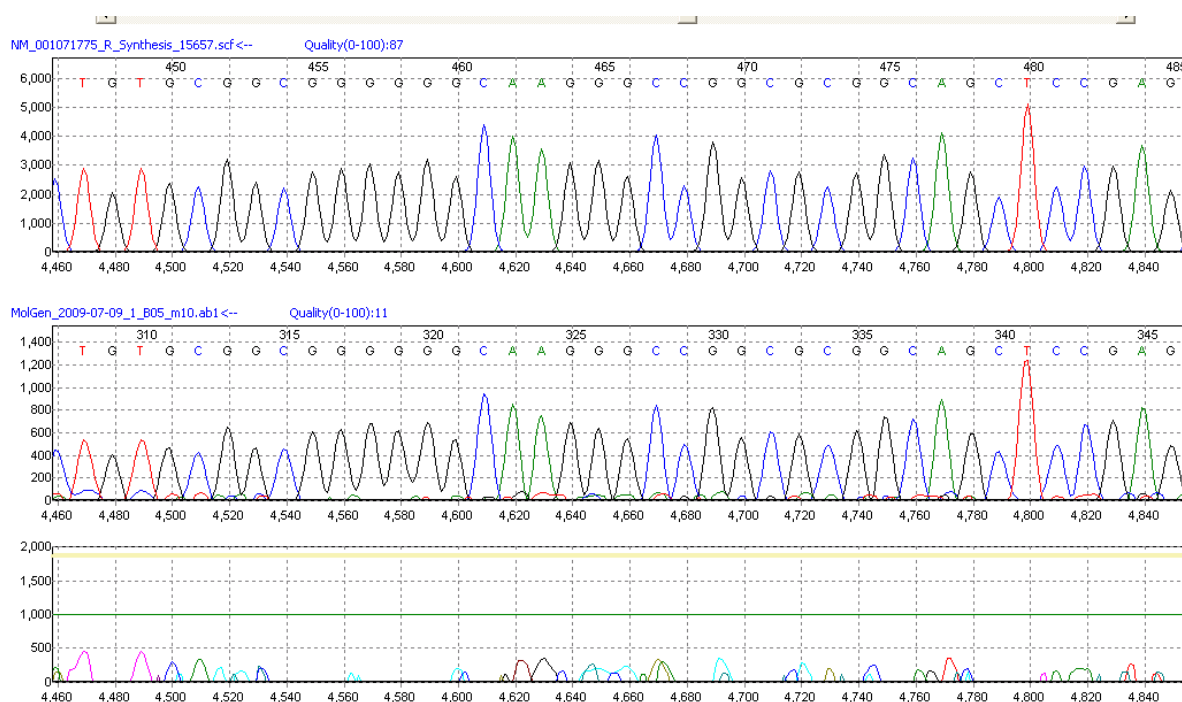


Figure 2.3 A sample of the sequence obtained for aldehyde oxidase in the middle frame.

The reference sequence is shown in the top frame of the Mutation Surveyor display and the lower frame contains analysis of the difference between these two traces - the green line indicates the threshold for significant deviation and therefore the possible presence of a polymorphism / mutation.

2.5: The impact of TGNs on clinical practice

2.5.1 Measurement of TGNs and MeMP

TGN and methylated thiopurine metabolite (MeMP) concentrations were measured as the hydrolysed base in whole blood in a protocol adapted from the method of Dervieux and Boulieu³²⁸ in the PRL (GSTS Pathology at St Thomas' Hospital, London). For clinical purposes, the PRL uses the therapeutic range 200-400 pmol/ 8×10^8 RBC for TGNs.

Briefly this method comprises: blood samples were collected into EDTA tubes, with erythrocyte counts obtained from each sample, so that drug concentrations could be normalised to 8×10^8 RBCs. After this analysis, 0.5 mL of blood was transferred into a microfuge tube. 100 μ l of dithiothreitol (DTT) 100 mg/ml was added and the sample rapidly deproteinised using 250 μ l of 15% perchloric acid. The deproteinised samples were then centrifuged at 12000xg for 2 min. The supernatants was transferred to a screw cap microfuge tube and heated for 1 h at 100°C to hydrolyse all the thiopurine nucleotides to their bases. After cooling, 2 μ l is then injected onto a Waters UPLC-reverse phase system with an Acquity column (C18, size 2.1x100 mm, 1.7 μ m). TGNs and MeMP were analysed simultaneously, and detected at 341 nm and 304 nm respectively by retention time and maximal absorbance.

2.6 Co-treatment with allopurinol and azathioprine

2.6.1 Thiopurine dosing and monitoring on combination treatment

TPMT activity, TGN and MeMP levels were measured by the PRL, with TPMT activity measured in all patients prior to first thiopurine prescription. TPMT activity in the range 11-25pmol/h/mgHb was considered intermediate (representing heterozygous deficiency with a target single agent AZA dose of 1-1.5mg/kg) whilst activity >25pmol/h/mgHb was considered to represent normal TPMT metabolism (with a target single agent AZA dose of 2-2.5mg/kg). Table 2.4 documents the dosing protocol for combination treatment according to TPMT result. Allopurinol was given at 100mg per day. Those patients with TPMT activity >35pmol/h/mgHb were considered to be at high risk of predominant methylation and were considered for primary therapy with combination AZA and allopurinol.

Blood monitoring following thiopurine initiation was undertaken in a standard manner and consisted of FBC and LFT testing at weeks 2, 4, 8 and 12 after initiation of combination therapy and TGN/MeMP levels were assessed at 4 weeks to permit dose optimisation. Blood monitoring was then undertaken every 3 months.

Table 2.4: Dosing strategy for low dose thiopurine and allopurinol

TPMT genotype	TPMT phenotype pmol/h/mgHb	AZA dose with 100mg Allopurinol	MP dose with 100mg Allopurinol
wild-type	≥ 26	0.5-0.8 mg/kg	0.25-0.5 mg/kg
heterozygous	11-25	0.25-0.5 mg/kg	0.125-0.25 mg/kg
homozygous	0-10	Avoid	Avoid

Blood results, particularly liver function tests, full blood counts and TGN and MeMP levels were compared before and after the addition of allopurinol.

2.7 A pharmacogenetic index to predict clinical outcome on azathioprine therapy

Four markers considered to predict non-response to thiopurine therapy were measured in the same prospectively recruited cohort of patients starting therapy with azathioprine for their IBD. These markers were: TPMT > 35 pmol/h/mgHb, *AOXI* 3404G, *ABCB5* c.343A>G and the presence of the insertion allele for sHLA-G. High TPMT activity has been established in the literature as a cause on non-response to thiopurines. *AOXI* 3404G and *ABCB5* c.343A>G were found, in the above work, to be associated with non-response to thiopurines. Finally, the presence of the insertion allele for sHLA-G has also been found to predict non-response in parallel work by a colleague in the PRL.

2.7.1 Methods used to determine presence of the insertion/deletion HLA-G polymorphism

HLA-G genotyping was done by Dr B. Baburajan and is included here for completeness.

The presence of the 14bp insertion/deletion in HLA-G exon 8 3'untranslated region was established using fluorescent PCR and size-based allelic discrimination using capillary electrophoresis on an ABI sequencer. Briefly, venous blood was collected in EDTA tubes and genomic DNA isolated using standard techniques. The primers: 5KBHLA-G, 5'-AGCTTCACAAGAATGAGGTGGAGC-3' and

PROHLA-G3, 5'-AATGAGTCCGGGTGGGTGAGCGA-3'

were used to amplify the HLA-G gene. The reaction mixture included recombinant *Taq* DNA polymerase recombinant (Invitrogen Ltd, Paisley, UK), 0.5 μ M of each primer, 2 μ g of genomic DNA reaction buffer (Roche, Basel, Switzerland) 1 \times ; each deoxynucleoside triphosphate (Roche) 0.2 mmol/l & MgCl₂ (Roche) 1.5 mmol/l. The thermo-cycling conditions were 94°C for 2 min, 25 cycles of 94°C for 30 s, 64°C for 60 s, and 72°C for 2 min, followed by 72°C for 10 min. The amplified products were visualized by electrophoresis on a 2.5% agarose gel (Invitrogen, Paisley, Scotland, UK) containing ethidium bromide (0.5 μ g/ml)

The details of the pharmacogenetic work which lead to the identification of additional markers is described in chapters 3 and 4. This information has been collated to establish whether measuring these additional markers allows more accurate risk stratification than measurement of TPMT alone.

Statistics were performed using Chi-square for trend using Instat version 3.0a for Macintosh, (Graphpad Software, San Diego, CA, USA www.graphpad.com).

2.8 Statistical Methods Used

The majority of the statistics used in this thesis involved the analysis of 2x2 contingency tables containing data about categorical values (generally the presence or absence of a particular SNP and its relationship to outcome such as: responder or non-responder, side effects or no side-effects). Where sample sizes were large enough Chi-square testing was applied. If any field in the contingency table contained a sample less than 5, then Fisher's Exact Test was applied in its place. This is because Chi-squared statistics produce an approximate probability, and its accuracy is affected by low sample sizes and very uneven distributions of the sample across the table. Fisher's Exact Test however is perfectly suited to this situation, (running into problems only with very large samples). In the analysis of the pharmacogenetic index, a number of different SNPs were being analysed against the categorical variable: responder or non-responder. Since this generated larger contingency tables, Chi-square for trend was applied. In larger contingency tables chi-square is thought to be applicable so long as 80% of the fields are expected to have more than 5 units in them. There is an extension to the Fisher's Exact Test which can be applied to larger contingency tables (the Freeman-Halton extension) but this again struggles with larger sample numbers and cannot be used at all if the total number of data points in the table is 120 or more.

In the analysis of blood count variables and TGNs the unpaired Student's t-test was used to compare means. This tests whether a normally-distributed variable such as mean cell volume differs between two populations. The unpaired t-test was used as we were comparing 2 different populations (rather than the same population under different conditions). If the data-sets failed the normality test then they were instead compared using Wilcoxon-Rank testing. Where the means of more than 2 sets were compared ANOVA (analysis of variance) was applied, again this is only applicable if the samples are normally distributed, where the data set failed the normality test then Kruskal-Wallis Testing was used in its place. These 2 tests are reported as significant if a difference is detected between any of the analysed groups. For example these tests reported that there was a significant difference in MCV according to TGN level, but on further analysis it was seen that the significant difference was only when those patients that were completely non-adherent were compared with those who were over-dosed on thiopurines.

Statistical analysis was performed using InStat version 3.0a for Macintosh, (Graphpad Software, San Diego, CA, USA www.graphpad.com). Haplotype analysis was performed using UNPHASED³²⁷. This analysis was performed by Dr Cathryn Lewis in the Genetics group at King's College London.

Chapter Three: The role of polymorphism in XDH, MOCOS and AOX1 as pharmacogenetic markers of clinical response to azathioprine treatment in inflammatory bowel disease

3.1 Introduction

According to the accepted model, three metabolic pathways compete to metabolise MP. Firstly the purine salvage pathway, beginning with hypoxanthine-guanine phosphoribosyltransferase, (HGPRT) which produces the desired end-product of thiopurine metabolism, thioguanine nucleotides, (TGNs) and secondly, two enzymes which compete against this process in order to inactivate MP: thiopurine methyltransferase (TPMT) and xanthine oxidase/dehydrogenase (XDH). These pathways compete indirectly, XDH being primarily responsible for first pass metabolism in the gut and liver. TPMT has been the subject of extensive pharmacogenetic study and is known to have pharmacogenetic importance in thiopurine therapy, see chapter 1. However, whilst inter-individual variability in XDH is also thought to contribute to variability in response to thiopurines, little work has been done to elucidate this relationship.

Alongside its key position in the purine salvage pathway, there are additional reasons to suppose that polymorphism of *XDH* could be relevant to clinical outcome on thiopurines. Blocking XDH activity (using the drug allopurinol) causes severe toxicity with standard-dose thiopurines, highlighting the important contribution of XDH to thiopurine catabolism. Also, genetic polymorphism in *XDH* is common, has functional consequences for the efficiency of the enzyme produced(23) and can influence azathioprine metabolite levels(24).

Molybdenum cofactor (MOCO) is a prerequisite for the action of XDH, aldehyde oxidase (AOX) and sulphite oxidase. MOCO deficiency is rapidly fatal due to the loss of sulphite oxidase activity(31), however, MOCO is further adapted (from the essential form required by sulphite oxidase) for use with XDH and AOX, by the enzyme molybdenum cofactor sulfurase (MOCOS). MOCOS deficiency (which has no effect on sulphite oxidase but creates deficiency of both XDH and AOX), is relatively benign, being associated only with a minor predisposition to the

development of renal calculi(32). The *MOCOS* gene is also subject to genetic polymorphism with the potential to affect its activity. Problems with the production of MOCO would have a knock-on effect on XDH and AOX activity and therefore also have the potential to influence the efficiency of AZA metabolism.

AOX is rarely mentioned in discussion of thiopurine metabolism but it has been shown to act on AZA, MP and their metabolites, contributing to thiopurine catabolism(18;34;37), (figure 1.2). The metabolites AOX produces have been the subject of very little study and their importance is not known. AOX occurs as a single isoform in humans, is widely distributed and has a broad range of substrates(33-35) suggesting a more extensive role than its known contribution to purine catabolism(36). Inter-individual variability in AOX activity has been documented(38;39), but the role of genetic polymorphism in this variability has not yet been established.

The work presented in this chapter seeks links between clinical outcome and genetic polymorphism in *XDH*, *MOCOS* and *AOX* in a well-defined prospective cohort of patients receiving AZA for IBD. It also includes the results of sequencing the exomic sequence of AOX in a subset of patients, to confirm the validity of the PCR method and seek linked polymorphism.

3.2 Methods

Samples originated from a prospective study of the pharmacogenetics of AZA in the treatment of IBD⁸⁷. 208 patients were included in the analysis of the original study, but only 192 were included in these genotyping studies, due to availability of adequate DNA for analysis. Patients all received 2mg/kg of AZA. Complete TPMT deficiency was an exclusion criterion, however none of these patients were encountered. Response was defined according to each patient's stated treatment goal. This was most commonly steroid withdrawal, which was defined as withdrawal of steroid treatment by 3 months and maintenance, steroid-free, for a further 3 months. If AZA was started for maintenance of remission, then remission had to be maintained for at least 6 months for treatment to be considered successful. Remission of active disease was defined by Harvey-Bradshaw index or Truelove and Witts criteria. Any patient that required escalation of treatment, either to surgery, biologic or alternative immunomodulator, was

considered to have experienced treatment failure. Adverse drug reactions were included only if they resulted in cessation of AZA treatment.

Known coding region SNPs in *XDH*, *MOCOS* and *AOXI* with a Caucasian minor allele frequency of at least 0.02 were selected for genotyping. SNPs that encoded a non-conservative change in amino-acid were preferred. For details of the SNPs selected see Methods. Patients were genotyped by real-time PCR using a Biorad MiniOpticon (Biorad, Hemel Hempstead, UK). For details of the PCR protocol see Methods.

A subgroup of patients was selected from the cohort described above for more complete assessment of polymorphism in *AOX* by sequencing of the entire exomic sequence of this gene. Five patients heterozygous for the SNP *AOX* 3404A>G by real-time testing and five who were wild-type for this gene were selected. All were non-responders to azathioprine despite therapeutic TGN levels and were wild-type for *TPMT**3C and *3A and *ITPA* c.94C>A variants. Details of the methods used for this sequencing are detailed above in the methods chapter 2.4.

3.2.1 Statistical Analysis

Details of the statistical analysis can be seen in the methods chapter above, section 2.8.

3.3 Results

192 patients were included in this pharmacogenetic analysis. The mean age was 39 years, (range 16-84) and 80 (42%) were male, 178 (93%) were Caucasian. 105 (55%) had Crohn's disease (CD), 86 (45%) ulcerative colitis (UC) and 1 had IBD-unclassified. There was a high rate of withdrawal due to ADRs: 77 of 192 (40%) patients. However, in those completing 6 months of treatment per protocol, clinical response rate (defined by indication, see above) was 71/115 (62%).

SNP genotyping was successful in greater than 99% of cases. All genotypes were in Hardy-Weinberg equilibrium and the allele frequencies measured in our cohort were similar to those reported in SNP databases, see table 3.1. SNPs *MOCOS* c.509T>C, c.1072A>G and c.359G>A

were linked. SNPs c.509T>C and c.1072A>G in absolute linkage disequilibrium ($r^2=1$, HapMap data), with both minor alleles occurring together, the linkage to SNP c.359G>A had an r^2 of 0.89 (HapMap data). These SNPs were therefore analysed together against outcome.

3.3.1 *XDH* and *MOCOS*

The relationship between genotype and clinical outcome (response and ADRs) is summarised in Table 3.2. The *XDH* 837T variant appeared to protect against the occurrence of ADRs although, whilst statistically significant, the association was weak ($p=0.048$, OR 0.23, 95% CI 0.05-1.05). This appeared to be due to an under-representation of atypical side effects (headache, flu-like symptoms / myalgia, rash, etc.) although this additional analysis just failed to reach statistical significance ($p=0.07$), perhaps due to the rarity of the *XDH* 837T SNP.

A similar protective effect was documented with the *MOCOS* 2107C variant, although again, this just failed to reach significance ($p=0.058$ in a recessive model, $p=0.13$ in a dominant model, OR 0.64, 95%CI 0.36-1.15). Removing those side effects which were already accounted for by *TPMT* polymorphism from the analysis strengthened the association between SNP *MOCOS* 2107A>C and protection against side effects, but this still just failed to reach significance ($p=0.078$ under a dominant model).

Patients with the haplotype of both protective variants (*MOCOS* c.2107A>C and *XDH* c.837C>T) did not experience ADRs [$n=7$, $p=0.019$ (Chi-square for trend)].

Restriction of the analysis to the Caucasian population had a minor impact on the significance of these associations (*XDH* 837T variant and ADRs $p=0.08$, OR 0.26, 95%CI 0.06-1.22, *MOCOS* 2107C variant and ADRs $p=0.056$ in recessive model, OR 0.26, 95%CI 0.36-1.20). Using haplotypes across all markers genotyped in *MOCOS* and *XDH* did not increase the signal strength ($p= 0.23$ and 0.24 , respectively).

There was no relationship seen between *XDH* or *MOCOS* genotype and successful outcomes on thiopurine therapy.

Table 3.1: Gene frequencies in our cohort compared with reported frequencies in SNP databases (<http://www.ncbi.nlm.nih.gov/SNP>)

SNP	Expected frequency	Documented frequency
XDH 2211C>T	0.31	0.25
XDH 3030C>T	0.23	0.23
XDH 837C>T	0.02	0.04
XDH 3717G>A	0.06	0.06
XDH 2107A>G	0.05	0.08
XDH 1936A>G	0.05	0.08
MOCOS 2107A>C	0.34	0.29
MOCOS 509T>C	0.03	0.06
MOCOS 1072A>G	0.03	0.06
MOCOS 2600T>G	0.10	0.10
MOCOS 359G>A	0.03	0.05
AOX 3404A>G	0.16	0.12

3.3.2 AOX1

The *AOX1* 3404G variant was associated with a lack of clinical response to AZA ($p=0.035$, OR 2.54, 95%CI 1.06-6.13, unchanged by excluding non-Caucasians $p=0.036$). The strength of this association was increased by excluding patients who were non-adherent to thiopurine treatment according to TGN level [$p=0.012$, (OR 6.94, 95%CI 1.58-30.43)]. TGN levels and TPMT activity were similar in those with and without the SNP ($p=0.46$, and $p=0.44$ respectively) and could not therefore account for this difference.

The *AOX1* 3404G variant did not predict the occurrence of ADRs.

3.3.3 Sequencing of AOX

The commercial real-time SNP assay was 100% accurate for the presence or absence of the SNP *AOX* c.3404A>G. No additional or linked polymorphism was detected in the exons of *AOX1*.

Table 3.2: The association between the selected SNPs in *XDH*, *MOCOS* & *AOX* and treatment outcome.

Responders are those defined as having a complete response (CR-therapeutic target reached with no steroid therapy, NR – therapeutic target not reached or alternative drug / surgery required to achieve it) and adverse drug reactions (ADRs) must have caused therapy to be discontinued. Statistics have been performed using the chi-squared test with a dominant model, to look for clinically relevant associations.

SNP	CR with SNP (%)	NR with SNP (%)	p-value	OR	95%CI	Tolerant with SNP (%)	ADRs with SNP (%)	p-value	OR	95%CI
<i>XDH</i> c.837C>T	7/71 (10)	5/44 (11)	0.79	1.17	0.35-3.95	12/115 (10)	2/77 (3)	0.048	0.23	0.05-1.05
<i>XDH</i> c.1936A>G	6/71 (8)	4/44 (9)	1.0	1.08	0.29-4.08	10/115 (9)	6/77 (8)	0.82	0.89	0.31-2.55
<i>XDH</i> c.2107A>G	6/71 (8)	2/44 (5)	0.71	0.52	0.10-2.68	8/115 (7)	5/77 (6)	0.89	0.93	0.29-2.95
<i>XDH</i> c.2211C>T	38/71 (53)	21/44 (48)	0.54	0.79	0.37-1.68	59/115 (51)	33/77 (43)	0.29	0.73	0.41-1.31
<i>XDH</i> c.3030C>T	25/71 (35)	17/44 (39)	0.71	1.16	0.53-2.52	42/115 (37)	33/77 (43)	0.38	1.3	0.72-2.35
<i>XDH</i> c.3717G>A	7/71 (10)	4/44 (9)	1.0	0.91	0.25-3.32	11/115 (10)	11/77 (14)	0.31	1.58	0.65-3.84
<i>MOCOS</i> c.509T>C, 1072A>G & 359G>A	13/71 (18)	6/44 (14)	0.43	0.7	0.25-2.01	19/115 (17)	7/77 (9)	0.14	0.51	0.20-1.27
<i>MOCOS</i> c.2107A>C	38/71 (54)	24/44 (55)	0.92	1.04	0.49-2.22	62/115 (54)	33/77 (43)	0.13 (0.058 recessive)	0.64	0.36-1.15
<i>MOCOS</i> c.2600T>G	12/71 (17)	10/44 (23)	0.43	1.45	0.57-3.70	22/115 (19)	18/77 (23)	0.47	1.29	0.64-2.61
<i>AOXI</i> c.3404A>G	12/71 (17)	15/44 (34)	0.035	2.54	1.06-6.13	27/115 (23)	13/77 (17)	0.26	0.66	0.32-1.38

3.4 Discussion

This work demonstrated that there was an association between the polymorphism *AOX 3404 A>G* and non-response to thiopurine therapy. There were also weak associations between polymorphism in *XDH* (*XDH 837C>T*) and *MOCOS* (*MOCOS 2107A>C*) and a reduced occurrence of side effects during thiopurine therapy.

The prediction and avoidance of thiopurine toxicity by detection of genetic polymorphism in TPMT has become a classic example of the usefulness of pharmacogenetics and is one of the few to be widely adopted in clinical practice⁸⁵. Polymorphism in *ITPA* also appears to account for a proportion of thiopurine toxicity^{87,151,168,329,330}, although unlike TPMT, there is no clear strategy for circumventing this, hence ITPase measurement has not translated to clinical practice in most

centres. Likewise, no other putative markers proposed in the literature have been translated into clinical markers and the majority of thiopurine non-response and toxicity remains unexplained.

If the *AOXI* 3404G variant adversely affects the activity of AOX, then the association between this SNP and lack of clinical response would suggest that AOX produces clinically active metabolites. 8-hydroxy-MP has been shown to be unable to slow the growth of rat sarcoma³³¹, but no functional data is available on any of the other AZA metabolites produced by AOX and neither has any functional work been done on immunomodulation in IBD. Alternatively, the *AOXI* 3404G variant could increase the activity of AOX, perhaps inactivating a high enough proportion of the ingested thiopurine to restrict TGN production. Against this, TGNs were normal in those with the *AOXI* 3404G variant in our cohort, but numbers in this subgroup were small and the result could have been confounded by variability in TGN measurements. Higher AOX activity might also increase breakdown of TGNs to thiouric acid.

Another mechanism by which reduced AOX activity could improve outcome on thiopurines relates to the order in which the available sites on the MP molecule are oxidised by AOX and XDH. XDH preferentially oxidises MP at the 8-position, then at the 2-position to form thio-uric acid³³². However during normal purine catabolism, hypoxanthine is initially oxidised at the 2-position, by XDH and AOX, to form xanthine, then at the 8 position to form uric acid. If AOX oxidises some MP at the 2-position first, this would form thioxanthine, a potent inhibitor of TPMT^{191,333} and a metabolite known to be produced in increased quantities when XDH activity is blocked^{334,335}. Thus patients with higher AOX activity would produce more TGNs and fewer methylated metabolites and have an improved response on thiopurines, whilst a patient that was partially deficient in AOX would produce less thioxanthine and therefore more methylated metabolites at the expense of TGN, a metabolite pattern which is known to be associated with an increased risk of non-response. The only study which has attempted to reproduce this effect of AOX could not demonstrate thioxanthine production¹⁷⁶, but this result conflicts with the excess of thioxanthine found in conditions of XDH inhibition in other studies (Blaker P, personal communication) and more work is required in this area. Thiouric acid, which is created from thioxanthine and is also hydroxylated at the 2 position, is also a TPMT inhibitor¹⁹¹. It is also

possible therefore, that reduced AOX activity could result in reduced thioric acid levels, increasing TPMT activity, leading to non-response.

Sequencing demonstrated that the commercial SNP assays used for the preceding chapters is an accurate way of establishing the presence of genetic polymorphism, specifically the *AOX* c.3404A>G SNP which was the most important finding in our analysis. At the time this work was performed, the SNP *AOX* c.3404A>G was the only coding SNP described in *AOX* and it remains the SNP with the highest frequency in the Caucasian population. The SNP rs3731722, His1297Arg, c.3890A>G, has been described since this work was completed. With a frequency of 0.0833 in the Caucasian population, this would be of interest for future pharmacogenetic work. The other mis-sense SNPs reported to date occur too infrequently to study in a cohort of our size, but might be amenable to study in larger cohorts, in particular, SNP rs143935618 Ile598Asn, c.1793T>A which occurs at a frequency of 0.0128 in the Caucasian population.

Although the findings in this study are based on data and genetic material collected from a large and carefully documented prospective cohort, the association between the described *XDH* and *MOCOS* SNPs and protection against ADRs during AZA treatment must be interpreted with caution, due to the borderline significance of the findings, and confirmation should be sought in other cohorts. This is particularly true as *XDH* c.837C>T does not encode an amino acid substitution. It is possible that the association is real, but that this specific SNP is not causal, but rather in linkage disequilibrium (LD) with another more important polymorphism. Within a 200 kbp region spanning from 50kbp upstream of the 5'UTR to 69kbp downstream of the 3'UTR for *XDH* HapMap contains 2 other SNPs (rs17011353 and rs17011359) in LD with SNP *XDH* c.837C>T with an $r^2 > 0.5$. Both of these SNPs are intronic but could be part of a regulatory or splicing element as seen with *ITPA*¹⁴⁹. For SNPs with a lower allele frequency the study may have been under-powered and numbers were not sufficient to define which side effects these variants are protective against. Additionally, as the majority of subjects were Caucasian, the findings are of uncertain significance for individuals from other ethnic groups.

It was anticipated that *XDH* and *MOCOS* SNPs would, by reducing inactivation of AZA to 6-TU, increase TGN production and therefore improve response or cause dose-dependent ADRs such as

myelotoxicity. The association with a reduction in ADRs however, suggests that thiopurine metabolites produced by XDH are toxic. A lot of work has been done in other contexts on the damaging effects of oxygen free radicals produced by XDH¹⁷⁷, an effect confirmed for thiopurine metabolites *in vitro*³³⁶ and supported by the successful use of allopurinol co-prescription alongside low dose AZA to circumvent hepatotoxicity in some patients⁹⁵.

In conclusion, the study in this chapter documents the discovery of a novel pharmacogenetic marker of non-response to AZA, the SNP *AOXI* 3404G. Polymorphism in *XDH* and *MOCOS* has also been demonstrated to be associated with a weak protective effect against ADRs. Whilst all these findings require replication in other cohorts, as more pharmacogenetic loci which affect success of thiopurine therapy are identified, it may be that, rather than checking a single locus (TPMT) before commencing thiopurine treatment, a panel of relevant enzymes or genes could be assessed, aiding drug selection and dosing, and better informing our monitoring and patient advice, see chapter 5.

Chapter Four: A study of polymorphism in MDR4 and MDR5 and clinical outcome on azathioprine in patients with inflammatory bowel disease

4.1 Introduction

The MDR transporters are trans-membrane pumps which export xenobiotics from cells. MDR1 has been the subject of extensive clinical work, analysing its relevance to outcomes on a variety of drugs, particularly in cancer treatment. However two very closely related pumps, MDR3 and MDR5 (encoded by the genes *ABCB4* & *ABCB5*) have been the subject of very little research and their relevance to clinical outcomes is not known.

4.2 Aims:

To establish whether common coding SNPs in *ABCB4* and *ABCB5* affect treatment outcome on thiopurines in IBD.

4.3 Methods:

Samples originated from a prospective study of the pharmacogenetics of AZA in the treatment of IBD⁸⁷. 208 patients were included in the analysis of the original study, but only 192 were included in these genotyping studies, due to availability of adequate DNA for analysis. Patients were all adults, gave written informed consent, had IBD diagnosed by standard criteria and received 2mg/kg of AZA. Complete TPMT deficiency was an exclusion criterion, however none of these patients were encountered. Ethical approval was obtained for both the original study (Guy's Hospital Research Ethics Committee, MREC 00/1/33) and the additional pharmacogenetic work (Bexley and Greenwich LREC, 06/Q0707/84).

Response was defined according to each patient's stated treatment goal. This was most commonly steroid withdrawal, which was defined as withdrawal of steroid treatment by 3 months and maintenance, steroid-free, for a further 3 months. If AZA was started for maintenance of remission, then remission had to be maintained for at least 6 months for treatment to be considered successful. Remission of active disease was defined by Harvey-Bradshaw index or Truelove and Witts criteria. Any patient that required escalation of treatment, either to surgery,

biologic or alternative immunomodulator was considered to have experienced treatment failure. Adverse drug reactions were included only if they resulted in cessation of AZA treatment.

In *ABCB4* there was one reported common non-synonymous coding SNP and 2 common synonymous SNPs with proposed functionality in other contexts³²⁶ which we selected for analysis. In *ABCB5* modelling has proposed 4 key polymorphism sites for drug resistance²⁵⁸, of these only 3 are common in the Caucasian population, and they were all selected for analysis. Detailed information on the selected SNPs is shown in the methods chapter.

DNA extraction had already been performed as part of the original study⁸⁷. However, additional DNA extraction was necessary from some patient samples to obtain sufficient DNA for study, see methods chapter above for further detail on DNA extraction methods. Patients were genotyped by real-time PCR, SNP probe details are shown in Table 3.1. More detailed methods, sample traces and statistical methods are included in the methods chapter above.

Table 4.1: The results of testing ABCB4& ABCB5 SNPs against treatment outcome on thiopurines.

Data for ADRs is not shown. * denotes significant associations

SNP	CR with SNP (%)	NR with SNP (%)	P-value	OR	95% CI
<i>ABCB4</i> <i>c.1954A>G</i>	10/76 (13)	7/45 (16)	0.79	1.22	0.42-3.5
<i>ABCB4</i> <i>c.711A>T</i>	26/76 (34)	13/43 (30)	0.69	0.83	0.37-1.86
<i>ABCB4</i> <i>c.175C>T</i>	21/76 (28)	13/43 (30)	0.83	1.11	0.49-2.54
<i>ABCB5</i> <i>c.1573G>A</i>	24/76 (32)	15/44 (34)	1	1.08	0.49-2.38
<i>ABCB5</i> <i>c.2T>C</i>	21/76 (28)	18/45 (40)	0.17	1.75	0.8-3.81
<i>ABCB5</i> <i>c.343A>G</i>	29/76 (38)	27/45 (60)	0.02*	2.43	1.14-5.17

4.4 Results:

The variants genotyped and their association with clinical response or adverse events are shown in Table 4.1. SNP *ABCB5* c.343A>G was associated with a lack of clinical response to thiopurine treatment (p=0.02, OR 2.43, 95%CI 1.14-5.17) and was associated with lower TGNs, mean difference = -40 pmol/8x10⁸ RBC, 95% CI: -75.7 to -3.3, p=0.033 (unpaired Student's t-test, with Welch correction for unequal variance).

No other SNP had a significant effect on clinical response. No SNP had a statistically significant association with ADRs, although the same SNP *ABCB5* c.343A>G showed a trend towards association with adverse events, (p=0.08, OR 1.7, 95%CI 0.96-3.03).

4.5 Discussion

The results of this study demonstrate that the SNP *ABCB5* c.343A>G is associated with lower TGN concentrations and associated lack of response to thiopurine therapy.

The role of the ABC transporters in drug-resistance is complex. There are many proposed mechanisms. Their hydrophilic pore joined to the ATP-dependent pump (which can extrude compounds against a concentration gradient) combine to create conditions for rapid drug efflux³³⁷. However, these pumps are also believed to possess floppase activity³³⁸ which prevents drug entry and to create a membrane potential difference which affects drug transport. ABC transporters have also been shown to have a role in cytokine release³³⁹ and the prevention of cell differentiation and apoptosis³³⁹.

There are no functional studies on *ABCB4* and *ABCB5* in this context and the precise mechanism by which polymorphism in *ABCB5* would confer drug resistance is not clear. The association with low TGN suggests that it is caused by efflux of AZA/MP and/or their metabolites from cells. In precisely which compartment this effect is occurring is also unclear. It could be a white cell phenomenon or due to extrusion from hepatic cells or total body excretion in the gut or kidney.

Recent work has suggested that the transporters responsible for the transport of thiopurines are actually in the ABCC subfamily of transporters³⁴⁰. However, ABCB transporters are responsible for the efflux of a wide variety of compounds and relevant metabolites could still be substrates for these ABCB transporters.

Despite the strength of using a large prospectively recruited cohort for this work, as with all newly identified pharmacogenetic associations, this warrants confirmation in other cohorts and a chance association cannot be excluded.

If these associations are confirmed, it is possible that the *ABCB5* c.343 genotype could be used alongside other markers of non-response to stratify an individual's likelihood of responding to thiopurine medication. As discussed for AOX in the preceding chapter, the marker could be used as part of a pharmacogenetic index seeking to predict more accurately an individual's chance of obtaining clinical remission on a thiopurine (see chapter 5). This information would be clinically useful, allowing physicians to make personalised drug prescriptions and prompting timely review of the efficacy of thiopurine therapy with the ultimate goal of improved clinical outcomes.

Chapter Five: A pharmacogenetic index using novel markers to stratify patients' risk of non-response to single agent thiopurine drugs

5.1 Introduction

Pharmacogenetics is the study of the impact of variation in candidate genes, most commonly drug metabolising enzymes, drug transporters and receptors, on clinical outcome during drug therapy. In pharmacogenomics by contrast, the whole genome is mined for association with outcome on drug therapy, with no attempt to target genes with predicted relevance to clinical outcome on therapy.

Both disciplines aim to improve treatment outcomes by facilitating personalised drug prescription based on an individual's genetic profile. Treatments could be modified to improve clinical responses or to minimise drug toxicity. This is particularly relevant where drugs have a narrow therapeutic window between potentially serious toxicity and the impact of non-response.

All three of these considerations are relevant to the use of thiopurines in IBD. The use of pre-treatment TPMT testing can prevent serious toxicity, assist tailored dosing and identify a group of patients for whom there is an increased risk of non-response and hepatotoxicity due to predominant methylation. However, this still leaves the majority of cases of non-response to thiopurines and the majority of toxicities experienced unexplained. The identification of other markers, which could be used alongside TPMT to improve on clinical outcome would be a major breakthrough in the use of thiopurine therapies.

The work described in this thesis and work of my colleagues Dr Azhar Ansari and Dr Bijay Baburajan has identified potential markers of non-response to thiopurine therapy. In this chapter I have collated all this data to present a pharmacogenetic index, using the available markers of non-response to stratify a patient's chance of response to thiopurine therapy before treatment begins.

In addition to the markers considered in the preceding chapters, this analysis incorporates a 14 base-pair insertion deletion polymorphism in the gene coding for soluble HLA-G. This polymorphism has already been associated with response to methotrexate in the context of rheumatological disorders²⁸³ and was being investigated for its impact on response to both methotrexate and azathioprine in IBD. HLA-G is a non-classical HLA protein, which has much less genetic variability than other HLA subtypes and exists at immunologically privileged sites, such as in the placenta, cornea, thymus^{284,285}. It inhibits both adaptive and innate immunity by inhibition of natural killer cells²⁸⁶, lymphocytes²⁸⁷ and dendritic cells²⁸⁸. HLA-G has been found to be up-regulated in a variety of pathological states such as cancer²⁸⁹ and expression appears to be enhanced by some viruses as a strategy for evasion of host immunity²⁸⁸. Induction of HLA-G is considered to be an important part of successful tolerance of transplanted organs²⁹⁰.

5.2 Methods

High TPMT activity is known to be associated with non-response to thiopurine therapy and this was confirmed in the prospective cohort described in the above chapters⁸⁷. In addition to this, the work described in chapters 3 & 4 above identified 2 new putative markers of non-response to thiopurines.

Work done by a colleague (Dr Bijay Baburajan) as part of his PhD thesis, identified a fourth marker of non-response to thiopurines, the presence of a 14bp insertion/deletion in HLA-G exon 8 3'untranslated region.

Since all this work had been done in the same prospectively recruited cohort of patients starting therapy with azathioprine for their IBD, it was possible to collate all this information in order to establish whether, used in combination knowledge of a panel of markers of non-response allowed more accurate risk stratification than measurement of TPMT alone.

The methods for genotyping are described above in the Methods chapter and the relevant results chapters (3 & 4).

Details of the statistical analysis can be seen in the methods chapter above, section 2.8.

5.3 Results

In patients with both genotype *AOXI* 3404G and TPMT activity >35pmol/h/mgHb only 33% (4/12) responded to treatment, compared to 44% (24/55) for those with either one and 86% (42/49) for those with two favourable markers ($p < 0.0001$, Chi-square for trend, unaffected by excluding non-Caucasians). SNP *ABCB5* c.343A>G was associated with a lack of clinical response to thiopurine treatment ($p = 0.02$, OR 2.43, 95%CI 1.14-5.17) and was associated with lower TGNs, mean difference = -40 pmol/8x10⁸ RBC, 95% CI: -75.7 to -3.3, $p = 0.033$ (unpaired Student's t-test, with Welch correction for unequal variance). When this marker is incorporated in the analysis then a further cumulative effect is demonstrated, with non-response being strongly associated with number of adverse predictors, $p < 0.0001$ (Chi-square for trend).

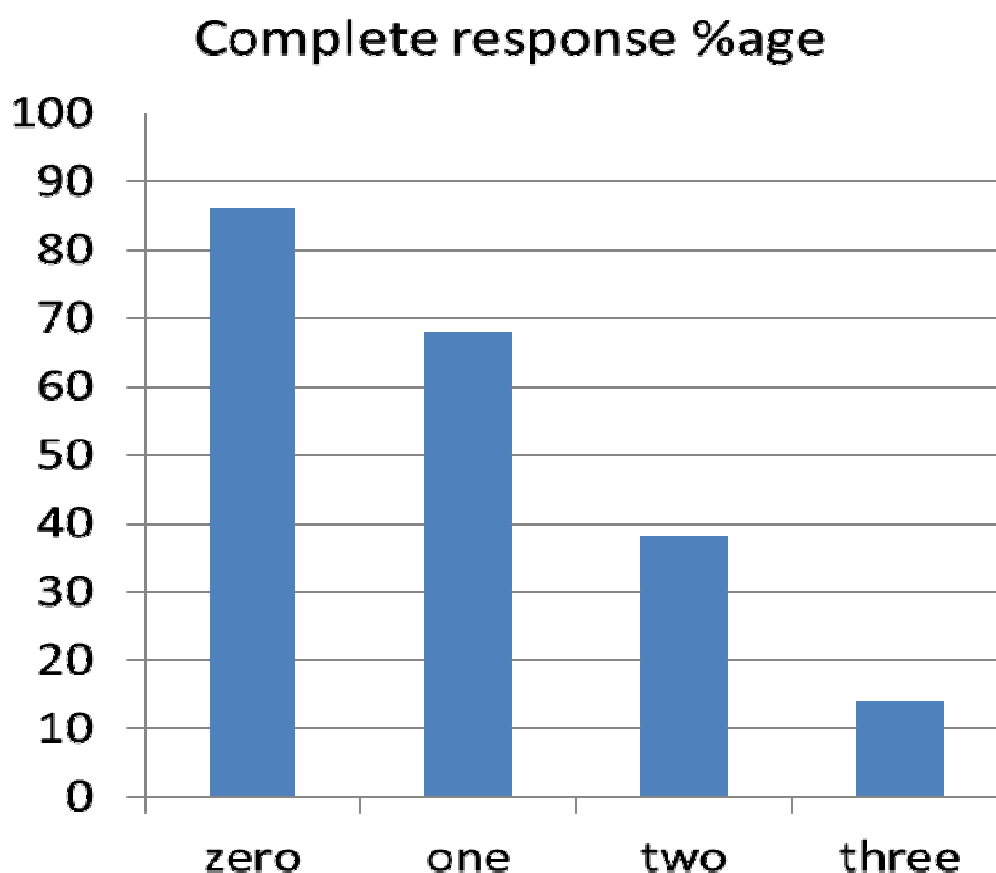


Figure 5.1 The cumulative influence of the three known pharmacogenetic markers of non-response on percentage complete clinical response to AZA.

Three markers: TPMT >35 pmol/h/mgHb, AOX c.3404A>G & ABCB5 c.343A>G. Patients classified according to the number of these markers which they carry.

When the results of the sHLA-G polymorphism is included in the place of ABCB-5, then a similar pattern is seen. See figure 5.2 Interestingly, adding both ABCB-5 and sHLA-G does not add further discrimination with those having 3 and 4 markers of non-response faring just as poorly, see figure 5.3

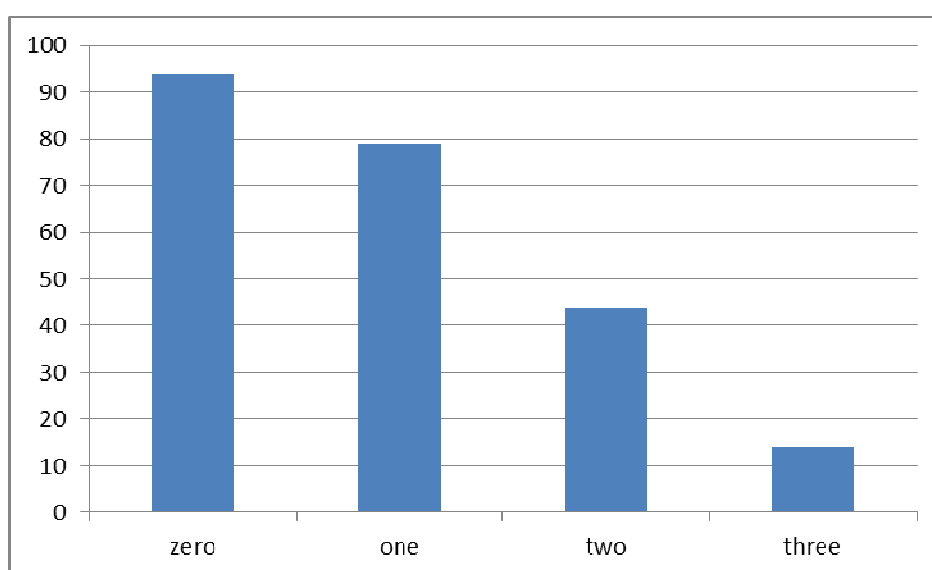


Figure 5.1 The accumulative influence of the three known pharmacogenetic markers of non-response on percentage complete clinical response to AZA.

Percentage response plotted against the number of markers of adverse outcome each patient has. Markers included are: TPMT >35 pmol/h/mgHb, AOX c.3404A>G & presence of the insertion allele for sHLA-G .

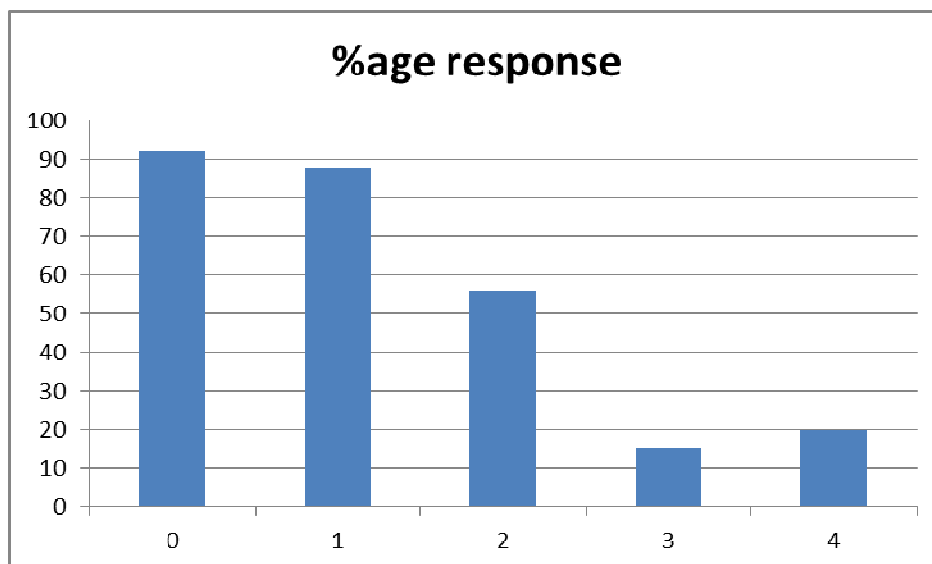


Figure 5.3 The accumulative influence of all four known pharmacogenetic markers of non-response on percentage complete clinical response to AZA.

Percentage response plotted against the number of markers of adverse outcome each patient has. Markers included are: TPMT >35 pmol/h/mgHb, AOX1 c.3404A>G, ABCB5 c.343A>G & presence of the insertion allele for sHLA-G

5.4 Discussion

This study demonstrates that certain novel non-TPMT pharmacogenetic markers add to the predictive value provided by TPMT testing in terms of clinical response on thiopurine therapy. The markers assessed were: coding SNP *AOX1* c.3404A>G, TPMT activity >35 pmol/h/mgHb, a 14bp insertion/deletion in sHLA-G exon 8 3' untranslated region and ABCB5 c.343A>G.

The chance of each individual responding to AZA treatment could be stratified to low, moderate or high probability according to the number of markers present. Such stratification could be done prior to initiation of thiopurine therapy, in the same way in which TPMT testing is currently already adopted. A low probability of response could indicate a prompt and critical review of treatment efficacy, with more rapid switch to an alternative immunosuppressive agent or even the first line use of reduced-dose azathioprine with allopurinol⁹⁴ or an alternative immunomodulator or biologic agent.

Use of alternative drugs as first line treatment, however, should be approached with caution, as some of these markers are likely to have an impact on the efficacy of a wide range of agents. For example, the sHLA-G insertion-deletion is known to also predict clinical outcome on methotrexate³⁴¹ and AOX breaks down methotrexate, producing an inactive 7-hydroxy metabolite²⁰⁶, raising the possibility that *AOXI* c.3404A>G could also affect response to methotrexate. The impact of ABCB-5 polymorphism on other immunomodulators is completely unknown.

Although the trends demonstrated are significant, the numbers in each group are small, particularly once patients with 3 or 4 markers are analysed. These results should be confirmed in other cohorts before any index would be ready for translation into clinical practice. However, these results would suggest that pharmacogenetic markers do still hold promise for translation into clinical practice and support the idea that personalised medicine is becoming an achievable goal.

Chapter Six: The use of thioguanine nucleotide monitoring to optimise clinical outcomes on thiopurine therapy in patients with inflammatory bowel disease

6.1 Introduction

AZA and MP remain central to the treatment of IBD, but they do not act directly but via the active end-product of their metabolism: thioguanine nucleotides (TGNs). The production of TGNs is competed against by the production of inactive methylated metabolites (MeMP) by the enzyme TPMT. TGN and MeMP levels can be easily measured in red blood cells, and monitoring of circulating TGNs has therefore been proposed as a way of optimising and individualising thiopurine treatment.

By monitoring TGN levels clinicians can detect non-adherence. TGNs also correlate with response to thiopurine treatment²⁹⁷ and disease activity^{305,342}, whilst high levels predict dose-dependent toxicity^{343,344}. This means that a therapeutic range can be established, allowing dosing to be optimised and individualised more effectively and detecting biochemical resistance (predominant methylation)^{295,296,305,343,345,346} which can be circumvented by co-prescription of allopurinol (see chapter 7). A recent paper addressing the utility of measuring TGNs in paediatric practice found that their use altered management in 36% of cases²⁹⁹.

Haematological indices such as mean cell volume (MCV), change in MCV, white blood cell (WBC) and lymphocyte counts are known to be affected by thiopurine therapy. This has led to the suggestion that these markers could be used to monitor treatment without the need for TGNs. However, although these markers correlate with TGN concentrations over large cohorts^{5,307,315-318}, there is considerable inter-individual variability which restricts their application to clinical practice^{294,299,318-320} even when incorporated into algorithms^{324,325}.

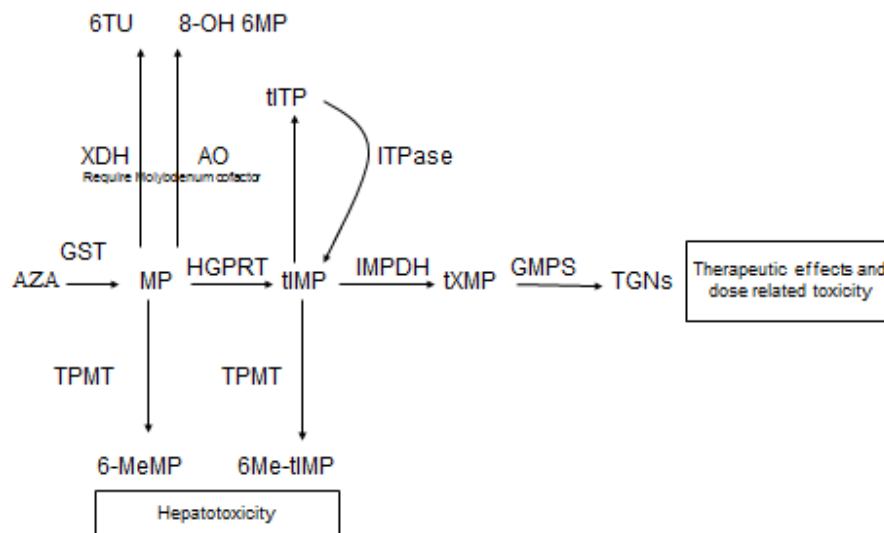


Figure 6.1 The metabolism of azathioprine showing the therapeutic/harmful effects of the 2 measured metabolites

Abbreviations:

Drugs: AZA – azathioprine, MP – mercaptopurine.

Metabolites: tIMP – thioinosine monophosphate, tXMP - thioxanthine monophosphate, TGNs – thioguanine nucleotides, tITP – thioinosine triphosphate,
 6-MeMP – 6-methylmercaptapurine, 6-Me-tIMP – 6-methyl thiopurine monophosphate, 8-OH 6-MP – 8-hydroxy mercaptopurine, 6TU – 6-thiouric acid

Enzymes: TPMT – thiopurine methyltransferase, XDH – xanthine oxidase / dehydrogenase, AO – aldehyde oxidase, ITPase – inosine triphosphatase, IMPDH – inosine monophosphate dehydrogenase, HGPRT – hypoxanthine-guanine phosphoribosyltransferase, GMPS – guanosine monophosphate synthetase.

The American Gastroenterological Association advocate using TGNs to monitor thiopurine use²⁹³ and British paediatric²⁹¹ and European guidelines²⁹² support their use for patients with suboptimal response to thiopurine drugs. However, many centres still manage their thiopurine patients without metabolite monitoring and many clinicians perceive that measuring TGNs would not change their practice or improve patients' outcome.

The aim of this study was to determine the impact of TGN monitoring on treatment decision making and clinical outcome in patients in the specialist IBD clinic at GSTT.

6.2 Methods:

Patients who had TGNs measured to monitor either AZA or MP treatment were identified from clinical records and from the Purine Research Laboratory (PRL) database. Only those patients attending the specialist IBD clinic at Guy's and St Thomas' NHS Foundation Trust were eligible for inclusion. In a proportion of patients, multiple TGN measurements had been taken. Patients receiving t(h)ioguanine or thiopurine/allopurinol co-treatment at the index TGN were excluded. In each case, clinical records and laboratory results were reviewed retrospectively to record data on demographics, type/extent of IBD, indication for treatment, thiopurine dose and toxicity

Clinical response was established from review of the notes and electronic patient records and in addition to the assessment of the treating physician, steroid-free remission rates at 6 months from index TGN were recorded in those patients where the TGN result dictated optimisation of thiopurine therapy (rather than recourse to surgery or biologic).

Any documentation of a change in management based on the TGN level was noted and management decisions divided into those which were appropriate (according to the TGN result) and those which were different from that dictated by the TGN result. Assessment of non-response to thiopurines is a key indication for TGN measurement. Therefore, a separate analysis of the impact of the TGN result on the management of non-responders was made. Since full blood count (FBC) indices have been proposed as surrogate markers of TGN values, these were assessed against TGN results in order to calculate the usefulness of FBC parameters in predicting TGN results using sensitivity/specificity analysis.

TGN and methylated thiopurine metabolite (MeMP) concentrations were measured as the hydrolysed base in whole blood according to the method of Dervieux and Boulieu³²⁸ in the PRL (GSTS Pathology at St Thomas' Hospital, London). For clinical purposes, the PRL uses the therapeutic range 200-400 pmol/8x10⁸RBC for TGNs. More detailed laboratory methods are provided in chapter 2.

Statistical analysis was performed using InStat version 3.0a for Macintosh, (Graphpad Software, San Diego, CA, USA www.graphpad.com). Using Chi-square and Fisher's Exact test, variance in means was tested by ANOVA where results were normally distributed, or alternatively by Kruskal-Wallis.

6.3 Results:

189 patients were identified, several of these had had multiple TGN levels measured and in total 608 TGN results were available for analysis. Patients' ages ranged from 12 to 83 years, (median 38 years), 103 were female, 134 had CD, 50 UC and 5 IBD-unclassified. At their index TGN measurement, 15 patients were on concomitant biologic therapy with either infliximab or adalimumab.

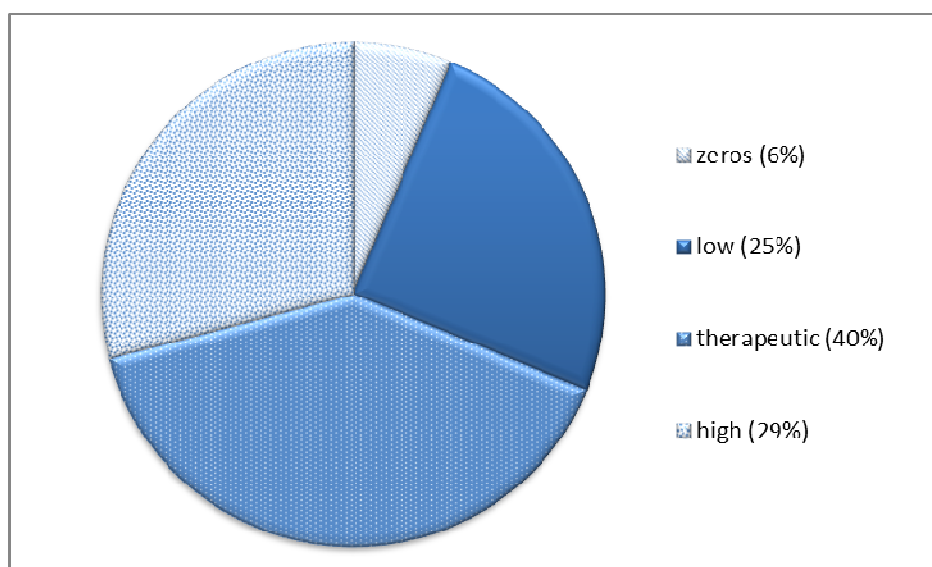


Figure 6.2: Distribution of patients, according to their initial TGN levels relative to our therapeutic range of 200-400pmol/8x10⁸RBC. "Zeros" are patients with no detectable TGNs or methylated metabolites.

Of the 189 patients, only 75 (40%) had TGN levels in the therapeutic range. 12 patients (6%) had zero detectable thiopurine metabolites, indicating non-adherence. 47 (25%) had sub-therapeutic TGNs indicating either under-dosing (n=39) or thiopurine resistance (n=8), and 55 (29%) had high TGN levels suggesting that their dose of thiopurine was too high. An overview of these index TGN results is provided in figure 6.2.

TGN levels predicted response to therapy with response rates varying from 84% in those who had TGNs in the therapeutic range to just 18% in those with no detectable TGNs (indicating non-adherence), see figure 6.3. Remission rate was equivalent in those with TGNs in the range 200-250 pmol/8x10⁸RBC (9/13) and those with TGNs in the range 350-400 pmol/8x10⁸RBC (14/17), confirming that the therapeutic range is appropriate (p=0.4).

6.3.1 Non-adherence

12 (6%) of patients were non-adherent at the time of their index TGN measurement with zero detectable TGN and MeMP levels. One of these patients had been started on a biologic at the same time as the thiopurine so that response to the thiopurine agent could not be accurately established. Of the remaining 11 patients on thiopurine monotherapy, 9 were non-responders. These results allowed non-adherence to be addressed with patients, prompting useful conversations about the importance and safety of their therapy and allowing alternative treatment approaches and a time-frame for thiopurine withdrawal to be discussed. Concerns about being on thiopurines at conception and during pregnancy were also raised. Health care workers are often concerned about approaching patients over issues of non-adherence but, in this study, patients generally admitted that they were not taking their therapy and the interaction prompted by this was a positive one. When all TGN measurements (not just the first on record for each patient) were included, 8% of results were zero, indicating variability in adherence over time. The two commonest reasons for patients to discontinue their thiopurine were: starting infliximab therapy (4/15) and attempting to conceive (4/15).

6.3.2 TGN concentrations below and above the therapeutic range

At the time of the index TGN measurement, 47/189 patients (25%) had sub-therapeutic TGN level, (Table 6.1). This led to 18 dose increases, (9 of which were in patients in complete remission) and 2 patients being switched to combination low dose AZA with allopurinol co-therapy, due to high MeMP levels, indicating preferential methylation / biochemical resistance to thiopurines.

55/189 patients (29%) had TGN concentrations above the upper end of therapeutic range (400 pmol/ 8×10^8 RBC), 2 of whom had extremely high TGNs >1000 pmol/ 8×10^8 RBC (Table 6.1). In these patients, dose reductions were, in practice, triggered by a TGN >550 pmol/ 8×10^8 RBC. None of the 30 patients with TGN in the range 400-550 pmol/ 8×10^8 had a dose reduction. In contrast, dose reductions were made in 14/24 patients with a TGN greater than 550 pmol/ 8×10^8 RBC ($p=0.0001$, Fisher Exact Test). At review, 7/7 patients in remission at the time of dose-reduction remained in remission, one patient continued to be steroid-dependent and the remaining patients had additional changes in their management (e.g. starting biologic) making the effect of thiopurine dose-reduction impossible to analyse in isolation.

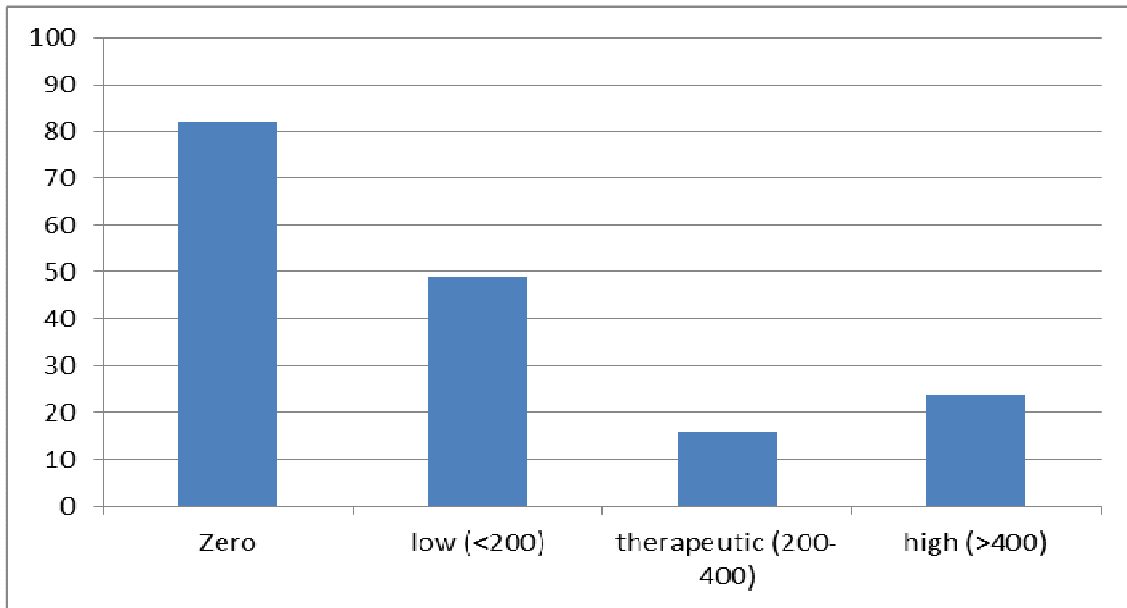


Figure 6.3: Percentage of patients unresponsive to thiopurine therapy in each TGN result classification: zero TGNs (non-adherent), low TGNs (<200pmol/8x10⁸ RBC), therapeutic TGNs (200-400pmol/8x10⁸ RBC) or high TGNs (>400pmol/8x10⁸ RBC).

6.3.3 Non-responders

53/189 patients were non-responders to thiopurine monotherapy at the time of their index TGN, 39 (74%) of these had management decisions prompted by the TGN result. 12 incidences of non-adherence were detected, 13 patients had a TGN-directed dose change and 3 patients were switched to allopurinol combination therapy on the basis of the TGN result. An additional 4 patients had changes made after a follow-up TGN measurement – 2 dose changes and 2 switch to AZA and allopurinol combination treatment. This brings the total with a change of treatment directed by TGN to 43/53 (81%). Most non-responders in whom no change was made were either lost to follow up or had entered remission at the time of their review. If non-responders had simply had their AZA dose increased at the time of index TGN, this would have been appropriate for 11 patients. However, 11 non-responders with existing high TGNs would have been dose-escalated inappropriately, as would 8 patients with predominant methylation, meaning that this strategy would potentially have caused more harm than good. In this cohort, 7/8 non-responders with preferential methylation (by ratio) avoided dose-escalation and its associated complications as a result of TGN monitoring. The one patient where dose-escalation was attempted despite preferential methylation developed hepatotoxicity. 11 non-responders went directly to a change in treatment plan including 6 undergoing surgery for stricturing disease. Blind dose escalation in this group could have delayed effective therapy whilst thiopurine dosing was altered inappropriately and then monitored for 3-4 months to await the effect of the dose change.

Where appropriate response to the TGN result was the only change in treatment, 18/20 (90%) patients had an improved clinical outcome versus 7/21 (33%) where the treatment decision was counter to that indicated by the TGN level ($p < 0.001$, Chi-square). If the treatment decisions directed purely at optimisation of thiopurine therapy (rather than an escalation to surgery or biologic) are considered, then where thiopurines were appropriately optimised, 14/20 patients were in steroid-free remission at 6 months and a further 3 patients were slowly weaning from long-term steroid treatment. This compares to only 3/10 patients achieving steroid-free remission for whom management decisions were contrary to that indicated by the TGN result ($p = 0.037$ or 0.003 if slow steroid-weaners are included as treatment successes).

6.3.4 Methylated metabolites

At the index TGN measurement, 21/177 (12%) adherent patients demonstrated preferential thiopurine methylation by ratio (MeMP/TGN >11). 5/177 had a MeMP greater than 5700 pmol/ 8×10^8 RBC (4 of these 5 also had a MeMP/TGN ratio >11).

Predominant methylation, as defined by MeMP/TGN ratio >11, was associated with TGN concentrations below the target range ($p < 0.001$, Chi-square) but not with clinical response to thiopurines ($p = 0.47$). Abnormal liver function tests were documented in 5/21 (24%) of patients with predominant methylation by ratio and 1/5 (20%) with high absolute MeMP levels (> 5700 pmol/ 8×10^8 RBC).

2 patients could not have the clinical response to thiopurine determined in isolation from other treatments. Amongst non-responders, 2/8 were switched to combination treatment with allopurinol (one after an inappropriate dose-increment caused abnormal liver function tests), 3 patients went rapidly to surgery and 3 continued on thiopurine monotherapy. In predominant methylators who had a good clinical response to their thiopurine at the time of their index TGN measurement, no action was taken in 6/11 cases. However, in the other 5 cases, 1 patient had their treatment stopped, 2 had dose adjustments and 2 were switched to low dose thiopurine in combination with allopurinol.

6.3.5 Predicting TGN and clinical outcome from full blood count indices

Table 6.1: Index thioguanine nucleotide concentrations and haematological indices in patients receiving azathioprine or mercaptopurine for the treatment of IBD.

TGN concentration pmol/8x10⁸RBC	number (%)	mean MCV fl (range)	mean WBC (range)	mean lymphocyte count (range)
Zero	12 (6)	89* (74-98)	6.7 (4.9-12.4)	1.6** (0.9-2.6)
Low (<200)	47 (25)	93 (69-118)	6.5 (2.7-14.9)	1.3 (0.4-2.9)
Normal (200-400)	75 (40)	94 (74-112)	6.7 (3.5-12.0)	1.3 (0.3-5.8)
High (>400)	55 (29)	96* (78-105)	6.0 (2.4-14.3)	1.1** (0.2-2.1)

Significant differences: *p=0.03, ANOVA, **p=0.02, Kruskal-Wallis

Table 6.2: Summary of all TGN results and full blood count indices.

TGN concentration pmol/8x10⁸RBC	number (%)	mean MCV fl (range)	mean WBC (range)	mean lymphocyte count (range)
Zero	46 (8)	88 (67-117)	9.0 (3.6-14.3)	1.5 (0.5-2.6)
Low (<200)	136 (22)	91 (69-118)	6.7 (2.7-14.9)	1.3 (0.2-5)
Normal (200-400)	260 (43)	94 (69-113)	6.5 (1.5-17.4)	1.2 (0.2-5.8)
High (>400)	166 (27)	97 (78-119)	6.1 (1.7-19.7)	1.1 (0.2-2.4)

A comparison between TGN and full blood count indices is shown in Table 6.1 for the index TGN result and in Table 6.2 for all available TGN results in these patients. Table 6.3 shows the rates of non-response to therapy at the time of the index TGN, divided according to whether the patients were non-adherent by TGN measurement or whether they had sub-therapeutic, therapeutic or high TGN levels. (Response data excludes patients receiving concurrent infliximab/adalimumab or any levels taken on patients that had received less than 4 months of

thiopurine treatment i.e. all patients where response to thiopurines could not be accurately judged).

Table 6.3: The relationship between first TGN concentration taken for each patient and clinical response at that time to azathioprine/mercaptopurine.

TGN concentration pmol/8x10 ⁸ RBC	Non-Responders (%)
Zero	9/11 (82)
low (<200)	22/45 (49)
normal (200-400)	11/67 (16)
high (>400)	11/46 (24)

When MCV and lymphocyte counts were compared across all four groups there was a significant difference between the MCV and lymphocyte count in those with TGN levels above the therapeutic range and those who were non-adherent ($p=0.03$, ANOVA and $p=0.02$, Kruskal-Wallis respectively). There was no significant difference in MCV or lymphocyte count between those with TGN in the target range and any other group. There was no statistically significant difference in total white cell counts across all groups ($p=0.4$, ANOVA). In terms of the raw data, the haematological indices were remarkably similar across all TGN groups (Table 6.2). Using haematological indices with previously published cut off values to predict TGN level revealed that these markers have poor sensitivity and specificity and are not suitable surrogate markers. Using $WBC < 4 \times 10^9$ cells/L to predict therapeutic or high TGN levels gives a specificity of 91.4% but a sensitivity of only 8.7%. Meanwhile, using an MCV cut off of 95fl gives a sensitivity of 53% and specificity of 58%.

Waljee *et al*³²⁴ suggested that the MCV/WBC ratio, using a cut of value of 12, predicts response to thiopurine treatment as accurately as TGN. In the study group presented here, there was a separation in response rate using this published cut off, 22/40 with a ratio less than 12 were non-responders, whilst only 33/124 were non-responders in group with a ratio above 12 ($p=0.001$,

Fisher Exact Test). This ratio predicted therapeutic or high TGN with a sensitivity and specificity of 75% and 25%. Within the current data set, it performed slightly better using a cut off of 20, which improved the sensitivity and specificity to 72% and 79% respectively).

6.4 Discussion

The results of this study show that TGNs are useful in clinical practice, identifying not just non-adherence, but also issues of dosing and preferential methylation. Where patients were unresponsive to thiopurines despite adequate TGN concentration, availability of this information allowed rapid decisions about treatment change.

In non-responders TGN measurement directed therapy, providing vital information regarding the reason for an individual's non-response and thereby facilitating personalised treatment decisions. If TGNs had not been measured and a strategy of blind dose-increment had been employed, this would have been likely to benefit 14/53 (26%) patients where TGN results demonstrated simple under-dosing. However, in 22/53 individuals who already had adequate or even high TGN concentrations, and in 8/53 demonstrating predominant methylation, it would have been potentially harmful, delaying effective treatment decisions whilst waiting another 4 months for an unnecessary dose-change to take effect or even causing toxicity. In the 9 non-adherent individuals, dose-increments would have had no effect and the issues causing non-adherence and therefore unsuccessful treatment would not have been explored. This raises an interesting point regarding patients given a historical label of non-response. Rather than abandoning thiopurines as a treatment option in this group, a retriial could be attempted with TGN measurements at 4 weeks and 4 months to ensure that they were not unresponsive due to issues of adherence, under-dosing or predominant methylation, all of which can be successfully circumvented with the use of TGNs.

In responders, the TGN measurements also provided useful information, detecting non-adherence and dosing issues, including cases with extremely high TGN concentrations, where toxicity was likely to have been avoided by timely dose-reduction.

Methylated metabolites provided additional information and identified the previously described group of patients who are biochemically resistant to thiopurines⁶⁴. Consistent with the literature, this study also demonstrated that individuals with hypermethylation had a high incidence of abnormal liver function tests and lower TGN levels. In reviewing these results it became clear that not all clinicians were responding appropriately to the information from methylated metabolites, resulting in missed and delayed opportunities to optimise therapy. As a result, a virtual clinic has been developed at GSTT in which all TGN and MeMP results are reviewed to ensure they are appropriately acted upon, and a handbook for clinicians produced in the IBD clinic providing practical advice on interpretation of the TGN and MeMP results. An overview of the guidance given on the interpretation of TGN/MeMP results is presented in Table 6.4.

Retrospective collection of clinical outcome data in this cohort introduces the possibility of a bias in the interpretation of results. However, remission rate was not our primary endpoint and we have attempted to make this assessment as objective as possible by introducing an analysis of steroid-free remission rates at 6 months, alongside the more global and subjective measure of the treating physician's assessment.

The only prospective study which addressed the utility of TGN measurement in clinical practice³⁴⁷ failed to show a higher remission rate in the group with TGN monitoring. However, this study had several limitations. Of particular concern was the short study period (16 weeks) and the extensive co-prescription of steroid which made the contribution of the thiopurine difficult to assess (all patients received steroid until 12 weeks, with 56% still on steroids when clinical outcome was measured at 16 weeks). Additionally, it would be difficult for a statistically significant difference in response rate to emerge where the control group received an average of 2.7mg/kg whilst the dose in the treatment group was not permitted to exceed 3mg/kg. This study did show that TGN monitoring could predict toxicity and was able to confirm the existence of a biochemically resistant group in whom dose-escalation did not increase TGN concentrations^{94,330,347}. A recent report of the use of TGNs in paediatric clinical practice supports the fact that measurement aids clinical decision making and improved patient outcomes²⁹⁹.

200-400 pmol/8x10⁸RBC was chosen as the therapeutic range in the PRL. There is a lack of evidence regarding the upper limit of the range although this figure is consistent with that used in other trials³⁴⁷. The lower limit derives from the literature and the PRL experience of TGN monitoring⁸⁷, although 200 pmol/8x10⁸RBC is slightly lower than the cut off used in other studies. We attempted to address this by comparing remission rates between those with a TGN between 200 and 250 pmol/8x10⁸RBC and those between 350 and 400 pmol/8x10⁸RBC and found no significant difference, suggesting that the therapeutic range is appropriate, although the sample is probably under-powered to draw a definitive conclusion. There is little data in the literature which addresses the question of how to respond to moderately high or low TGN concentrations or what should be done with a patient with a good response to treatment but TGNs outside the target range. Long term complications of thiopurine use, particularly the occurrence of malignancy, may relate to high TGN levels³⁴⁸ and since the experience presented here suggests that if TGNs are greater than 550pmol/8x10⁸RBC, dose-reduction can be undertaken without compromising clinical benefit, we would recommend that such patients have their dose of thiopurine adjusted. The effect of dose-reduction with TGN between 400 and 550 pmol/8x10⁸RBC could not be assessed as no dose-reduction was attempted on patients with TGNs within this range. In patients who have a good clinical response to thiopurines despite TGNs below the target range, it is usual practice to cautiously increase the thiopurine dose and repeat levels. However, whilst it is logical to presume that this increases the chances of these patients remaining in remission, there is no evidence base to support this practice.

Those patients with a therapeutic TGN concentration had no significant difference in any blood count parameter, even when compared to those who were non-adherent. Blood count indices predicted TGN with very poor sensitivity and specificity. The WBC/MCV ratio performed slightly better but still failed to reach a sensitivity or specificity which would replace TGN in clinical practice. MCV, WBC, and lymphocyte counts varied substantially within all TGN ranges, some individuals maintaining completely normal full blood counts despite therapeutic or even high TGN levels. Together, these findings suggest that blood count indices are not an adequate replacement for TGN monitoring in clinical practice. An additional advantage of TGN monitoring is that concentrations reach a steady state 4 weeks^{87,300} after therapy is initiated. This allows optimisation of dosing long before clinical response is assessed at 3-4 months, which we

anticipate would shorten the time to achieving remission. Changes in surrogate markers of response such as lymphocyte count and MCV evolve over the same time-frame as clinical response and so would not contribute to achieving a more rapid remission.

TGN measurements cost approximately £30 at the PRL. If restricted to use in non-responders, each TGN result should direct a change in therapy. In reality, 39/53 non-responders had a change in their treatment made on the basis of the TGN result, which represents a cost of £41 per treatment change (£56 if those who would have benefited from blind dose-increments are also excluded). This represents very good value for money. Comparison with the measurement of TPMT activity, which is widely adopted to support the use of thiopurines in IBD, reveals that, although the test costs the same, £300 will be spent on TPMT testing per change in treatment (if the starting dose is titrated down to 50% for heterozygotes) and £9000 if TPMT testing is used only to save a homozygous patient from potentially fatal overdose.

Not using TGN measurements in clinical practice also has costs. Patients may unnecessarily lose one of the few established treatment options for IBD, by not having their reasons for non-response explored. They may experience avoidable toxicity or relapse / develop progressive disease due to suboptimal use of thiopurines. They may also experience avoidable treatment escalation to biologic or surgery. For the hospital there are also the financial implications of a patient developing serious toxicity or requiring treatment escalation to biologics or surgery. If even a small proportion of patients could be salvaged and made to respond to thiopurine monotherapy, this would represent a significant cost saving.

In summary, therefore, in our practice, TGN monitoring guides clinical decision-making and appears to improve clinical outcomes. Checking TGN concentrations in non-responders is cost-effective and in each case should prompt a change in treatment. Testing TGNs in all patients on thiopurines will confirm continuing adherence, identify under- and over-dosing and predominant methylation. Responders with TGN concentrations >550 pmol/ 8×10^8 RBC can safely have their dose of thiopurine reduced. The results of this study support the measurement of TGN and MeMP concentrations in all thiopurine-treated patients with IBD, at 4 weeks after treatment

initiation or following any change in dose, in any cases of non-response to thiopurines and as a periodic check (6-12 monthly) in patients in established remission.

Table 6.4: Use of TGN and MeMP levels in clinical practice

TGN level pmol/8x10⁸RBC	Interpretation	Action required
Zero	non-adherence	Discussion with patient
Low (<200)	MeMP:TGN <11 under-dosed MeMP:TGN ≥11 hypermethylation	Dose increment & recheck TGN in 4 weeks Switch to low dose AZA & Allopurinol (see chapter 7)
Normal (200-400)	adequate dose	In responders no action required. For non-responders – recheck TGN, seek alternative explanation for symptoms, consider switch to alternative therapy e.g. methotrexate
High (>400)	over-dose	In responders consider dose-reduction, particularly if TGN>550. In non-responders, recheck TGN, seek alternative explanation for symptoms, consider switch to alternative therapy e.g. methotrexate

Chapter Seven: Optimising thiopurine outcomes by the co-prescription of allopurinol

7.1 Introduction

Thiopurines remain first line treatment for IBD and have a growing role as concomitant immunomodulation alongside biologic therapy. However, many patients are unable to tolerate conventional thiopurine treatment and a further proportion of eligible patients will not respond to thiopurine therapy. Any strategy which could overcome these problems would, by ensuring the greatest possible number of patients could benefit from thiopurines, be an important addition to our treatment armamentarium.

Allopurinol, an off-licence drug, which is widely used in the treatment of gout, inhibits xanthine oxidase/dehydrogenase (XDH) and therefore alters the metabolism of thiopurines. It was originally developed alongside thiopurines, for exactly this reason, but, when the early studies in leukaemia failed to demonstrate improved outcomes, thiopurine and allopurinol co-prescription was abandoned³⁴⁹. Decades later, combination treatment was trialled in the context of renal transplant immunosuppression where improved outcomes were demonstrated, with improved graft survival³⁵⁰ and optimisation of thiopurine metabolite profiles³⁵¹.

In the IBD literature a subgroup of patients who predominantly methylate thiopurines has been characterised⁶⁴, these patients are at risk of both hepatotoxicity⁹⁵ and non-response during treatment with thiopurines^{94,190}. The discovery that this phenomenon could be circumvented by the co-prescription of allopurinol resulted in a resurgence of interest in this treatment combination³⁵².

Allopurinol blocks the activity of XDH, a major contributor to thiopurine catabolism, responsible for a significant first pass metabolism in the gut and liver, see figure 7.1. Blocking this first pass metabolism increases the proportion of ingested thiopurine that will be converted to TGNs and the dose of administered thiopurine must therefore be reduced to approximately 25% of the usual

dose, in order to avoid dose-related toxicity, especially myelotoxicity¹⁹⁰ (see Table 7.1). The mechanism by which allopurinol also reduces the production of methylated metabolites is the subject of much debate, but new data from our laboratory suggests that it may be a result of higher thioxanthine levels achieved in the presence of allopurinol, inhibiting TPMT activity⁹⁶.

This Chapter documents a new large cohort of patients with IBD prescribed thiopurine / allopurinol co-treatment, and describes indications for co-treatment, treatment outcome and toxicities encountered.

7.2 Aims:

This study aimed to establish the outcome of attempts to overcome contra-indications for / problems with thiopurine monotherapy with a strategy of using azathioprine and allopurinol co-prescription. The rate of success overcoming these contra-indications was the primary outcome measure, however overall remission rates were also to be calculated.

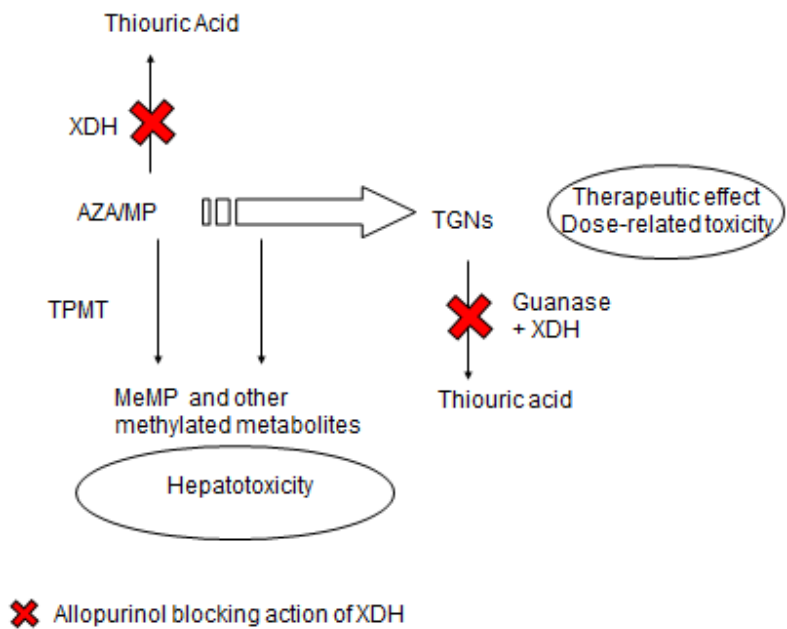


Figure 7.1 The effect of allopurinol on azathioprine metabolism.

7.3 Methods

Combination treatment was considered in patients experiencing hepatotoxicity or other “atypical” side effects on thiopurine monotherapy. It was also used for those who were achieving a sub-optimal response (with a thiopurine metabolite profile suggesting either hyper-methylation or under-dosing which could not be corrected due to intolerance of higher doses). This group included a few patients with a historical label of non-response to thiopurine monotherapy and a TPMT activity >35 pmol/h/mgHb. Patients were identified who had been switched as a result of a ratio of MeMP:TGN >11 , predicting adverse outcome, but not currently experiencing a loss of response or toxicity. Finally, patients with a TPMT greater than 35 pmol/h/mgHb (the activity threshold previously demonstrated to predict non-response⁸⁷) were considered for combination treatment as first line thiopurine therapy.

The protocol for dosing and monitoring of combination treatment is detailed in the methods section above.

The primary outcome measure for this study was whether or not co-prescription with allopurinol could successfully overcome whatever problem had been encountered on thiopurine monotherapy. In some cases this was a failure to achieve clinical remission, but in the majority was drug intolerance, normalisation of LFTs or normalisation of the thiopurine metabolite profile.

In order to confirm that combination therapy was effective in achieving good clinical outcomes in all patients, clinical remission rates for the whole group were also calculated as a secondary endpoint. This was a retrospective analysis of results from clinical practice and therefore formal clinical disease activity measures (such as the Harvey Bradshaw Index) were not generally available. Clinical response was therefore assessed at one year of treatment, on the basis of the treating physician’s global assessment (according to each patient’s indication for treatment). The use of steroids, biologics or surgery was interpreted as an indication of treatment failure. Patients who had been on treatment for long enough to assess outcome (greater than 4 months) but not yet for one complete year were included in a secondary analysis. ADRs must have been significant

enough to require cessation of combination therapy and be considered to be possibly related to combination therapy by the treating physician.

Laboratory methods for determination of TGN/MeMP results are detailed in the methods chapter above. Statistical methods are also detailed in the methods chapter, at section 2.8.

7.4 Results

110 patients being treated with a combination of low dose thiopurine and allopurinol were identified, of which 109 had adequate information available for analysis of clinical outcome on combination therapy. 69 patients had CD, 33 had a diagnosis of UC and 4 patients had IBD-unclassified (IBD-U). 3 patients were treated for orofacial granulomatosis (OFG) and one for eosinophilic colitis. The male:female ratio was 51:59, age range 20-84 years, disease duration 1-34 years. Follow up was for a mean of 16 months (range 0.5-47 months)

There were 4 main indications for co-prescription: predominant methylation found on TGN monitoring (n=27 non-responders, n=8 responders), hepatotoxicity (n=24), other “atypical” side effects (n=28), and primary treatment due to high TPMT (n=23). The majority of patients had failed on single agent thiopurine therapy due to either non-response or ADRs (failure on azathioprine n=69, on mercaptopurine n=8, having tried both as monotherapy n=12) 20 patients had no recorded prior exposure to thiopurine monotherapy. Three patients were initiated on combination therapy on the basis of high TPMT activity, but had medical records of a historical exposure to single agent therapy.

In patients with TPMT activity in the normal range, single agent AZA doses ranged from 0.6-2.5 mg/kg, average 1.9mg/kg, MP doses ranged from 0.37-1.77mg/kg, average 1.03mg/kg, (lower doses due to ADRs restricting dose-escalation). In combination with 100mg of allopurinol, AZA doses ranged from 0.16-1.0mg/kg, average 0.54mg/kg,

7.4.1 Success in overcoming problem with monotherapy

Overall, 64/78 (82%) patients treated with AZA and allopurinol combination therapy successfully overcame the problem preventing successful treatment with single agent thiopurine. This analysis does not include the whole cohort, as patients were excluded if they were given

combination treatment first line, lost to follow up or if clinical response was the outcome measure, but could not be accurately assessed due to concomitant biologic therapy. In one instance, a patient was excluded due to inadequate duration of combination treatment to accurately assess clinical response. If those prescribed combination treatment as their first line therapy (on the basis of high TPMT activity) were included, with steroid-free remission at one year as their outcome measure, then 78/96 (81%) of patients successfully circumvented the problem encountered on thiopurine monotherapy.

7.4.2 Overall Clinical Response Rate

When a more rigorous endpoint of remission at one year of treatment was applied, then the overall clinical response rate was 39/63 (62%). If patients that had not yet completed a full year on combination therapy (but where treatment was for longer than 4 months, allowing an assessment of response) were included in the outcome analysis, then 59/83 (71%) of all patients, where the effect of thiopurine could be studied in isolation, achieved clinical remission.

Lastly, if only those patients that had previously failed on thiopurine monotherapy are included then 39/59 (66%) were in remission at one year.

7.4.3 Thioguanine nucleotide and methylated metabolite levels

During combination treatment TGN levels increased from a median of 213 to 397 pmol/ 8×10^8 RBC, $p < 0.0001$. In one case there appears to have been issues of compliance with combination therapy with zero detectable TGN levels. A few patients did have a drop in TGN after co-prescription, which is likely to reflect a cautious dosing strategy.

MeMP levels dropped from a median of 3559 pmol/ 8×10^8 RBC during thiopurine monotherapy to 115 pmol/ 8×10^8 RBC during combination treatment, $p < 0.0001$. Paired metabolite levels before and after the switch to combination therapy are shown in Figure 7.2 (TGN) and Figure 7.3 (MeMP).

7.4.4 TPMT activity

The median TPMT activity for the whole cohort was higher than the general population at 39.5pmol/h/mgHb. This could be skewed by the deliberate selection of a subgroup due to high pre-treatment TPMT activity. Hence, the analysis was repeated without this group – giving a median TPMT of 37 pmol/h/mgHb, still above the general population median.

7.4.5 Blood counts

Various full blood count indices have been considered to be suitable surrogate markers of TGN concentration. Total white blood cell counts were statistically lower on combination therapy than on single agent thiopurine therapy, although the difference was numerically small [6.4 vs. 6.9 (p=0.02)]. Interestingly, this was not, as might be anticipated, due to a reduction in lymphocyte count, which was unchanged (p=0.27) but instead due to a reduction in neutrophil count [from 4.7 to 4.3, (p=0.02)], which could represent improved disease control or a reduction in steroid dosage.

Similarly, MCV measurements were statistically significantly higher on co-therapy than on single agent thiopurines, but with a small numerical difference (mean of 92.3 vs. 92.6, p<0.0001). The MCV/WBC ratio, also thought to be a suitable surrogate marker of thiopurine responsiveness increased a small but statistically significant amount, from 15.1 to 16.8 (p=0.002).

7.4.6 ADRs during combination therapy

The literature contains no reports of adverse events on combination thiopurine and allopurinol therapy, aside from those caused by thiopurine dosing errors. However, in our series there were a total of thirteen adverse events reported which were serious enough to require an alteration in treatment. Three patients developed mild, self-limiting rashes attributable to allopurinol, but there were no cases of toxic epidermal necrolysis. Two patients developed abnormal liver function tests attributed to the allopurinol. One of these patients was diagnosed with early primary sclerosing cholangitis (PSC) during work up for this but the improvement in his LFTs when returned to single agent thiopurine was considered to indicate that allopurinol was contributory. The other patient had a colectomy for refractory UC and discontinued thiopurine

therapy with subsequent resolution of his LFT abnormalities. One patient continued to take full dose AZA alongside allopurinol, this was detected by TGN monitoring before any change in blood counts occurred and combination therapy was stopped.

In addition, six patients experienced “atypical” side effects commonly encountered on thiopurine monotherapy, predominantly nausea and vomiting. These ADRs all resolved when thiopurines were discontinued. Five of these represented a recurrence of the side effect which had precluded thiopurine monotherapy whilst a final patient discontinued combination treatment as it was considered to be contributing to a deterioration in the control of their chronic obstructive pulmonary disease.

7.4.7 Results according to indication for combination treatment

7.4.7.1 Hepatotoxicity

20/25 patients for whom the indication for combination treatment was hepatotoxicity could tolerate combination therapy with normalisation of their LFTs. One discontinued therapy immediately due to a recurrence of the nausea also experienced on single agent thiopurine and one was lost to follow up. Three patients had an alternative (or possibly additional) explanation for abnormal LFTs (1 had genetic haemochromatosis and 2 were diagnosed with early PSC). These patients initially all continued on combination treatment to minimise the risk of super-added thiopurine-induced hepatotoxicity. However, one of the PSC patients later went back to single agent AZA as they believed the combination therapy coincided with a worsening of their LFT results. They did experience some improvement in his LFTs, suggesting that the allopurinol may indeed have been contributory.

In most published studies of drug related hepatotoxicity, transaminases have to reach twice the upper limit of normal before hepatotoxicity is diagnosed. However, in this clinical experience, 9/21 patients were switched before this endpoint was reached. Nevertheless, alanine aminotransferase (ALT) was significantly reduced by the switch to combination treatment, from a mean of 151 IU/L to 30 IU/L (mean difference 124 IU/L, 95%CI 61 to 187, $p=0.0006$). The same was also true for both gamma glutaryl transferase (GGT) [which dropped from a mean of

163 IU/L to 49 IU/L, (mean difference 133 IU/L, 95%CI 48-217, p=0.005)] and ALP [mean reduced from 134 IU/L to 76 IU/L, (mean difference 50 IU/L, 95%CI 8-93, p=0.02)].

7.4.7.2 Other ADRs

This group contained 28 patients with non-hepatotoxic side effects on monotherapy. These included flu-like symptoms, fatigue, gastrointestinal disturbance, rash, alopecia, tremor, headaches, myalgia/arthralgia, myelotoxicity and frequent respiratory infections. Most patients in this group experienced multiple adverse effects but, despite this, 24/28 (86%) were able to tolerate co-treatment with allopurinol. Two patients stopped co-treatment due to a recurrence of the side effects they had experienced on monotherapy (arthralgia, nausea and fatigue) and one patient discontinued combination therapy due to abnormal LFTs. One patient progressed rapidly to colectomy for uncontrolled UC after which their thiopurine therapy was no longer required.

In those patients where a judgement about the clinical outcome of combination treatment could be made, 11/20 (55%) achieved a clinical remission at 1 year (or the latest available assessment if treatment duration was for less than one year). If only those completing a full year of therapy were included in this analysis, the figure was 47%. (Patients discontinuing treatment due to side effects were included in this analysis as treatment failures).

7.4.7.3 Non-response to single agent thiopurine

This group includes 27 patients, 18 of whom demonstrated predominant methylation on TGN monitoring. An additional 6 patients had a historical label of non-response to azathioprine alongside a high TPMT activity and 3 patients were partial responders in whom dose-optimisation could not be achieved due to side effects.

In those for whom a judgement about the clinical outcome of combination treatment could be made in isolation from other treatment changes 10/17 (59%) achieved clinical remission at one year, this figure rose to 13/20 (65%) when those doing well, but yet to reach a full year of therapy, were included. Again, those unable to tolerate combination treatment due to side effects were included in the analysis as treatment failures.

7.4.7.4 Combination therapy as a first line treatment

This group included 23 patients, 5 of whom could not be accurately assessed for clinical response to combination therapy (1 concomitant biologic, 2 inadequate duration of treatment and 2 non-adherent by TGN). Of the remaining 18 patients, 14 (78%) achieved clinical remission on combination treatment.

7.4.7.5 Response to an adverse metabolite profile

This group contained 8 patients, 7 of whom improved their thiopurine metabolite profile and maintained clinical remission (one was also on a biologic throughout). One patient developed a rash on allopurinol and was transferred back to single agent treatment without any loss of response. The rash resolved on monotherapy suggesting that allopurinol was the causal agent.

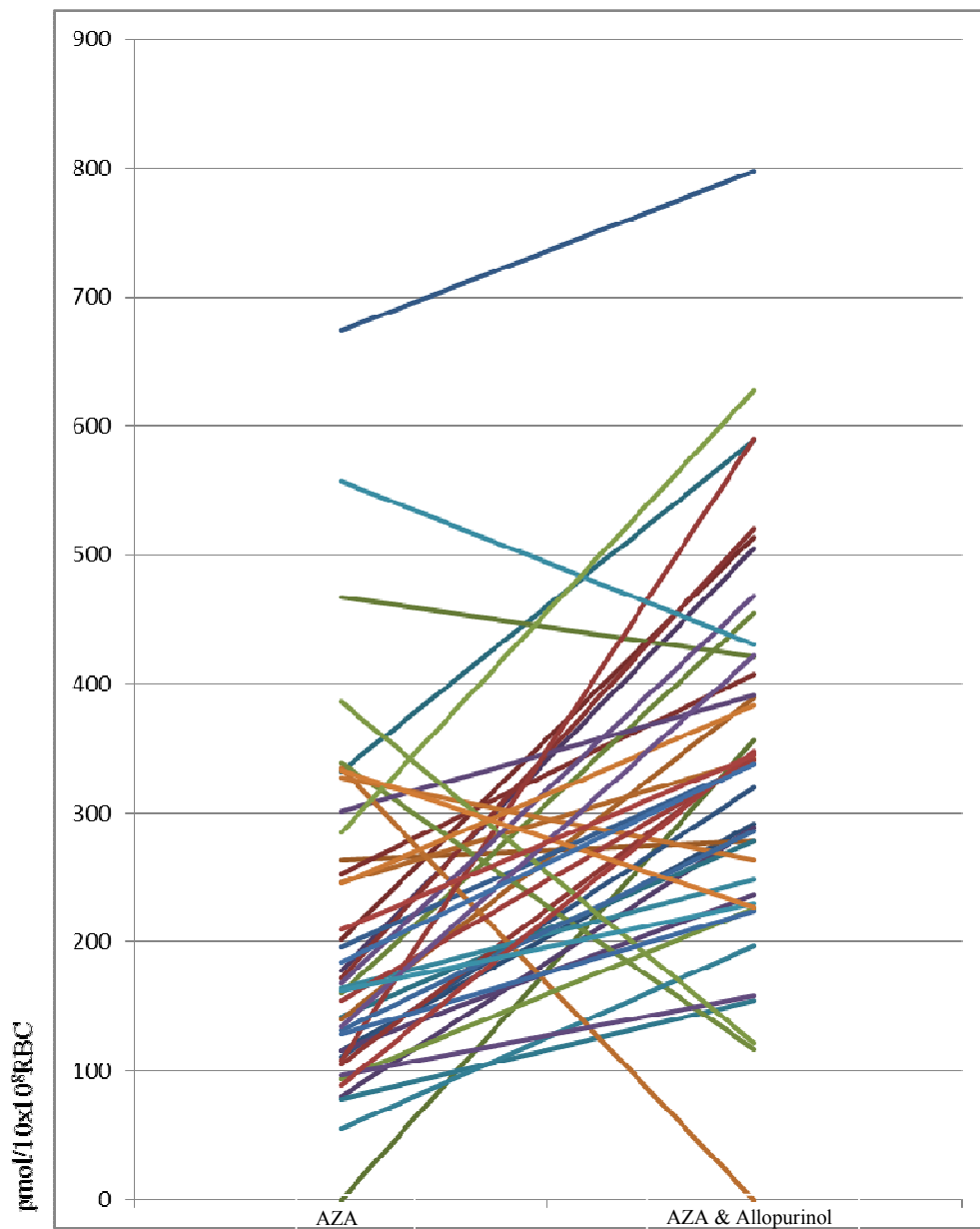


Figure 7.2: The effect of co-prescription of allopurinol on TGN levels pmol/10x10⁸RBC.

This figure excludes the patient who did not reduce his AZA dose appropriately, and therefore had extremely high TGN levels. Values on the left are those on monotherapy, those on the right are on combination treatment. Only those subjects with paired values are shown.

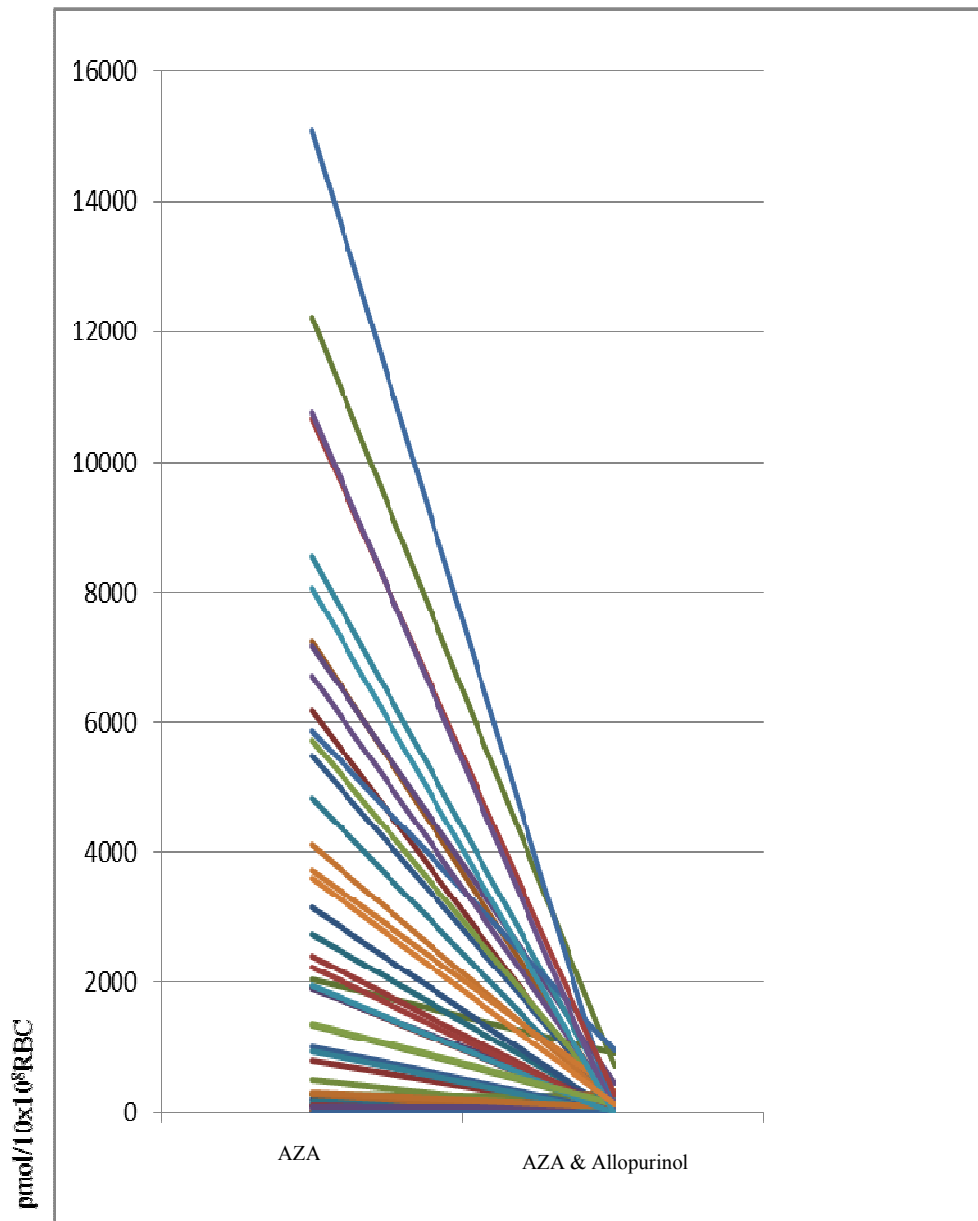


Figure 7.3 The effect of allopurinol co-prescription on Methylated metabolite levels pmol/10x10⁸RBC.

Values on the left are those on monotherapy, those on the right are on combination treatment. Only those subjects with paired values are shown.

7.5 Discussion

This study has demonstrated that co-prescription of allopurinol alongside appropriately reduced doses of AZA or MP circumvents not just hepatotoxicity on single agent thiopurine, but also other side effects. This emerging role for combination therapy is important, as atypical side effects are responsible for a large number of patients losing thiopurines as a treatment option. The study also demonstrated that the majority of these patients tolerated combination treatment and achieved clinical remission, providing an important treatment option both for patients starting thiopurine therapy and those with a historical label of thiopurine intolerance.

The study also demonstrated that combination therapy could achieve a clinical response for a significant number of those failing to respond to thiopurine monotherapy. This is important both for patients failing to achieve remission on single agent thiopurine and also those on thiopurines alongside biologic therapy. Combination therapy could therefore recapture clinical response in a significant proportion of patients in whom thiopurine therapy would otherwise have been abandoned.

Using figures from the literature, we have calculated the theoretical size of this missed opportunity. In a cohort of 207 patients with IBD⁸⁷ initiating thiopurine therapy, 60 patients had their thiopurine discontinued as a result of non-specific side effects, 8 due to hepatitis and 32 were non-responders with a TPMT activity greater than 35pmol/h/mgHb (a surrogate marker of hypermethylation). This is a total of 100 patients, nearly half of the cohort, that may have benefitted from co-treatment with allopurinol. From the results of this study, it can be predicted that 53 of these would achieve remission if offered combination therapy with allopurinol as an alternative treatment. This represents 26% of the whole original cohort, an extremely significant proportion of our patients. This analysis could be skewed by a high side effect rate, but even using a lower rate of 7.2 % (10% total side effects¹⁴ with 2.8% experiencing side effects unsuitable for combination therapy i.e. pancreatitis and myelotoxicity) would still leave 12% of all patients started on AZA, who could be rescued from thiopurine failure to 1 year remission on combination therapy.

The use of combination therapy for hepatotoxicity was originally restricted to those patients with high methylated metabolite levels. However, combination treatment could theoretically provide additional benefits in those with liver disease, by reducing doses of thiopurine for first pass metabolism, altering the balance of other thiopurine metabolites, blocking the production of oxygen free radicals¹⁹⁰ and by raising hypoxanthine levels which provides purines, through salvage pathways, for cellular repair¹⁸⁷. This study also reports (for the first time) the development of abnormal liver function in 2/109 patients attributable to allopurinol co-treatment, and practitioners must be aware of this possibility.

Our understanding of most ADRs encountered on thiopurines is very limited. Myelotoxicity is known to be dose-related in most instances and to result from high TGNs. However, even “atypical” ADRs appear to be dose-related to some extent. Many patients tolerate thiopurines at low doses but are unable to tolerate escalation to their target dose or therapeutic TGN level, limiting response. Nausea and the myalgic flu-like syndrome fall into this category and it is postulated that these result from an accumulation of alternative thiopurine metabolites (thioITP being specifically implicated in the flu-like syndrome^{87,353}). The significantly reduced parent drug concentration and/or altered balance of metabolites produced by combination treatment is thought to be the mechanism by which these ADRs are circumvented

Whilst using combination therapy, the standard FBC and LFT monitoring regimen which was used in the department for patients on monotherapy was also adopted for combination treatment. Blood monitoring remains at least as important on combination therapy and practitioners should be aware that dose-adjustments whilst on allopurinol have a much larger impact on TGNs and clinical outcome. Dose-adjustments should also, therefore, be 25-33% of those usually attempted on monotherapy. Safety concerns over the combination of allopurinol and AZA in the literature relate to inadvertent co-prescription of these two drugs without suitable dose reduction of the thiopurine. Correct dosing (see Table 7.1), supported by TGN measurement, should avoid this problem. Measurement of TGN/MeMP levels facilitates safe, personalised dose-adjustment. TGN's were therefore checked at 4 weeks after initiation, and 4 weeks after any dose-change on combination therapy. They were also used to investigate inadequate response and 6-12 monthly

in those established on therapy. The 4 week check avoided a potentially serious ADR due to over-dosing in this cohort.

Side effects reported for allopurinol include rash, gastro-intestinal disturbance, malaise, headache, vertigo, drowsiness, visual and taste disturbances, hypertension, alopecia, paraesthesia and neuropathy, gynaecomastia and blood disorders. Although previous reports of the use of combination therapy in the literature have not encountered any toxicity associated with allopurinol, 2 cases of hepatotoxicity possibly related to the use of allopurinol were encountered in this study, as well as 2 cases of self-limiting rash. Rash is the most significant side effect of allopurinol therapy and whilst most are mild and self-limiting, allopurinol is the commonest drug associated with the development of toxic epidermal necrolysis (TEN)³⁵⁴. There appears to be a pharmacogenetic basis for this with an association demonstrated with the HLA-B*5801 allele. TEN is more common in individuals of Asian parentage, particularly among the Han Chinese population^{355,356}. Since TEN carries a significant mortality³⁵⁷, pre-treatment pharmacogenetic testing for this HLA-type has been proposed by some groups³⁵⁶ although TEN remains a very rare occurrence³⁵⁴. The dose of 100mg, as used in this study, is also thought to be relatively safe³⁵⁴.

First line combination treatment was adopted in the IBD service at GSTT for patients with very high TPMT activity in an attempt to reduce the occurrence of ADRs and non-response in this high risk group. Subsequent work on this area in the PRL has highlighted that the reasons for patients developing predominant methylation are much more complex than a simple elevation of TPMT activity and combination treatment on the basis of high TPMT activity alone is no longer used. Instead, combination treatment is initiated once predominant methylation is demonstrated on TGN monitoring (or for adverse effects). As more is uncovered about the reasons for predominant methylation and a more comprehensive pre-treatment risk assessment can be performed, it is likely that first line combination treatment will be introduced more widely for a sub-group of patients. A head-to-head prospective trial of single agent thiopurine against combination therapy is needed to establish whether all patients might be better off on combination therapy from the outset, in view of the high response and relatively low side effect profile reported here and in other studies of combination therapy.

Whilst blocking XDH with allopurinol would logically increase TGNs (both by decreasing TGN breakdown and reducing first pass metabolism – see figure 7.1) and therefore necessitate a thiopurine dose reduction, it is not clear why it has such a profound effect on methylated metabolite levels. Allopurinol and its active metabolite oxypurinol are not known to alter TPMT activity⁹⁴, although published data is lacking. Early work from the PRL has, however, demonstrated that thioxanthine inhibits TPMT activity and increased production of thioxanthine, due to the presence of allopurinol, could account for the effect of combination treatment on methylated metabolites.⁹⁶ The lower thiopurine doses permitted by co-administration of allopurinol could result in reduced methylation, if TPMT is unable to efficiently work at these lower concentrations. This could additionally explain the partial reversal of predominant methylation achieved by splitting the dose of thiopurine drugs^{358,359}.

In conclusion, combination treatment with low dose thiopurine and allopurinol is a safe and effective option for patients that would not otherwise be able to benefit from thiopurine treatment, whether due to intolerance or lack of clinical response. Treatment is cheap and can be monitored in the same way as standard AZA treatment. TGN/MeMP measurements identify suitable candidates for co-treatment and are essential to guide personalised adjustments to dosing and to avoid dose-related toxicity and accidental over-dosing.

Conclusions:

In this thesis I have explored the impact of genetic polymorphism, in several novel targets, on success of thiopurine treatment. SNPs in xanthine oxidase/dehydrogenase (*XDH*) and the final enzymatic step which activates its essential cofactor (molybdenum cofactor sulfurase, *MOCOS*) are shown to protect against side effects on AZA. Polymorphism in aldehyde oxidase (*AOX*) and multi-drug resistance protein 5 (*ABCB5*), on the other hand, are shown to predict a lack of response to thiopurine treatment. Sequencing *AOX* validated the real-time PCR results but suggested that there were no other coding SNPs likely to be contributory. The way in which these markers of non-response could be combined to produce a clinically applicable pharmacogenetic index is explored, and the hope expressed that these, and/or similar markers of response to thiopurine treatment, will soon be applicable to clinical practice improving patient experience and outcome on these drugs.

Clinical data supporting the optimisation of azathioprine therapy by the measurement of thioguanine nucleotide concentrations, alongside methylated metabolite levels are presented. These support the use of TGNs in the clinic to aid decision making and provide explanations for non-response to azathioprine. Additionally, co-prescription of allopurinol to optimise azathioprine response (for those who are intolerant of thiopurines or have biochemical resistance), is explored using analysis of the outcomes of the largest reported cohort in the literature. This data confirms the usefulness and safety of this strategy in a variety of contexts and confirms co-prescription as an essential tool in the treatment of IBD.

The conclusion of my thesis is that there is much that can be done to improve the outcome of patients receiving thiopurines. The studies here have focussed on IBD, but the impact on outcome should be relevant to the use of these drugs across many other disciplines, including treatment of leukemia and the prevention of transplant rejection. Strategies such as measurement of TGNs and the use of allopurinol co-prescription have already reached the bedside in some centres and should be adopted in a more widespread manner. The pharmacogenetic markers of response presented here are of interest, but their significance clearly needs replication in another cohort before they can be considered for incorporation into clinical practice. If this were to be achieved

however, this would be a major break-through, as the ability to stratify the chance of success on thiopurine agents, before starting therapy, could significantly shorten the time taken for a patient to achieve an important clinical remission and avoid futile therapy in patients with predictable drug resistance.

In a wider context, pharmacogenetics / pharmacogenomics and individualised prescribing is the subject of a huge amount of on-going research. Whilst very little of this has yet reached the bedside (even TPMT is still not routine in some centres), global interest in pharmacogenetics / pharmacogenomics is growing and the FDA is promoting translation of this research into clinical practice by putting information on known pharmacogenetic markers onto drug labels. This includes the use of TPMT testing prior to prescription of azathioprine.

<http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm>

What is needed to reap the rewards of all this work however, is a greater awareness and understanding of this area from clinicians in all areas of medical practice, so that each prescription becomes the result of an individualised assessment of risks and benefits, improving outcomes for our patients.

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