Liver inflammation in inflammatory bowel disease

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Liver inflammation in Inflammatory Bowel Disease

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Abstract

A third of patients with Inflammatory Bowel Disease (IBD) develop abnormal liver function tests (LFTs) at some point during their care. Over half of IBD patients are on thiopurines, which are not only a common cause of abnormal LFTs, but are also associated with a chronic liver disease, called nodular regenerative hyperplasia (NRH).

The aim of this thesis was to determine the mechanisms and risk factors by which liver inflammation occurs in IBD, especially in relation to thiopurines. This thesis achieves its aim, firstly, through a large cohort study of patients with abnormal LFTs in IBD. Secondly, a candidate gene analysis is performed to determine which genes predispose to thiopurine hepatotoxicity. Thirdly, an assay is developed to measure compounds of the Methionine Cycle using mass spectrometry, to assess their role in thiopurine hepatotoxicity and as potential future biomarkers. Lastly, magnetic resonance elastography (MRE) is assessed as a potential screening modality to diagnose NRH.

By providing novel answers as to the causes of liver inflammation in IBD, this thesis brings Medicine a step closer in preventing our patients from developing a common extraintestinal manifestation of their illness.

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List of abbreviations:

ADA- Adalimumab

ADC -Apparent diffusion coefficient

AIH – Autoimmune hepatitis

ALL – Acute lymphoblastic leukaemia

ALP – Alkaline Phosphatase

ALT – Alanine transaminase

AMP - Adenosine monophosphate

AMTCI - 4-amino s–methylthio carbonyl imidazole

5-ASA - 5-aminosalicylic acid

AZA- Azathioprine

BMHT - Betain-homocysteine methyltransferase

CD - Crohn's Disease

CI – Confidence interval

CMV - Cytomegalovirus

CV - Coefficient variance

CβS - Cystathionine- β-synthase

Cγl - Cystathione-γ-lyase

CSF - Cerebrospinal fluid

dTGTP - DeoxythioGTP

dNTPs - deoxynucleotides

DILI -Drug induced liver injury

DMSO - Dimethyl sulfoxide

dNTPs - Deoxynucleotides

DM – Dominant model

ECCO – European Crohn's and Colitis Organisation

EBV – Ebstein Barr Virus

EDTA - Ethylenediaminetetracetic acid

ERCP – Endoscopic Retrograde Pancreatography

Gln – Glutamine

Glu – Glutamate

GSTT – Guy's and St Thomas' Hospital

HCL – Hydrochloric acid

HELLP - Haemolysis, Elevated liver enzymes and Low platelets

HVPG – Hepatic venous pressure gradients

γGC - γ-glutamyl cysteine

γGCS - γ-glutamyl cysteine synthetase

γGCT - γ-glutamyl cyclotransferase

GI - Gastrointestinal

GMP - Guanosine monophosphate

GSH - Glutathione synthetase

GST - Glutathione-s-transferase

GWAS – Genome wide association studies

HBI – Harvey Bradshaw Index

Hep B SAg – Hepatitis B surface antigen

Hep B CAb – Hepatitis B core antibody

HLA – Human Leukocyte Antigen

HCys - Homocysteine

HIV – Human immunodeficiency virus

HPLC – High performance liquid chromatography

HPRT - Hypoxanthine phosphoribosyl transferase

IBD - Inflammatory Bowel Disease

IFX - Infliximab

Ig - Immunoglobulin

IAC – IgG4-associated cholangiopathy

IMP - inosine monophosphate

IMPDH - Inosine monophosphate dehydrogenase

ITP – Inosine triphosphate

LOR – Loss of response

LC-MS/MS - Liquid chromatography – tandem mass spectrometry

LD – Linkage disequilibrium

LDTA - Low dose thiopurine with allopurinol

LFT – Liver function tests

MAT - Methionine adenosyltransferase

MeMP - Methylmercaptopurine

MeMPR - methylmercaptopurine ribosibe

MRM - Multiple reaction monitoring

MS - Methione synthase

MS – Mass Spectroscopy

MTHF - Methyltetrahydrofolate

MTHFR - 5,10 MTHF reductase

MP - Mercaptopurine

MPT - Mitochondrial permeability transitions

MRCP – Magnetic Resonance Cholangio Pancreatography

MTX - Methotrexate

MW – Molecular weight

NAFLD – Non-Alcoholic fatty liver disease

NASH – Non-Alcoholic Steatohepatitis

NICE – National Institute for Clinical Excellence

NRH – Nodular regenerative hyperplasia

OR - Odds ratio

PCR - Polymerase Chain Reaction

PSC – Primary sclerosing cholangitis

PG - Pyroglutamic acid

RBCs – Red Blood Cells

Rcf – Relative centrifugal force

RM – Recessive model

ROI – Region of interest

SAM – S-adenosyl methionine

SAH - s-adenosyl homocysteine

SAHH - s-adenosyl homocysteine hydroxlase

SCCAI – Simple Clinical Colitis Activity Index

SECs – Sinusoidal endothelial cells

SNP – Single nucleotide polymorphism

TAE - Tris-acetate -EDTA buffer

TG - Tioguanine

TGDP – Thioguanine diphosphate

TGMP- Thioguanine monophosphate

TGTP – Thioguanine triphosphate

TGN - thioguanine nucleotide

THF - Tetrahydrofolate

TIMP - thioinosinic acid

TNF -Tumour Necrosis Factor

TPMT - Thiopurine methyl transferase

TUA - thiouric acid

UC - Ulcerative Colitis

ULN – Upper limit of normal

WB – Whole Blood

WBCs – White Blood Cells

WBD – Weight-based dosing

XDH – Xanthine dehydrogenase

XO - Xanthine oxidase

XOD - xanthine oxidase dehydrogenase

Chapter 1: Literature Review

1.1 Definition of IBD, epidemiology and pathology

Inflammatory bowel disease (IBD) is largely made up of two chronic disease processes, ulcerative colitis (UC) and Crohn's Disease (CD). Whilst CD can affect all parts of the gastrointestinal (GI) tract and layers of the bowel wall, UC affects the mucosa of the colon only. Although IBD was recognised as early as the 17th century, classification based on 2 distinct phenotypes came much later, with UC being described in the 19th century as an IBD affecting the colon only. It was not until the 1930s that Burrill Bernard Crohn published several cases of "Terminal ileitis: a new clinical entity," describing Crohn's disease for the first time.[1]

UC is more prevalent in females, in non-smokers, and has a bimodal age distribution affecting adults in their 20s and 60s.[2, 3] CD occurs affects mostly young children or adults in their 20s, with a strong predisposition towards smokers. Both types present with chronic diarrhoea and abdominal pain. Rectal bleeding is more common in UC, as it almost always affects the bowel distally in a diffusely erythematous manner as opposed to CD, which causes patchy inflammation occurring throughout the GI tract.[4] The behaviour of CD, as described in the Montreal classification, can be stricturing (B2), penetrating (B3) or neither (B1).[5] As CD affects all layers of the bowel wall, it leads to narrowing or stricturing of the lumen. Fistulas can develop, leading to abscess formation and collections, a phenotype referred to as Penetrating Disease. Through this process, perianal disease can occur in a subset of patients causing fistulas through the walls and muscles around the rectum, to other organs such as the vagina and bladder. As UC affects the mucosa of the colon only, this penetrating and fistulating behaviour is not seen. However, severe flares of UC risk dilatation of the colon, subsequent perforation and death through abdominal sepsis.

Both conditions can be distinguished histologically as well as by their presentation and behaviour. Granulomas are a hallmark of CD which are not seen in UC. In CD, inflammatory cells can be found outside the colon, such as the ileum, although backwash ileitis can occur in severe extensive UC.

The increased incidence of IBD follows a South-to-North, East-to-West pattern of distribution and therefore is more commonly seen in developed countries, globally affecting 5-500 individuals per 100,000 with 1-20 per 100,000 new cases yearly.[6, 7] The reason for the geographical variation is uncertain but may represent dietary differences or a genetic predisposition.[8, 9] Over 200 genes have been found which may increase the likelihood of getting IBD, with the concordance being around 20%.[10-15]

1.2 Natural history of IBD

For UC, the severity of disease correlates with the location. Patients with extensive UC are more likely to have flares of disease than those where inflammation is limited to the rectum.[16] At diagnosis, the location of the disease is evenly distributed with a third having proctitis or proctosigmoiditis, a third having left sided colitis and a third having extensive or pancolitis.[16] In any year, a third of patients will have flares, a third will remain in remission, whilst another third will have mild to moderate symptoms.[17] Patients who are admitted to hospital with flares of their disease have a 20% risk of colectomy, which confers a 10% mortality if performed as an emergency.[18] Given the primary goal of treatment is to prevent surgery, emphasis is placed on preventing hospital admissions by treating flares early. On the other hand, an early colectomy, in a patient who needs it, is better than a colectomy in a patient who has already perforated. The Truelove and Witts criteria predicts patients who need intravenous steroids whilst the Travis Criteria predicts patients who need escalation from steroids to intravenous ciclosporin.[19, 20] The lifetime risk of a colectomy in UC is 15-20%, whereas for extensive disease, it increases to 20-25%.[18, 21]

The management of CD is different and depends on the location and behaviour of the disease. Again, the aim is to prevent the need for surgery which risks short bowel syndrome and nutritional failure. Around 50% of patients with CD will need surgery at some point in the course of their disease and 70% of those patients will require a second operation within 5 years.[22] Patients with stricturing disease can present with symptoms of bowel obstruction. Treatment depends on the degree of inflammation of the stricture, which if present, could be amenable to medical therapy. Alternatively, strictures which are fibrostenotic, with little or no inflammation, will either need resection, stricturoplasty or endoscopic dilatation depending on the location and length of the stricture. Penetrating disease, presenting as abscesses, may require antibiotics and drainage prior to medical therapy. Similarly, perianal disease may require either laying open or seton insertion to allow for healing. Medical therapies can downstage the degree of inflammation enough to allow for a more limited operation but often a small resection is required followed by preventative medical therapy to prevent disease recurrence.

1.3 Treatments and goals of therapy

There has been a shift in the thinking for treatment of IBD. Traditionally, there was a "step-up" approach to medical therapy, although increasingly as new biologics become available, a "top down" approach is preferred for particular patients.[23] Treatment is increasingly aimed at achieving both sustained clinical remission and mucosal healing, thereby altering the natural history of the disease and especially resection-free survival.[24, 25]

Mesalazines or 5 -aminosalicyclic acids (5-ASAs) are generally the first-line of therapy in IBD and avoid many of the side effects of its predecessor, sulfasalazine. Sulfasalazine is reduced by colonic bacteria to its active moiety 5-ASA and, sulphapyridine, a compound responsible for many of its side effects – hence why 5-ASAs are now used on their own. There is some evidence supporting their use at inducing remission in mild CD, but no evidence for them maintaining remission; remission rates in UC are 21%.[26,27] Rectal preparations are also available allowing for the treatment of more distal disease topically and, in fact, the combination of both rectal and oral preparations have been shown to be superior to a single route alone.[28] Mesalazines are generally well tolerated although there are risks of mesalazine-induced nephrotoxicity. Mesalazine-induced hepatotoxicity is also reported although rare in comparison to hepatotoxicity from thiopurines.[29]

Thiopurines (namely azathioprine (AZA), mercaptopurine (MP) and less so tioguanine (TG)) are the commonest drug therapy used in the treatment of IBD and the first-line treatment for mild to moderate CD. Despite being used initially for the treatment of acute leukaemia and then renal allograft rejection, they have found their home in the treatment of IBD more so than any other medical condition. However, there are risks associated with thiopurines including the development of lymphoma.[30]

Biologics have revolutionised the management of IBD. As the National Institute of Clinical Excellence (NICE) agrees to fund more biologics, clinicians have more choice as to which biologics to use for certain patients, such that treatment can be individualised (based either on the patient's history or the immunology of their disease). Anti-tumour necrosis factor α (TNFα) monoclonal antibody drugs such as adalimumab (ADA) and infliximab (IFX), have efficacy in induction and maintenance of remission for both moderate to severe CD and UC.[31, 32] However, 10-30% of patients do not respond to anti-TNF (primary non-responders) and 50% of patients who initially respond, eventually lose response with time (secondary loss of response (LOR)) due to the formation of antibodies against the drug. This immunogenicity can be prevented by the concomitant use of thiopurines as demonstrated

in the SONIC trials (Study of Biologic and Immunomodulator Naïve Patients in CD).[33] Switching between anti-TNF drugs or out of class at the time of LOR can avoid treatment failures.[34, 35]

There are also risks associated with anti-TNF agents including lymphoma and opportunistic infections, both of which are increased with concomitant therapy.[36, 37] This led to other molecules being targeted in the hope of reducing those risks. Vedolizumab, a humanised monoclonal antibody to α4β7 integrins, is thought to be safer because of its gut selectivity whilst also being shown to an effective treatment in both UC and CD with clinical remission rates being 42% and 39% respectively at 1 year.[38] Other biologics include ustekinumab, an interleukin-12 and 23 antagonist with NICE agreement for use in CD and, golimumab, another anti-TNF with NICE agreement for its use in UC.[38] Patients with UC have only recently had access to tofacitinib, a Janus kinase inhibitor, and the first oral monoclonal antibody for IBD.

1.4 Liver inflammation in IBD

Abnormal liver function tests (LFTs) occur in 30% of patients with IBD at some point during their disease.[39] The most recognised cause is primary sclerosing cholangitis (PSC), a chronic inflammatory disease of the bile ducts causing stricturing, which can eventually progress to cirrhosis and liver failure.[40] The median survival without liver transplantation is 12 years and even less for patients who present with symptoms at diagnosis.[41] 90% of patients with PSC have IBD whilst 5% of patients with IBD have PSC, with PSC being more common in young males with UC. The onset of IBD occurs typically before that of PSC. The increased risk of cholangiocarcinoma is 160-fold with a 4-fold increase in colorectal cancer, hence these patients require regular cancer surveillance.[40] The gold standard for diagnosis of PSC is by endoscopic retrograde pancreatography (ERCP). However, non-invasive methods, using magnetic resonance cholangiopancreatography (MRCP), are usually preferred due to the complication rate associated with ERCP.[42] There is evidence to suggest that gut microbiota may play a role in the relationship between IBD and PSC, in that distinct gut flora have been found to be more prevalent in the GI tract of patients with PSC.[43] There is also likely be an autoimmune component given the findings that over 97% of patients with PSC have one or more autoantibodies detectable, and 81% have 3 or more.[44] Susceptibility loci have been found which confer a genetic predisposition to developing PSC and UC.[45] UC in PSC, has a distinct phenotype, with patients being more likely to have pancolitis and rectal sparing, and more likely to have backwash ileitis.[39]

Abnormal LFTs in IBD present a diagnostic challenge to Gastroenterologists. Failure to diagnose PSC could prevent the patient from receiving appropriate cancer surveillance. Alternatively, there are other causes for abnormal LFTs which need to be diagnosed before complications develop.[39] Small duct PSC is diagnosed histologically from a liver biopsy in patients who have cholestatic LFTs but where the cholangiogram is normal. 80% of such patients have IBD and the risk of cholangiocarcinoma is extremely low. Prognosis is good with only 12-23% developing PSC. Autoimmune hepatitis (AIH) occurs both on its own and as an overlap condition with PSC based on histology. Prognosis is variable, based on response to steroids and on whether either AIH or PSC become more dominant as the condition progresses.[46] IgG4-associated cholangiopathy (IAC) is more common in patients with IBD, particularly UC.[47] Elevated IgG4 levels, distinct histology and radiology, and response to steroids are the hallmarks of IAC.[48] Patients can present with obstructive jaundice earlier than in PSC but respond well to steroids with reversal of stricturing.[49] Both secondary hepatic amyloidosis and chronic liver granulomatosis can occur in active CD, as a feature of metastatic CD or secondary to sulfasalazine treatment.[50, 51]

Gallstones are twice as likely in CD compared to the general population. 13-34% of patients with an ileal resection or ileitis have gallstones.[52] This may reflect changes in bile acid absorption or reduced gall bladder motility.[53] Non-alcoholic fatty liver disease (NAFLD) occurs in 8.2% of IBD patients with risk factors being obesity (Odds ratio (OR) 2.1), steroid use (OR 3.7), previous small bowel surgery (OR 3.7) and hypertension (OR 3.5) when compared to IBD patients without NAFLD. Of all the IBD patients who have abnormal liver biopsies, 50% have been found to have steatosis.[54]

The prevalence of hepatitis B and C in patients with IBD has been shown to be equal to that of the general population.[55] Hepatitis B core antibody (cAb) positivity in one Spanish cohort was found to be 7.1% in patients with CD and 8% in patients with UC. Hepatitis C was detected in 2.3% of patients with CD and in 1.3% of patients with UC. The same study showed that reactivation of hepatitis B in surface antigen (sAg) positive patients exposed to IFX, occurred in 36% with two thirds developing liver failure.[56] Reactivation of Hepatitis B in sAg negative, cAb positive patients is rare but has been described. There is disagreement as to whether thiopurines on their own increase the risk of reactivation. If patients are on steroids with thiopurines, they should be on prophylaxis.[57] European Crohn's and Colitis Organisation (ECCO) guidelines advise all patients with hepatitis B who are on thiopurines, to have antiviral prophylaxis, whilst patients with normal serology should be vaccinated.[58, 59] On the other hand, reactivation of hepatitis C does not occur with immunosuppressive treatment.

1.5 Drug-induced hepatotoxicity

Drugs are a common cause of hepatotoxicity in patients with IBD. Drug-induced liver injury (DILI) affects 19.1 per 100,000 people in the US and accounts for 6.8 - 13% of all causes of acute liver failure.[60] The commonest causes are co-amoxyclav and nonsteroidal anti-inflammatory drugs whilst a United States (US) registry study of acute liver failure due to DILI found that isoniazid, followed by trimethoprim, phenytoin and herbal medicines were most prevalent.[60, 61]

The true incidence of DILI is uncertain because what constitutes a DILI is not universally defined. Poor reporting is also contributory. DILI can be idiosyncratic (unpredictable or dose-independent) or intrinsic (predictable or dose-dependent). The injury is expressed as one of 3 different biochemical patterns: hepatocellular, cholestatic or mixed. This is dependent on whether alanine transaminase (ALT) or alkaline phosphatase (ALP) are raised more than the other (Table 1.5.1). The point in time at which the DILI is classified as one of these 3 patterns is not defined. Therefore, a DILI that is hepatocellular at diagnosis, may well become cholestatic later.

DILI can also be immune or non-immune with the former presenting with fever, rashes and eosinophilia on blood tests. Immune mediated drug reactions have an earlier onset of between 1 and 6 weeks, and there is typically rapid re-injury on re-challenge. Non-immune mediated liver injury occurs at any time up to a year.

Histology is not necessary to diagnose DILI as it can show a variety of patterns. Some drugs, such as anti-TNF agents, cause a drug-induced autoimmune hepatitis which can respond to steroids.[62]

DILI is reported to be more common in females, patients with HIV, the obese and in the elderly.[63, 64] Alcohol is known to be a risk for paracetamol-induced liver injury although there is no evidence that underlying liver disease itself increases the risk.[65] Patients with hepatitis B and C are more at risk of DILI from anti-retroviral and anti-tuberculous treatment; NAFLD can be aggravated by methotrexate (MTX) and steroids.[66, 67]

Genes are also thought to play a role. Genome wide association studies (GWAS) in a cohort of patients with DILI to co-amoxiclav, confirmed susceptibility for a particular Human leukocyte antigen (HLA) class II gene, although the positive predicted value for these single nucleotide polymorphisms (SNPs) was only 0.1%.[68] A much better association was found between the HLA B*5701 allele and an 80-fold increase to the risk of flucloxacillin DILI.[69]

Hepatitis E has been proposed as the actual cause for many suspected DILIs in 3 to 13% of cases. In one study 50 out of 318 patients with suspected DILI tested positive for hepatitis E IgG.[70]

The mean time from stopping the drug to the resolution of jaundice is 38 days for cholestatic DILI and 30 days for hepatocellular DILI.[71] Hy's law states that where DILI causes an elevation of bilirubin to 3 x upper limit of normal (ULN), there is a 10% mortality.[72] MiR-122 has been suggested as a candidate biomarker for paracetamol DILI and other markers may help diagnose other types of DILI.[71]

Drug-induced autoimmune-like hepatitides are important to distinguish from other types of DILI since they can be treated with steroids. These present with raised immunoglobulins and positive anti-smooth muscle antibodies and are typically associated with drugs such as minocycline, nitrofurantoin and anti-TNF agents. Unlike idiopathic AIH, there is a lack of chronicity histologically, and they do not relapse following resolution of liver biochemistry.[73]

Table 1.5.1: The 3 main patterns of DILI as determined by the R ratio defined by the Council for International Organisations of Medical Sciences and modified by the Food and Drug Administration (FDA).[74]

Hepatocellular	Defined as a DILI where the R ratio ((ALT/ULN) ÷ (ALP/ULN)) is ≥ 5
Cholestatic	Defined as a DILI where the R ratio ((ALT/ULN) ÷ (ALP/ULN)) is ≤ 2
Mixed	Defined as a DILI where the R ratio ((ALT/ULN) ÷ (ALP/ULN)) is between 2 and 5

Dili is defined as ALT > 3 X ULN or ALP > 2X ULN. R is a ratio of the ALT to ALP relative to their respective upper limits of normal (ULN).

Most of the drugs used to treat IBD can cause hepatotoxicity. 5-ASAs caused hepatotoxicity in 2% of UC patients.[29, 75] In one US study, 14.3% of IBD patients treated with MTX for over 26 months developed hepatotoxicity.[76] However, very few patients (1 in 20) who develop hepatotoxicity on MTX, actually have significant fibrosis when biopsied.[77] IFX has also been shown to cause hepatotoxicity; Between 2003 and 2011, 34 cases in the US were described, with reversal of hepatotoxicity after stopping IFX.[78] However, IFX rarely caused jaundice and very rarely resulted in liver failure.[79]

A recent prospective cohort study of patients with IBD monitored for abnormal LFTs, graded liver injury as either hepatocellular or cholestatic according to 2 grades (different from Hy's criteria): grade 1 as an ALT up to 3 x ULN and grade 2 as an ALT > 3 x ULN for hepatocellular liver injury and grade 1 as an ALP 1-2.5 x ULN and grade 2 as an ALP > 2.5 x ULN for cholestatic liver injury.[80] The authors found that 7.6% of IBD patients developed a grade 1 cholestatic liver injury at some point when monitored over a 1-year period, compared to 26.3% who developed a grade 1 hepatocellular injury. Under half of such patients had persistence of the injury (defined as injury on > 1 measurement). The grade of liver injury and its persistence is shown in Table 1.5.2.

Table 1.5.2: Type and grade of liver injury seen in a cohort of 251 IBD patients when monitored over a year.[80]

Liver injury grade and type	Definition	% fitting grade	% Persistence
Grade 1 Hepatocellular	Up to 3 x ULN	26.3	11.2
Grade 2 Hepatocellular	>3 X ULN	2	0.4
Grade 1 Cholestasis	Up to 2.5 x ULN	7.6	3.2
Grade 2 Cholestasis	>2.5 X ULN	0	0

ULN: Upper limit of normal. Persistence defined as liver injury on > 1 measurement.

1.6 Thiopurines in IBD; their history and efficacy

Thiopurines were developed by Gertrude Elion in the 1940's as antimetabolites to nucleic acids and their efficacy first demonstrated by inducing remission in children with acute lymphoblastic leukaemia (ALL).[81] In 1962, AZA was then developed as an immunosuppressant to prevent renal transplant rejection and in the late 1960's as a steroid sparing agent in the treatment of IBD.

60% of IBD patients receive thiopurines with proven efficacy at maintaining steroid-free remission in UC and CD.[82] The role of thiopurines at inducing remission in UC remains controversial.[83] Thiopurines have been demonstrated to achieve steroid free remission in 53% of previously steroid dependent UC patients, hence why guidelines suggest they be used where patients have required 2 or more courses of steroids in a year.[23, 84] Thiopurines have also been demonstrated to both induce remission in CD and reduce the need for surgery by 40% and are therefore used earlier in the treatment of CD, where 5-ASA drugs have no proven role.[85]

AZA and MP also have efficacy in treating fistulating disease.[86] The TOPPIC (Trial of prevention of post-operative CD) study showed that MP reduced the frequency of post-operative recurrence, particularly in smokers where the recurrence rate was greater.[87]

The SONIC study showed that patients who were on both IFX and thiopurines had better outcomes than those on IFX alone.[33] The likely mechanism for this is by preventing the formation of anti-drug antibodies and LOR. Therefore, most patients who are on biologics are on concomitant thiopurines as well. A positive role for concomitant therapy has not been shown to be significant for golimumab and vedolizumab therapy.[88, 89]

However, a third of patients are unable to tolerate thiopurines due to adverse effects of flu-like symptoms, leukopenia (1-3-12.6%), hepatotoxicity (4%), pancreatitis (3%) and gastric intolerance (1.3-6%).[90] These side effects are seen between 2 and 4 weeks after commencing therapy and can be either idiosyncratic or dose dependant. Overall, cessation of treatment occurs in 9-25% of patients with no therapeutic benefit seen in 9-15%.[91] Where pancreatitis has occurred with AZA or MP, TG is an alternative therapy, metabolised via a different pathway, avoiding further pancreatitis.[92]

1.7 Thiopurine metabolism

All 3 thiopurines are prodrugs. Their metabolism results in the formation of TG nucleotides which are precursors for nucleic acid synthesis. Approximately 90% of AZA is metabolised to MP by sulfhydryl-containing compounds such as glutathione-s-transferase (GST).[93] The bioavailability of AZA is anything from 27-83% compared to that of MP, which is around 5-37%. AZA reaches peak plasma concentrations between 1-2 hours with a half-life of around an hour. MP is then transported intracellularly by SLC28 and SLC29 transporters to undergo three metabolic fates (Figure 1.7.2).

The first is the oxidation of MP to thiouric acid (TUA) by aldehyde oxidase and xanthine oxidase dehydrogenase (XOD). TUA is an inactive metabolite and excreted in the urine within 24 hours. XOD is absent from circulating bloods cells but is highly expressed in the liver and small intestine.[94] XOD deficiencies are autosomal recessively inherited with an incidence of around 1/70,000, and significant variations in XOD activity exist between racial and gender groups.[95, 96] Complete XOD deficiency results in severe toxicity on full dose AZA.[97]

The second fate is its breakdown into inactive methylmercaptopurine (MeMP) by thiopurine methyl transferase (TPMT), an enzyme which catalyses the s-methylation of aromatic compounds in pro- and eukaryotes. TPMT has a trimodal distribution of activity within the population. It was first appreciated in the 1980's that differences in TPMT activity account for the variability in tolerance to thiopurines. Most people have a "wild type" genotype for TPMT, meaning that they possess both normal alleles. Around 1 in 10 are carriers for a deficiency associated allele (heterozygotes) and 1 in 300 are homozygous variants and have complete TPMT deficiency.[98] Mutations most commonly occur in 3 genes: TPMT*2, TPMT*3A, and TPMT*3C.[99] The frequency of these variants is associated with ethnicity with variants for TPMT*3A being the most common in Caucasians.[100] TPMT*3C is more common in Asians whilst although TPMT*2 is rare, it is more frequently seen in patients of African origin. TPMT*3C is the ancestral allele, and on this background, the 2nd mutation, TPMT*3B occurred, giving rise to the TPMT*3A variant. Hence, TPMT*3B mutations will only occur alongside a TPMT*3C mutation, and results in the difference between TPMT*3A and *3C variants. With complete TPMT deficiency, no MeMP is produced thereby surrendering MP solely to its other metabolic fates, risking bone marrow toxicity due to the increased concentration of thioguanine nucleotides (TGNs) formed. Heterozygotes are also at risk of bone marrow toxicity, hence the importance of measuring TPMT before a patient starts treatment (Table 1.7). TPMT phenotyping gives a better reflection of TPMT status as it takes account of all TPMT genotypes and other factors such as red cell age which will alter TPMT activity.[101]

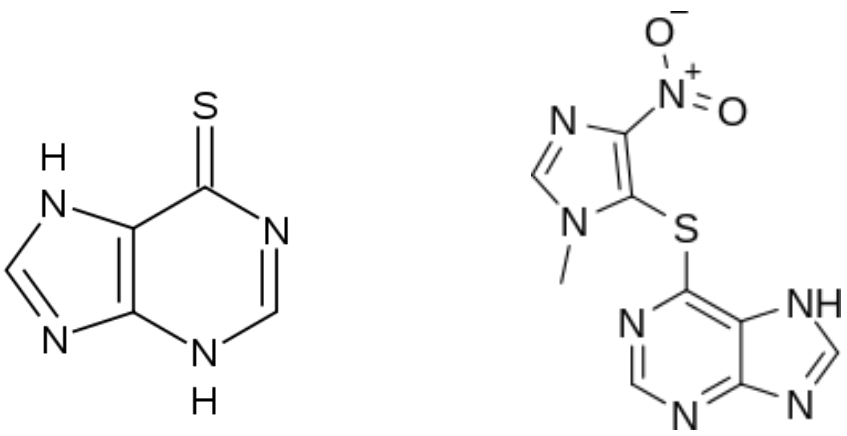
In the TOPIC study (Thiopurine response Optimization by Pharmacogenetic testing in Inflammatory bowel disease clinics), patients were randomised to either TPMT-based dosing or standard dosing at 1-5mg/kg for MP and 2-2.5mg/kg for AZA and both efficacy and side effects measured prospectively. Although TPMT genotyping did not alter efficacy, it did significantly reduce haematological adverse drug reactions compared to standard dosing for TPMT heterozygotes (22.9% versus 2.6%).[102] There were no differences in rates of hepatotoxicity between the two groups.

Table 1.7: TPMT can be classified by either TPMT genotype or by RBC TPMT activity, the latter being what is most commonly measured in the lab before patients start treatment and based on which thiopurines can then be appropriately dosed.

TPMT Genotype	RBC TPMT activity pmol/h/mgHb	Dose
Wild Type Normal	≥26	AZA 2-2.5 mg/kg MP 1-1.5 mg/kg
Heterozygote Intermediate	11-25	AZA 1-1.5 mg/kg MP 0.5-0.75 mg/kg
Homozygous variant Zero	≤10	Avoid or if using AZA consider 0.1-0.2mg/kg

MeMP metabolites are then broken down into 8-hydroxy-methylMP and 2,8, hydroxy-methylMP. The methyl donor for TPMT is s-adenosyl methionine (SAM) which is one of the chief methyl donors of the body and hence important in a variety of different methylation reactions. SAM is an essential compound for the TPMT assay as the methyl donor and has been associated with liver injury. It is therefore extremely relevant to this thesis and will be discussed in more detail in a Section 1.17.

The 3rd fate for MP is via the purine salvage pathway. This generates the nucleotide, 6-TGN, which carries the sulphur moiety so particular to thiopurines, into the backbone of DNA preventing DNA replication and hence production of the most rapidly dividing cells such as white blood cells (WBCs).



Mercaptopurine Azathioprine

Figure 1.7.1: Shows MP and AZA molecular structures. Both have a sulphur or thiol moiety making them extremely reactive with other organic molecules. AZA has an additional imidazole ring, the molecule so specific to purines compared to pyrimidines.

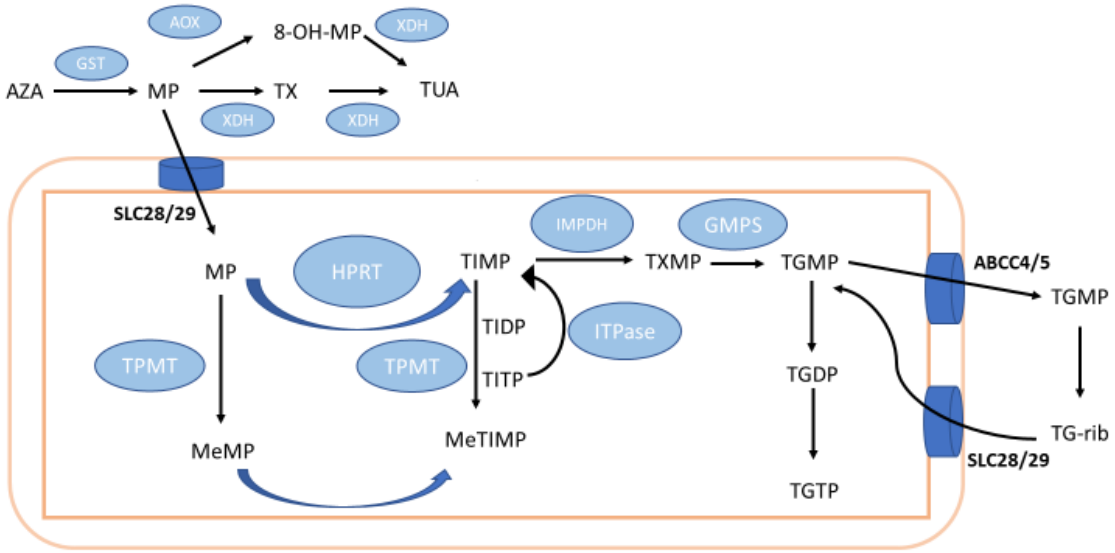


Figure 1.7.2: The 3 metabolic fates for MP after intracellular uptake by SLC nucleoside transporters.

The purine salvage pathway involves the formation of TGNs (TGMP, TGDP, TGTP) through the conversion of MP to the ribonucleotide, thioinosinic monophosphate (TIMP) by hypoxanthine phosphoribosyl-transferase (HPRT). Inosine monophosphate dehydrogenase (IMPDH) converts TIMP into thioxanthine monophosphate (TXMP). Guanosine monophosphate synthetase (GMPS) converts TXMP into thioguanylic acids which then form the TGNs. TGNs are the active metabolites of thiopurines which incorporate the sulphur moiety into the phosphoribonucleotide backbone of DNA preventing DNA unwinding. ATP-binding cassette sub-family C members 4 and 5 (ABCC4, ABCC5) have been implicated in the cellular efflux of TGMP, the activity of which may vary depending on individual genotypic variances. AZA: azathioprine; MP: mercaptopurine; GST: glutathione-S-transferase; TX: thioxanthine; TUA: thiouric Acid; AOX: aldehyde oxidase; XDH: xanthine dehydrogenase; OH-MP: 8-hydroxy-mercaptopurine hydroxy-mercaptopurine; HPRT: ; MeMP: methylmercaptopurine; TPMT: thiopurine methyl transferase; MeTIMP: methylthioinosine monophosphate; TIDP: thioinosine diphosphate; TITP: thioinosine triphosphate; ITPase: inosine triphosphate pyrophosphatase; TGMP: thioguanine monophosphate; TGDP: thioguanine diphosphate; TGTP: thioguanine triphosphate; TG-rib: thioguanine riboside.

Lastly, TG has also been shown to be effective at inducing and maintaining clinical remission in both CD and UC.[103, 104] There are concerns regarding the development of nodular regenerative hyperplasia (NRH), a chronic non-cirrhotic liver disease. However, studies which suggested this risk were in patients on high doses of TG (>40mg). A cohort study of 54 patients from Guy's and St Thomas' Hospital (GSTT) (median follow-up of 16 months) found no NRH and TG successfully circumvented many of the intolerances (e.g pancreatitis) associated with conventional thiopurines. Response rates at 6 months were 59%.[105] The bioavailability of TG is 14-46% and, due to rapid intracellular transport, the drug is undetectable at 6 hours.[106]

1.8 Mechanisms of thiopurine action

This section outlines a number of proposed mechanisms by which thiopurines could affect the immune system. The theory that this occurs only through the insertion of "rogue nucleotides" into the DNA of immune cells, preventing DNA replication, is an oversimplification.[107, 108] DeoxythioGTP (dTGTP), is in fact a good substrate for DNA polymerase and is able to become fully incorporated into DNA, although the stability of base pairing appears less and the risk of mutations more than with standard base pairing.[109 110]

A more likely mechanism is that the replacement of the single oxygen atom of guanine by sulphur increases the susceptibility of the base to several potential biochemical reactions due to its propensity to undergo methylation. Indeed, SAM methylates 6-TG bases into DNA 6-meTG.[111] This new configuration is more susceptible to post mismatch repair (MMR) processing, arresting the cell in G2 phase of the cell cycle.[112] This theory is confirmed by the fact that defective MMR processes afford cells a degree of resistance against thiopurine-mediated cytotoxicity.[113] Additionally, DNA 6-meTG is more vulnerable to oxidation by ultraviolet light generating highly damaging reactive oxidative species (ROS) which causes collateral damage to normal DNA bases; [111, 114, 115] this might explain the increased risk of skin cancers in patients on thiopurines.

The second mechanism proposed is through purine starvation by methylated metabolites. For example, MeTIMP is an inhibitor of phosphoribosyl pyrophosphate (PRPP) amidotransferase, the enzyme required for *de novo* purine synthesis to form TIMP.[116] This inhibition results in a delay of cells in the S phase of the cell cycle, thereby suppressing cell growth. This may explain why the formation of methylated metabolites in thiopurine metabolism could cause myelosuppression independently of high TGNs.

The third potential mechanism involves the inhibition of telomerase by dTGTP. Telomerase is essentially the DNA polymerase responsible for the maintenance of telomeres at the end of chromosomes. Telomerase is especially active in cancer cells. Numerous nucleotide analogues inhibit telomerase hence their use as anti-tumour and anti-viral agents. dTGTP has a free hydroxyl group and when incorporated into DNA, makes extension by telomerase impossible.[117] Indeed, telomere length has been found to be shortened in IBD patients on AZA compared to IBD patients not on AZA.[118] dTGTP also competes with other endogenous nucleotides such as GTP for Rac 1 binding causing T cell apoptosis;[119] this has been illustrated *in vivo* where AZA caused the presence of apoptotic T cells in the lamina propria of IBD patients.[120]

There is further evidence that thiopurines cause immunosuppression. MP has been shown to impair *in vitro* differentiation of dendritic cells, reducing the pro-inflammatory proteins IL-23 and CCR7, whilst increasing the anti-inflammatory cytokine, IL-10.[121] AZA has been shown to affect the phenotype of macrophages by altering the surface expression of RM3/1, a macrophage differentiation antigen.[122] Mouse models have confirmed that ^{3}H thymidine labelled monocytes and macrophages are reduced in a dose-dependent fashion in response to AZA.[123] More recent studies *in vivo* have shown that IBD patients on thiopurines have lower CD3-negative lymphocyte counts with significant reductions in natural killer and B cells.[124]

1.9 Metabolite monitoring

Initially, drug monitoring involved measuring plasma MP and urinary excretion of TUA. However, neither correlated with clinical response and were only useful for monitoring adherence.[125] An assay was then developed which measured RBC TGNs from whole blood.[126] Several studies have confirmed that RBC TGNs and MeMP are not significantly altered by the timing of blood sampling.[127] In fact, the stability of these metabolites means that the measurement of RBC TGNs and MeMP represent the accumulation of metabolites over a 4 week period.

The benefits of measuring these metabolites were first shown in patients with leukaemia where the efficacy from escalating doses of MP was associated with higher TGNs.[128] In 2005, Roblin et al compared the benefit of achieving TGNs > 250 pmol/8 X 10^8 RBC in steroid-dependent IBD patients on AZA. Those patients who did not have TGNs optimised to > 250 pmol/8 X 10^8 RBC had significantly lower clinical remission rates.[129] In 2006, a meta-analysis compared the clinical remission rates of patients with TGNs > 230-260 pmol/8 X 10^8 RBC compared to < 230 pmol/8 X 10^8 RBC showing them

to be 62% and 36% respectively. The difference in TGNs between patients with active disease versus patients in clinical remission was 66 pmol/8 X 10^8 RBC.[130] A larger cohort study confirmed a favourable relationship between TGNs above a particular value, and clinical response.[131]

However, a prospective study of 113 patients showed no relationship between TGNs and sustained clinical remission.[132] A smaller study of 57 patients randomized to either an adapted dose of AZA according to TGNs (therapeutic range 230-450 pmol/8 X 10^8) or normal dosing, showed no significant difference in clinical response although this study could be critiqued for being underpowered.[133]

What might explain the discrepancy between these studies is how TGNs are measured which is as a composite of TGMP (Thioguanine monophosphate), TGDP (Thioguanine diphosphate) and TGTP (Thioguanine triphosphate) because the conventional assay, using acid hydrolysis HPLC analysis, degrades these individual TGNs to the nucleobases. Therefore, TGTP is not measured directly but reported as the hydrolysed base. Ion-pair HPLC with fluorescence detection can measure each of the TGNs. The relative concentrations of each of the TGNs are shown in Table 1.9 with 80% of the TGN pool being TGTP. Neurath el al measured the different TGNs individually showing that levels of TGDP > 15% correlated with a lack of clinical response to AZA, whereas the higher the TGTP, the more likely the clinical response.[134] This may explain why some patients have therapeutic TGNs but lack response, if in fact, TGDP is > 15%, and why some studies differ on the efficacy of optimizing TGNs. The problem with measuring individual TGNs is their instability at storage temperatures with one study demonstrating that individual TGN levels reduced by 53% when stored at 22°C and by 90% when stored at 4°C, over a 7-day period.[135]

Whilst the debate continues as to whether metabolite measurements improve outcomes, it is without doubt that they allow for the detection of poor adherence, thiopurine-refractory disease and patients at risk of adverse effects. Hepatotoxicity and myelosuppression were found to be related to both high TGNs and MeMP levels (> 5700 pmol/8 X 10^8 RBC have been associated with a 3-fold risk of hepatotoxicity whilst TGNs > 450 pmol/8 x 10^8 RBC and MeMP > 11,450 pmol/8 X 10^8 RBC increase the risk of myelotoxicity).[136,137]

Table 1.9: HPLC-LCMS can be used to separate TGNs into its 3 constituent nucleobases the proportions of which may impact on the clinical efficacy of thiopurines.

TGN	% of TGN pool measured by HPLC- LCMS
TGMP	Trace
TGDP	16%
TGTP	80%

Finally, another issue regarding metabolite monitoring is that although metabolites are measured in RBCs, RBCs are not the target cell for the drug. It would make more sense to measure the metabolites within WBCs. Metabolites have been shown to significantly vary between cell types and between studies. For example, one study showed that TGNs were 31xhigher in leukocytes than in RBCs whilst another found that leukocyte TGNs were only 2-fold higher and that increases in leukocyte TGN correlated well with increases in RBC TGN.[138] One study showed that MeMP in leukocytes were undetectable whilst another study found detectable levels of MeMP in the leukocytes from murine leukemic cell lines, which increased relative to the dose of MP.[139, 140] The reason why TGNs might not be as high in RBCs as in WBCs is that mature RBCs lack IMPDH and therefore are unable to metabolise MP to GMP. Instead RBC TGNs reflect what is absorbed from their extracellular environment during red cell maturation.[125] With TG, higher TGNs are measured.[105] This reflects the fact that RBCs are able to salvage TG directly to TGN via HPRT as opposed to the formation of TGNs from MP which requires IMPDH.

1.10 Thiopurine hypermethylation

Approximately 15-24% of IBD patients preferentially produce MeMP levels as a proportion of TGN when treated with AZA or MP.[131, 136] This is known as hypermethylation or "thiopurine shunting", the standard definition for which is an MeMP to TGN ratio > 11. MeMP:TGN ratios > 11 have been found to correlate with a lack of efficacy in the treatment of IBD and, as previously stated, MeMP levels > 5700 pmol/8 X 10^8 RBC have been associated with a 3-fold increased risk of hepatotoxicity whilst MeMP > 11,450 pmol/8 X 10^8 RBC increase the risk of myelotoxicity.[136, 137, 141] With hypermethylation, when the dose of AZA or MP increases, MeMP increases disproportionally, with TGN levels often remaining sub-therapeutic.

One might expect TPMT activity to be a predictor of hypermethylation and, of the patients who are more likely to suffer from reduced efficacy and adverse effects. Indeed, hypermethylation is not seen in low (completely deficient) and intermediate (carrier range) TPMT phenotypes and patients with an ultra-high TPMT phenotypes (>40 pmol/h/mgHb) are less likely to respond to treatment.[142] However, there is a very poor concordance between high TPMT and hypermethylation, suggesting that there may be other factors which play a role in the accumulation of MeMP.

There are generally 2 potential ways of treating hypermethylation. The first and most conventional approach is to reduce the dose of AZA or MP to 25-50% and add in allopurinol (low dose thiopurine with allopurinol (LDTA)).[143] The second and less orthodox approach, is to split the dose of AZA or MP to a twice daily dose. This has been shown to be successful in one study of 20 patients with MeMP > 7000 pmol/8 X 10^8 RBC. Dose splitting resulted in a significant reduction in MeMP (11,879 vs 5955 pmol/8 X 10^8 RBC) whilst not altering TGNs (250 vs 227 pmol/8 X 10^8 RBC).[144] I have replicated this outcome as part of my own research and added it as an appendix to this thesis. The rationale for dose splitting is based on the hypothesis that lowering the dose of thiopurines, reduces the concentration of MP to below the substrate affinity for TPMT, thereby reducing its methylating activity and the amount of MeMP produced. Indeed, one study showed that different metabolites of the purine salvage pathway formed at different concentrations of the drug and that MeMP was only measured once their concentration was high enough.[145]

1.11 Thiopurine hepatotoxicity

Liver injury through thiopurines is the central theme of this thesis. Hepatotoxicity from thiopurines affects 5% of all patients and up to 20% of hypermethylators, although a causal relationship between MeMP formation and hepatotoxicity remains unestablished.[143] A prospective study evaluated thiopurine-induced hepatotoxicity and concluded it as being either dose-dependent or dose independent (allergic/idiosyncratic).[146] Of a cohort of 161 patients, 13% developed abnormal LFTs (defined as > 50% ALT or ALP rise), occurring after a median of 85 days (2-951). Over 80% were hepatocellular, whilst the remainder were cholestatic. A third had their treatment withdrawn, whereas just under half continued on thiopurines at the full dose following a temporal adjustment, with subsequent resolution of hepatotoxicity. The main predictor of hepatotoxicity was the concomitant use of steroids whilst males were also more likely to develop drug-induced liver injury, as has been suggested by other studies.[147] In some patients, thiopurine hepatotoxicity only occurred after a long time into therapy, confirming that a cumulative effect of metabolites (or dose-dependent) can cause liver injury.[148]

Several models have been proposed for thiopurine hepatotoxicity. The most compelling model is a dose-dependent "de-energization" process leading to cell necrosis as demonstrated in rat hepatocytes incubated with different concentrations of AZA/MP.[149] During exposure to varying concentrations (0.1-5µmol/L) of AZA/MP, a progressive decline in hepatocyte viability, measured as LDH leakage, followed by cell necrosis, was seen over 4 days of incubation. This was accompanied by increased oxidised glutathione, reduced mitochondrial glutathione synthetase (GSH) compared to cellular GSH, ultrastructural evidence of mitochondrial injury and a decline in cellular ATP content. The addition of GSH and NAC protected against AZA-induced cell necrosis in previous studies. Consistent with this was that the depletion of GSH, prior to incubation with AZA, resulted in increased cell death. Through the addition of allopurinol and Trolox (a vitamin E analogue), xanthine concentrations and GSH levels were preserved, preventing hepatotoxicity. Hence a model of hepatotoxicity has been proposed whereby the release of reactive oxygen species (ROS) through XO mediated oxidative stress, depletes GSH and ATP, leading to mitochondrial damage and cell necrosis (Figure 1.11).

The dose-independent process for thiopurine hepatotoxicity might be explained through the release of formaldehyde. Formaldehyde has been associated with hepatotoxicity in rat livers through the depletion of glutathione with subsequent hepatocyte mitochondrial damage confirmed on electron microscopy [150, 151] The mechanism by which formaldehyde is released during thiopurine metabolism will be discussed in detail in Section 1.16, however in short, the demethylation of MeMP metabolites by cytochrome P450 has been proven to release formaldehyde in hepatocyte mitochondria.[152]

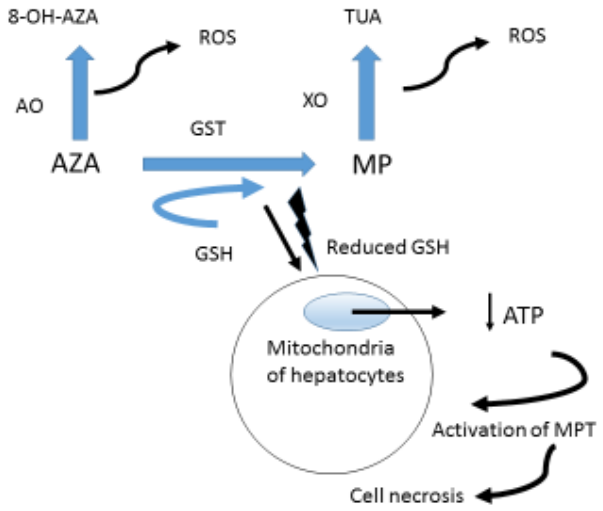


Figure 1.11: A proposed mechanism for thiopurine hepatotoxicity as suggested by Tapner et al.[149] The formation of ROS, through XO mediated oxidative stress as a result of thiopurine breakdown, and the conversion of AZA to MP by GST, leads to a depletion of GSH and ATP, resulting in the death of mitochondria within hepatocytes and subsequent liver injury. Interestingly, XO concentrations measured in liver tissue taken from the open liver biopsies or hepatectomies of 189 patients, have been shown to be higher in males, suggesting that hepatotoxicity from thiopurines may be more likely in males.[153] AZA: azathioprine; MP: mercaptopurine; AO: aldehyde oxidase; 8-OH-AZA: 8-hydroxy-Azathioprine; TUA: thiouric acid; ROS: reactive oxygen species; GST: glutathione s-transferase; GSH: reduced glutathione; ATP: adenosine triphosphate; MPT: mitochondrial permeability transitions.

In addition to the dose-dependent and dose-independent hepatotoxicity, there is also a link between thiopurines and a rare chronic liver disease called nodular regenerative hyperplasia (NRH), another central theme of this thesis. Again, depletion of GSH has been proposed as the mechanism by which sinusoidal damage and NRH occurs as a result of long-term thiopurine use.[154]

Indeed, reduced glutathione appears to be a central feature to thiopurine hepatotoxicity and is a common mechanism by which other drugs cause liver injury too, most notably paracetamol. Another potential mechanism by which glutathione is reduced during thiopurine metabolism will be discussed in Section 1.17, when the role of the TPMT cofactor, SAM, in hepatotoxicity is discussed.

1.12 Nodular regenerative hyperplasia (NRH)

NRH is a type of non-cirrhotic liver disease occurring due to sinusoidal damage and disruption of the hepatic microcirculation. This leads to ischaemia and atrophy of local liver parenchyma followed by compensatory hyperplasia in tissue which remain normally vascularised (Figure 1.12.1). 50-70% of patients with NRH present with abnormal LFTs only, whilst others may present with the features of portal hypertension such as varices or ascites.[155, 156] Prognosis is variable with very few patients requiring liver transplantation.[157-159]

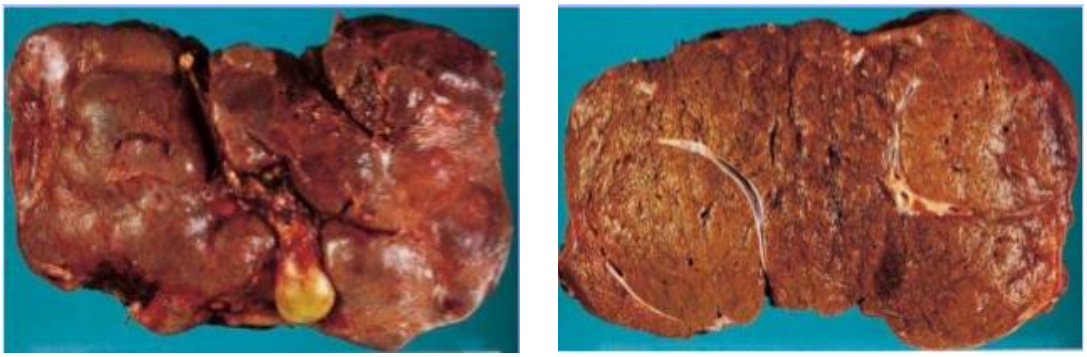


Figure 1.12.1: The gross appearances and cut surface of a liver with NRH. Whilst the gross appearances are similar to that of cirrhosis, the cut surface shows less defined nodular transformation compared to that seen in cirrhosis with no fibrous septa.[160]

Although 0.5-2.6% of the population have been shown on autopsy studies to have NRH, drugs, especially thiopurines, are the major cause in younger age-groups.[161] The mechanism by which thiopurines cause NRH is poorly understood. The only mechanism proposed is through the depletion of GSH causing sinusoidal endothelial cell (SECs) injury, identical to how thiopurines injure hepatocytes as outlined in Figure 1.11. Pre-incubation of SECs in GSH likewise prevented GSH depletion and toxicity. One study found that murine sinusoidal endothelial cells (SECs) were in fact more vulnerable to toxicity (reduced cell viability) when incubated in AZA, than hepatocytes.[154] This endothelial cell toxicity leads to microcirculation damage, local ischaemia and veno-occlusive disease, the pathological hallmark of NRH (Figure 1.12.2).

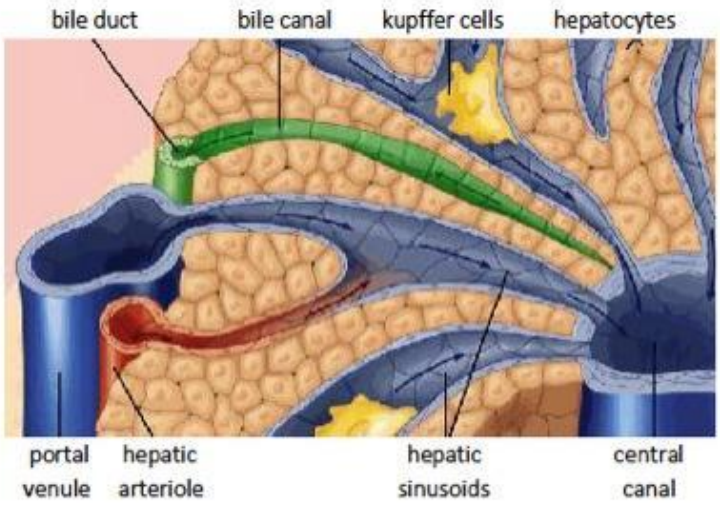


Figure 1.12.2: Sinusoids are the microcirculation of the liver and arise from tributaries of the portal vein and hepatic artery. They drain into the central canal and then finally the hepatic vein. They are separated from hepatocytes by the Space of Disse and lined by sinusoidal endothelial cells (SECs) which are prone to damage when exposed to drugs such as AZA and TG.[149] This leads to sinusoidal dilatation and local ischaemia of liver parenchyma, followed by hyperplasia and nodular transformation of normally vascularised tissue. *Image obtained from studyblue.com*.

Concerns regarding TG and NRH were first raised following a study in children with acute lymphocytic leukaemia who were randomised to receive either 75 mg MP or 60 mg of TG.[162] 20% of patients in the TG arm developed veno-occlusive disease (VOD or sinusoidal obstruction), and their dose was subsequently reduced with no recurrence of VOD. However, of those who continued TG, 4% (of whom 95% were male) developed signs of portal hypertension such as splenomegaly and varices.[163]

3 case reports of patients with IBD on TG diagnosed with NRH were published leading to further safety concerns.[164] In 2003, 111 patients on a median dose of 40 mg TG for a median of 10 months, were grouped according to abnormal (n=29) or normal platelet counts and LFTs.[165] Most patients had received at least a year of AZA, MP or both, prior to switching to TG. Thrombocytopenia occurred in isolation as did abnormal LFTs with the transaminases raised more than the ALP. Of those patients with abnormal blood tests (LFTs or platelets), 17 patients underwent liver biopsy and 75% were found to have features of NRH on biopsy. 11% of those patients with normal blood tests were also biopsied and 55% were found to have NRH. The conclusions were that NRH can develop silently in the context of normal blood tests, but more commonly will present with thrombocytopenia, abnormal LFTs or both.

A multicentre internet-based reporting study on TG causing suspected NRH was published in 2007.[166] Centres were asked to report patients on TG who developed abnormal LFTs and NRH. The results showed a prevalence of 14.5% having abnormal LFTs. Of the entire cohort, 60 patients had a liver biopsy, of whom 27% had histopathological confirmation of NRH. The median duration of treatment was 118 weeks (range 21-169 weeks) and the median dose was 40 mg. Age was felt to be a significant contributor for the diagnosis of NRH along with abnormality of LFTs and/or FBC (sensitivity of 68.8%) and an abnormal ultrasound of the liver. Both CT and MRI failed to show differences between the NRH and the non-NRH groups.

The same year, a prospective study of 28 patients after 30 months of TG treatment, at a median dose of 19.5 mg, demonstrated only 2 patients being equivocal for NRH on liver biopsy.[167] The conclusions were that NRH was probably TGN-dependent. The same group performed a retrospective study on thiopurine-naïve IBD patients who had had liver biopsies performed at the time of surgery.[168] 6% had histological evidence of NRH, a similar percentage to that seen due to TG.

The risks of NRH in patients taking AZA have also been assessed. Between 1994 and 2005, a prospective study by the GETAID group (Groupe d'Etude Therapeutique, Belgium, France, Switzerland) found 37 patients with biopsy-proven NRH.[169] Significantly more patients with CD had NRH compared to UC (31 vs 6), and significantly more were male (81%). The median duration of treatment was 48 months (range 6-187) at a mean dose of 2 mg/kg/day. Indications for biopsy included clinical signs of portal hypertension (N=12), radiological suggestion of liver disease (N=4) and abnormal biochemistry (N=21). In terms of blood tests, 5% had no abnormalities, whilst 14% had liver abnormalities only, 11% had thrombocytopenia only and 70% had both. 84% had portal hypertension either radiologically or endoscopically. Significant risk factors after multivariate analysis were male gender and stricturing CD.

There are conflicting conclusions from these seminal studies as to the prevalence of NRH in thiopurine patients. Indeed, from our own cohort at GSTT of 54 patients on TG, no NRH was diagnosed, despite 11 patients having liver biopsies, although TGNs were lower (1071 versus 15,680 pmol per 8 x 10^8 RBC) and duration of treatment shorter (16 versus 70 months) compared to other studies.[105] The variation in prevalence of NRH between studies most likely lies in the difficulty that clinicians have in diagnosing it. Diagnosing NRH non-invasively remains a challenge and so liver biopsy remains the gold standard, although both MRI, fibroscan and portal pressure measurements have all been assessed for comparative sensitivity and specificity. One study performed both gadolinium-enhanced MRI and liver biopsies in 45 patients after at least 8 weeks of 40-60mg TG.[170] Eight patients had evidence of NRH on biopsy whilst eight were equivocal. The sensitivity and specificity of gadolinium-enhanced MRI was

77% and 72% respectively. Fibroscan, which grades fibrosis in patients with liver diseases leading to cirrhosis, is comparatively poor at diagnosing NRH.[171] Transjugular portal pressure measurements can show a mixed picture with hepatic venous pressure gradients (HVPG) tending to be mildly elevated. HVPG range varies from 5-10mmHg in keeping with a presinusoidal/sinusoidal pathology due to a combination of obliterative venopathy and sinusoidal obstruction.[172] Although variable, it does help exclude cirrhotic causes of portal hypertension where HVPG would be higher. Even liver biopsy, via the percutaneous or transjugular approach, is prone to inter-observer disagreement and the risk of fragmentation.[169] Buster et al discuss a case typical of patients presenting with NRH.[173] Their patient had cholestatic LFTs 8 years before suffering a variceal bleed. The patient underwent more than one indeterminate liver biopsy. It was not until the patient had significant portal hypertension, that NRH was diagnosed on biopsy. This raises issues regarding the sensitivity of liver biopsies and when to perform one.

Buster et al also describe a case of NRH developing in a patient on AZA for immunosuppression following liver transplantation, who was a heterozygote for TPMT*3C and ITPA (94C>A).[173] Breen et al describe 2 further cases of NRH developing in TPMT*3A heterozygotes.[174] The role played by TPMT genotypes in the development of NRH is an interesting one. Since NRH is thought to be related to high TGNs, TPMT heterozygotes could be more at risk. Although these patients were wild type for methyltetrahydrofolate (MTHFR) genotypes, MTHFR and homocysteine (HCys) have also been implicated in the development of NRH. One case report describes a patient with a triad of hip necrosis, portal vein thrombosis and NRH with associated hyperhomocysteinaemia due to an MTHFR mutation for 677C>T.[175]

The potential for reversibility in some patients raises the possibility of there being a “point of no return” in the complications which ensue from NRH.[173] In a study of patients who were on AZA after orthotopic liver transplantation, 9 patients developed NRH (median 64 months of treatment).[176] After stopping AZA, whilst 5 patients exhibited reversibility, either histologically or biochemically, 4 patients progressed requiring repeat transplantation.

Finally, it is reported that 8% of HIV positive patients on didanosine develop NRH with an OR of 3.4 (95% CI, 1.5-8).[177] Didanosine is also a purine analogue and shown to cause oxidative stress and mitochondrial dysfunction with subsequent hepatotoxicity in a similar way to thiopurines.[178] NRH is also associated with oxaliplatin chemotherapy and other antiretroviral agents.[179]

1.13 Use of allopurinol co-therapy to prevent hepatotoxicity

Allopurinol was developed by Gertrude Elion to increase the bioavailability of MP by preventing its degradation to TUA.[180] Only later was allopurinol's mechanism of action extrapolated to treating hyperuricaemia and gout. Allopurinol has a short half-life (3 to 4 hours), a fact that runs contrary to its ability to cause sustained reductions in uric acid levels. However, it was discovered that allopurinol is not only a competitive inhibitor of XDH but is itself oxidised to form oxypurinol, a compound with a much longer half-life of 18-30 hours due to is reabsorption in kidney tubules. Since oxypurinol is not absorbed orally, but allopurinol is, allopurinol acts as the ideal oral prodrug.

Allopurinol-thiopurine co-therapy showed no enhancement in efficacy in the treatment of childhood leukaemia but instead resulted in toxicity.[81] Indeed, TGNs were found to be significantly higher in renal transplant patients on allopurinol-thiopurine co-therapy compared to patients on AZA alone; this correlated with reductions in WBCs.[181]

By inhibiting XDH, more MP is available to be metabolised by HPRT to form TIMP and later TGNs; hence the rise seen in TGNs on co-therapy. As far more TGNs form, so the dose of AZA (or MP) needs reducing to 25-50% of the original dose.[182] This approach prevents hypermethylation, as the reduction in dose results in far less MP available to undergo methylation by TPMT to form MeMP. Instead, shunting towards TGNs becomes far more efficient with minimal or even no methylation to MeMP or oxidation to TUA.

Running contrary to this hypothesis is the observation that in TPMT deficient patients, the dose of AZA needs to be reduced to 10% suggesting that 90% of MP is methylated in TPMT wild types. However, adding in allopurinol to TPMT wild types needs a reduction to 25% suggesting that 75% of MP is oxidised to TUA. Therefore, there must be overlap in the actions of allopurinol to inhibit both TPMT and XDH. Several studies have looked at this both *in vitro* and *in vivo*, the hypothesis being that the formation of thioxanthine (TX) and oxypurinol, inhibit TPMT. Allopurinol results in a rise in both compounds, hence indirectly inhibiting TPMT (Figure 1.13).[183]

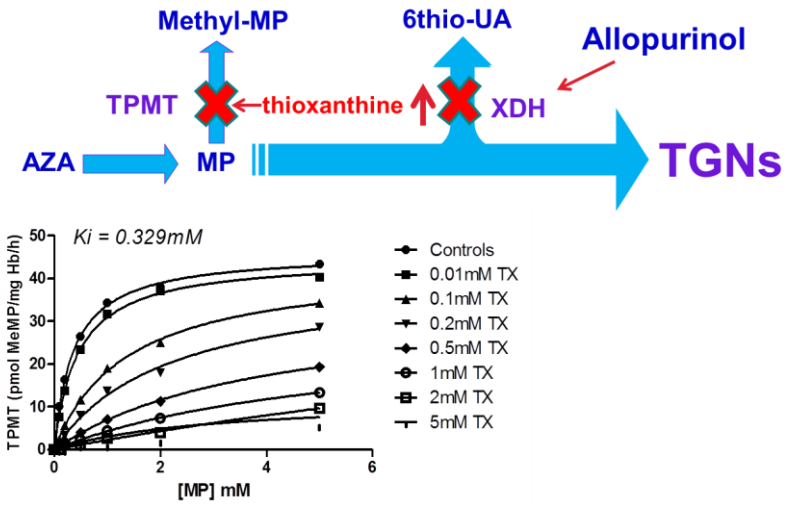


Figure 1.13: The Purine Laboratory at St Thomas' Hospital showed that allopurinol indirectly inhibits TPMT. This occurs via TX accumulation, a potent TPMT inhibitor. Increasing concentrations of TX correlated with reductions in TPMT activity relative to increasing concentrations of MP, as shown in the figure. Allopurinol thereby allows the dose of AZA and MP to be reduced by 25%, forming the same concentration of TGN but little or no MeMP.[183, 184] TX: thioxanthine; Methyl-MP: Methylmercaptopurine; 6thio-UA: 6 thiouric acid; XDH: Xanthine dehydrogenase.

The combination of low dose thiopurine (25-50% of the original dose) with 100 mg allopurinol (LDTA) has been shown to successfully correct hypermethylation, reducing the formation of MeMP significantly, and bypassing side effects such as hepatotoxicity.[183,185] It also improves the efficacy of thiopurines in patients who had been previously poor responders by allowing for the optimisation of TGNs without risking hypermethylation.[182]

Some groups suggest LDTA can be used first-line in all thiopurine-naïve patients with IBD, whereas others only advocate LDTA in patients with a high TPMT (>35 pmol/h/mgHb) where the likelihood of hypermethylation can be pre-empted, although the correlation between high TPMT and hypermethylation is tenuous. In a recently published study, clinical response rates in patients on LDTA were 79% with CD and 64% with UC. 89% of patients who had previously had side effects with monotherapy were able to overcome these with LDTA.[186]

LDTA may not be advisable in all patients as there are potential risks with allopurinol. Firstly, there is no safety data to advocate allopurinol use in pregnancy. Secondly, allopurinol can cause severe skin reactions such as Stevens-Johnson syndrome and toxic epidermal necrolysis to which patients possessing the HLA-B*5801 allele, are particularly vulnerable.[187]

1.14 Pharmacogenetics of thiopurine metabolism

Pharmacogenetics is the study of the association between variability in drug response, drug toxicity and polymorphisms in genes in order to adapt drugs to a patient's specific genetic background and therefore make them more efficacious and safe.[188] Prospective observational studies have shown that adverse drug reactions account for 6.5% of hospital admissions and cost the NHS £466m per year.[189]

It has been proposed that genes contribute to 40% of inter-individual variation in treatment outcomes.[190] A polymorphism is the existence of 2 or more alleles or sequence variants within a given population. A single nucleotide polymorphism (SNP) is an exchange, insertion or deletion at a particular locus within a DNA sequence. SNPs can occur either within coding sequences (or exons) or in between coding sequences (introns). Exonic SNPs are less common than intronic SNPs (1 in every 2500 bases) but as they affect amino acid sequences and hence potentially protein function, they tend to have the greatest impact.[191]

This section highlights the SNPs that have so far been found relevant to thiopurine metabolism and how we might use them in practice. Obviously, pharmacogenetics in the form of TPMT testing has already been in clinical practice for some time. However, new SNPs associated with the risk of pancreatitis from AZA, may be used in clinical practice soon. Genome wide assay studies (GWAS) have proven a strong association within the class II HLA region at rs2647087 (HLA-DQA1*02:01-HLA-DRB1*07:01) with homozygous variants having a 17% risk of AZA pancreatitis and heterozygotes having a 9% risk.[192] Testing for NUDT15 variants will soon be commonplace in clinical practice. Although the incidence of these variants is higher in East Asians, they have also been shown to increase the risk of thiopurine-induced myelosuppression in those of European Ancestry.[193-195] NUDT15 phosphorylates TGTP and TdGTP preventing their incorporation into DNA and hence their immunosuppressive effects – homozygous variants for NUDT15 have been shown to reduce leukocyte counts by 88% from baseline.[193]

Other SNPs which have been found relevant include GST, the enzyme which converts AZA to MP. One of three SNPs located on the GST-M1 gene, has been associated with a complete absence of enzyme activity.[196] This GST-M1 null genotype has been shown to be protective against leukopenias and is carried in 50% of people.[197] This may explain the variation of tolerability for AZA and why some 60% of patients find MP more tolerable.[198]

The 3 most common genetic variants for TPMT have already been discussed (see Section 1.7): TPMT*2,*3A and *3C (the allelic frequencies in Caucasians are 0.17%, 4.5% and 0.4% respectively). [199] These TPMT alleles, coded for on chromosome 6, culminate to produce a trimodal distribution of activity and ultimately a different response and adverse effect profile.[200, 201]

The MTHFR gene is also polymorphic, with SNPs being shown to reduce TPMT activity of patients who should ordinarily be within the wild type range. MTHFR is the rate determining enzyme of the Folate Cycle converting 5,10 MTHFR to 5-MTHFR which, together with methione synthase (MS), convert HCys to methionine with vitamin B12 as the cofactor. The most studied variants are 677 C>T and 1298 A>C located on chromosome 1. These two variants reduce the activity of MTHFR resulting in lower folate levels, higher HCys, reduced SAM and increased SAH.[202] These findings bear relevance to Section 1.17 where SAM, SAH and the Methionine Cycle are discussed in greater depth. In one study, patients with acute lymphoblastic leukaemia (ALL) on thiopurines, who carried variants for both MTHFR and TPMT, were more likely to suffer bone marrow toxicity.[203]

XDH and XO are both inter-convertible forms of the same enzyme according to its redox state. In the centre of the enzyme is a molybdenum cofactor which is the site at which oxidation of MP to TUA occurs. The enzyme is a competitor with TPMT for the metabolism of MP. It is therefore no wonder that enzyme deficient patients (classic xanthinuria type 1) should be prone to significant toxicity from AZA.[97] Whilst measurements of XDH activity have shown little inter-individual variation, direct activity as measured in liver autopsy samples have demonstrated a four-fold inter-individual variation in activity with 20% of men and 27% of females having low activity.[153, 204] *In vitro* studies have shown 8 SNPs; variants for the XDH and molybdenum co-factor sulfurase enzyme (XDH c.837C>T and MOCOS c.2107A) protected against some adverse effects from AZA in patients with IBD.[205] A variant for AOX1 (AOX1 c.3404A > G) predicted a lack of response to AZA. Combining knowledge of these 3 variants with TPMT activity, allowed for the chances of responding to AZA to be divided into 2 groups where one group had an 86% chance of responding and the other only 33%.

The HPRT gene is located on the X chromosome, hence variants are only expressed in males. In Lesch-Nyhan Syndrome, patients lack HPRT activity and have been shown to be especially resistant to AZA treatment.[206] In one study in children with ALL, there was a 10-fold variation in HPRT activity with children being less responsive to AZA if they had low activity.[207]

IMPDH activity is encoded by 2 genes; Intra-individual variation in activity is low and hence unlikely to explain variations in thiopurine metabolism.[208]

ITPase (Inosine triphosphate pyrophosphatase) plays a role in preventing the accumulation of harmful rogue nucleotides such as deoxy ITP being incorporated into DNA and disrupting the function of normal cellular processes.[209] Low activity is associated with the accumulation of ITP in RBCs and has been associated with multiple birth defects in mice.[210] In humans, ITPase activity is genetically determined. In Caucasians, 2 SNPs have been found (c.94C>A and IVS2 +21A>C) which result in a low activity through misplicing of the ITPA gene.[211] Homozygous variants for c.94C>A lack any ITPase

activity whilst activity is reduced to 22.5% in heterozygotes. Meanwhile, heterozygotes for IVS2 +21A>C have 60% activity.[209] The relevance of ITPA variants to thiopurines is that ITPase degrades the thioinosine nucleotides (TIDP and TITP) to TIMP which is then the substrate for IMPDH to form TGNs. Hence, lower ITPase activity might result in lower TGNs and reduced efficacy and, as there is an increased thioinosine nucleotide pool for methylation, more side effects may occur. Indeed, ITPA c.94C>A variants were associated with pancreatitis, flu-like illness and early treatment failure in a cohort of patients with Crohn's disease.[212, 213] However, this relationship has not been replicated in further studies.[214]

SNPs within genes coding for transmembrane cellular transporter pumps, which govern the influx and efflux of thiopurine metabolites, have been identified as playing potential roles for determining metabolite profiles. The importance of transporter pumps is highlighted by studies on human T-cell lymphoblastic cell lines, where reduced MP uptake conferred a resistance to MP.[215] SLC28 and SLC29 transporters import molecules down and against the concentration gradient respectively. Although the genetic variation in the SLC transporter family has been shown to be lower than other transporter families, a variant of SLC38A1 has been found relevant to gemcitabine treatment in that tumour cells expressing the variant were found to have a lower affinity for uptake of gemcitabine.[216]

ATP-binding cassette (ABC) transporters are energy-dependent exporter pumps and highly expressed in the colon and small intestine. Variants for ABCB1 have been associated with a lack of response to AZA in CD as well as in ALL, potentially through increasing the efflux of AZA metabolites.[217, 218] ABCB5 shares similar homology to ABCB1. The ABCB5 c.343A>G variant was associated with lack of response and lower TGNs in candidate gene analyses whilst GWAS performed in our lab associated this variant with a weakly significant propensity to hypermethylation.[219]

Point-of-care genetic testing kits could offer IBD patients immediate information on the risks and benefits of thiopurine treatment according to detected polymorphisms interpretable either alone or in combination with others, offering exciting opportunities to improve treatment outcomes.

1.15 Non-genetic factors influencing thiopurine metabolism

Non-genetic factors, such as drugs (other than allopurinol), alter metabolite profiles. 5-ASAs have been shown to shunt metabolite formation from MeMP to TGNs, reducing the risk of hypermethylation in a dose-dependent fashion.[220] In so doing, they can increase the risks of leukopenia but also improve rates of efficacy. This has been shown to occur through the inhibition of TPMT.[221, 222]

Combination treatment with MTX and MP, both antimetabolites which inhibit *de novo* purine synthesis, has been shown to be better than MP alone at reducing circulating leukaemic cells in the treatment of acute leukaemia.[223] Although not used in clinical practice for IBD, there are case reports of this combination being used successfully for several dermatological conditions.[224] High MTX polyglutamate levels correlate with high TGNs, confirming a synergistic relationship between the two *in vivo*.[225] *In vitro* studies confirmed that TPMT activity was significantly reduced once MTX infusions were started, through MTX binding to and inhibiting TPMT.[226]

Ribavarin, an antiviral acting through IMPDH inhibition, has been reported to cause severe bone marrow suppression in a patient co-prescribed with AZA.[227] IMPDH activity has been shown *in vivo* to correspond inversely with RBC MeTIMP levels, although this did not correspond with changes in TGNs.[228]

TGNs measured before and after commencing IFX, demonstrated that IFX was associated with a significant increase in TGNs.[229]

Diuretics and non-steroidal anti-inflammatory drugs (NSAIDs) inhibit TPMT albeit weakly, although the clinical relevance of this has not been established.[230, 231]

1.16 CYP1A2 polymorphisms in demethylation of thiopurines

Demethylation is the process by which MeMPs are converted back to MP (Figure 1.16), a reaction which results in hepatotoxicity. This reaction was first demonstrated by Sarcione et al in 1960.[232] Rats injected with radiolabelled MeMP (MeMP-S^{35}) produced as a urinary metabolite, MP- S^{35}, in equal amounts as rats injected with MP- S^{35} produced MeMP- S^{35}, suggesting that the major catabolic pathways for MP and MeMP are in divergent directions. This was confirmed in humans by Elion et al when TUA was measurable in the urine excreted from patients given MeMP.[233]

CYP450 enzymes were shown to catalyse this demethylation. Studies showed that the demethylation of methylmercaptopurine riboside could be inhibited by up to 50% through pre-treatment of rat liver microsomes with ementine dihydrochloride, an inhibitor of CYP450.[234] The Purine Laboratory at GSTT has also confirmed the demethylation of MeMP in human liver microsomes mediated by CYP1A2 enzymes. Blaker et al measured the formation of MP from MeMP using specific recombinant human liver cytochromes incubated with and without β-Nicotinamide adenine dinucleotide 2-phosphate (NADPH), an essential cofactor for the reaction to occur. MP was only measurable in cytochromes CYP1A2 and CYP2C9 incubated in NADPH. The findings suggest that a balance between TPMT and demethylating enzymes are responsible for the variation in metabolite profiles and a focus for potential thiopurine drug interactions.

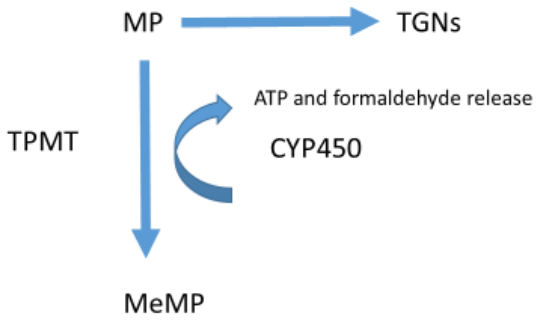


Figure 1.16: The demethylation pathway of MeMP under the influence of CYP450 enzymes with the resulting formation of ATP and formaldehyde. MeMP: Methylmercaptopurine;MP: Mercaptopurine; TPMT: Thiopurine methyltransferase; TGNs: Thioguanine nucleotides; ATP: Adenosine triphosphate.

Smoking has been shown in other models to induce CYP450 enzyme activity. One study on smoking and olanzapine, an antipsychotic drug metabolised by CYP450, demonstrated that smoking induced a six fold increase in CYP450 activity causing much lower levels of the drug.[235-237] This suggests that smoking could upregulate the demethylation process thereby reducing MeMP and increasing TGNs. In smokers, with particular CYP450 polymorphisms, this demethylation process may be upregulated. As the efficacy of thiopurines correlate with higher TGNs, this hypothesis could explain why in the TOPPIC study, smokers stood to benefit more in terms of lower levels of recurrence, from being on thiopurines, than non-smokers.[87]

The demethylation of drug metabolites by cytochrome P450 has been proven to release formaldehyde in hepatocyte mitochondria.[152] Formaldehyde has been associated with hepatotoxicity in rat livers through the depletion of glutathione.[150, 151]

There are several CYP1A2 polymorphisms, of these CYP1A2*F and CYP1A2*1C are the most relevant. Their effect on the CYP1A2 gene and relative allele frequencies are summarised in Table 1.16. This demethylation process may be an explanation for thiopurine hepatotoxicity through the release of formaldehyde, particularly in hypermethylators. If so, CYP1A2 variants and smoking may be factors in predisposing certain patients to thiopurine hepatotoxicity.

Table 1.16: The MAF for the 2 commonest CYP1A2 variants and their effect on the activity of the gene.

CYP450 polymorphism	Minor Allele Frequency	Activity of the CYP1A2 gene
CYP1A2*F	0.37	Increased
CYP1A2*C	0.2	Decreased

The CYP1A2*F variant has been associated with an altered phenotype through an increase in CYP1A2 enzyme activity (ultra-rapid metabolizer). This altered phenotype is observed only in the presence of an inducer such as smoking or heavy coffee consumption.[238]

1.17 SAM, SAH and Homocysteine and relevance to hepatotoxicity and hypermethylation

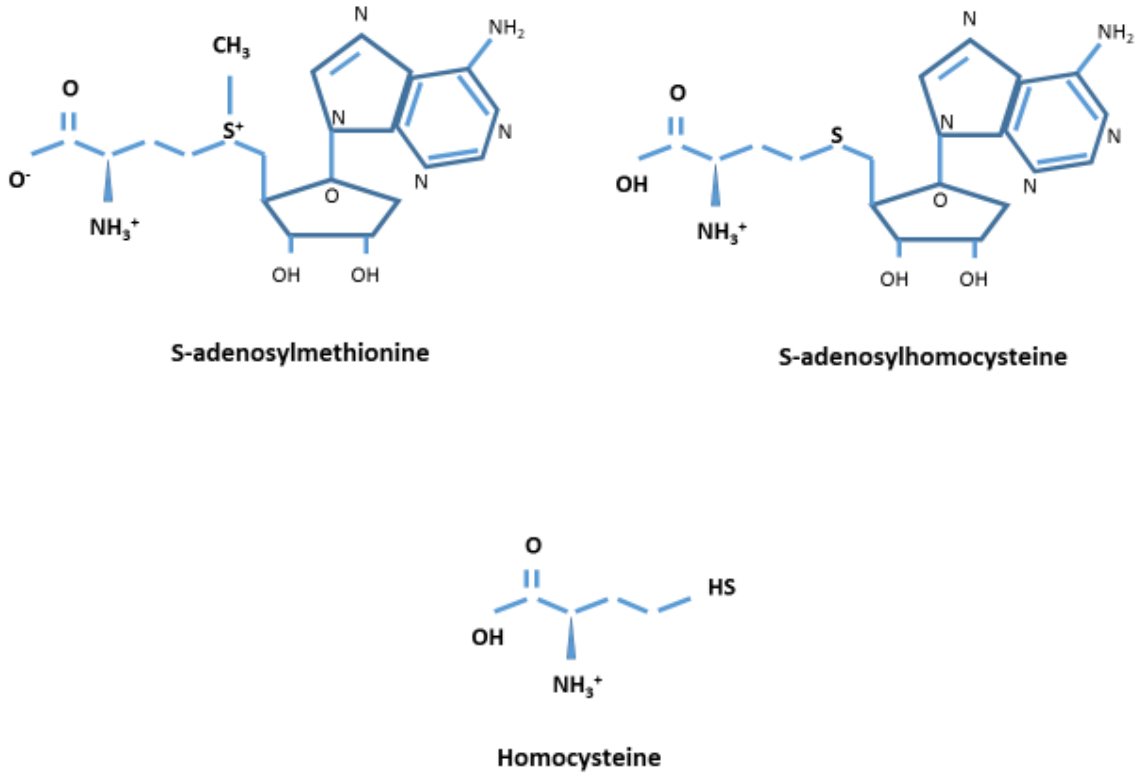


Figure 1.17.1: The structures of SAM, SAH and homocysteine

S-adenosyl methionine (SAM) is the chief methyl donor owing to its unique sulfonium centre and second to only ATP in terms of the number of reactions it is involved in. SAM is a metabolically pleotropic molecule, participating in 3 types of biochemical reaction, mainly in the liver: Transulfuration, transmethylation, and aminopropylation.[239] SAM is vital for the synthesis of a variety of compounds such as histones, nucleic acids, neurotransmitters and hormones. Conversely, SAM's by-product, s-adenosyl homocysteine (SAH), is a DNA methylase inhibitor, thereby competing with SAM but with a higher affinity for the methyltransferase active site.[240]

This section describes the 3 biochemical reactions of SAM and SAH and how concentrations of both compounds are regulated by the folate-dependent Methionine Cycle. The evidence for the relationship between the Methionine Cycle and liver disease is also reviewed. Lastly, difficulties in the measurement of SAM and SAH are discussed.

Transulfuration, transmethylation, and aminopropylation

SAH is hydroxylated by s-adenosyl homocysteine hydrolase (SAHH) to form homocysteine (HCys) and adenosine.[241] HCys is then the substrate for 2 competitive reactions. The first is re-methylation to form methionine, obtaining a methyl group from methyltetrahydrofolate (MTHF), catalysed by methionine synthetase (MS); methionine is then converted to SAM by the enzyme methionine adenosyltransferase (MAT). The second is transulfuration to form cysteine, catalysed by cystathionine- β-synthase (CβS) and cystathione-γ-lyase (cγl); cysteine is then metabolised to form glutathione synthetase (GSH) in the liver (Figure 1.17.2).

Aminopropylation synthesises polyamines via SAM decarboxylase, essential for an array of different biological processes from maintaining membrane potential to controlling cellular pH and volume. However, little SAM is used up for polyamine synthesis.[242]

The MTHFR Cycle provides the methyl group for the re-methylation of HCys to methionine. 5-MTHF is formed from 5,10-methyltetrahydrofolate (5,10 -MTHF) in a reaction catalysed by MTHF reductase (MTHFR). Folic acid is essential for the replenishment of 5,10 –MTHF through the formation of tetrahydrofolate (THF).[243]

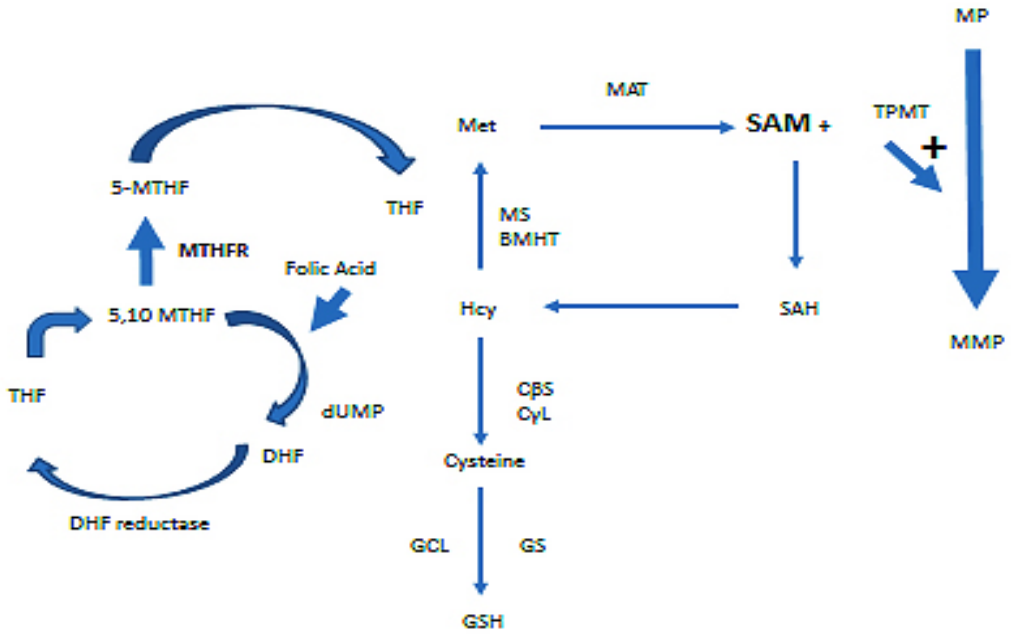


Figure 1.17.2: The transulfuration and transmethylation reactions, the effects on TPMT and the Methionine Cycle, and the formation of GSH. Transulfuration is the transfer of sulphur from methionine to form cysteine and GSH via Hycs. Transmethylation is where SAM acts as the methyl donor with TPMT to methylate MP to form MeMP with SAH as the by-product. The Methionine Cycle is also highlighted with the formation of THF, the enzyme which methylates HCys to form methionine within the Methionine Cycle. MP: mercaptopurine; MeMP: methylmercaptopurine; SAM: S-adenosylmethionine; TPMT: thiopurine methyltransferase; SAH: S-adenosyl homocysteine; Hcy:Homocysteine; MET:Methionine; THF:Tetrahydrofolate; 5-MTHF: 5-methytetrahydrofolate; DHF; Dihydrofolate, dUMP: deoxyuridinemonophosphate; GCL: glutamate cysteine ligase; GS: GSH synthetase; GSH: Glutathione synthetase; CβS: cystathionine- β-synthase; cγl: cystathione-γ-lyase; MS: Methionine synthetase; BMHT: betain-homocysteine methyltransferase.

Homeostasis of the Methionine Cycle

There are thought to be 2 intracellular pools of SAM formation, one more active pool in the cytosol, and another less active pool in mitochondria. It is likely that SAM remains in the cell of its origin rather than effluxing out into the extracellular space and being shared between cells.[242] The tissue content of SAM is both organ-specific and methionine-dependent. Concentrations in liver tissue of rats range from 50 to 100 nmol/g but increase to 650 nmol/g in diets containing excess methionine.

The homeostasis of this cycle is regulated both by the compounds and the enzymes within it. Both genetic variants of enzymes and dietary deficiencies therefore affect it. Homeostasis of the relative concentrations of SAM and SAH is important, since a decrease in the SAM:SAH ratio, either by a reduction in SAM or an elevation of SAH, can reduce methyltransferase activity.[240] For example, SAH is normally at a several-fold lower concentration than SAM, but elevated HCys can raise intracellular SAH sufficiently to reduce the conversion of SAM to SAH.[244] A rise in the concentration of SAH will also upregulate CβS activity, decrease betain-homocysteine methyltransferase (BMHT) and increase the activity of MTHFR.[245-247]

Low SAM concentrations were found in patients with MTHFR deficiency, emphasising the importance of the Methionine Cycle in SAM and SAH homeostasis. Genetic variants in any of the main enzymes give rise to hyperhomocysteinaemia.[248] The commonest variant involves the C>T substitution at nucleotide 677 of the MTHFR gene. Homozygous variants for MTHFR 677 C>T have been found to have hyperhomocysteinaemia and HCys concentrations were found to significantly different between 677 C>T variants.[249] Also, homozygous variants for both the CβS (I278T and G307S occur in 24% and 31% of the population respectively) and the adenosine deaminase gene, lead to increases in HCys and SAH.[250, 251]

By contrast, drugs and nutritional deficiencies that affect SAM concentrations, through decreasing MS activity, result in increased HCys and SAH.[252, 253]

Table 1.17.1: Significant differences in concentrations of HCys (measured in µmol/L) occur amongst control and cancer patients between 3 variants for C677T MTHFR.[249]

	CC	CT	TT
Controls (N= 100)	9.01 ± 2.76	9.71 ± 2.34	17.56 ± 10.81
Cancer patients (N=93)	11.82 ± 3.09	12.45 ± 4.46	16.88 ± 9.25

The Methionine Cycle and disease

Alterations in SAM and SAH concentrations and the SAM:SAH ratio, also referred to as "methylation potential", have been associated with particular disease states. SAH is lower than SAM because SAH is very quickly converted to HCys. In end-stage renal failure, HCys concentrations increase, resulting in an elevation in SAH.[254] In renal failure, the SAM:SAH ratio is therefore low.[242] Alterations of SAM and SAH, leading to a low ratio, have been shown to inhibit transmethylation reactions in a number of tissues. This has been proven in models of TPMT where TPMT is inhibited in uraemic patients; despite increasing concentrations of SAM, TPMT failed to increase in uraemic patients, whereas it did increase in controls.[255] Hyperhomocysteinaemia has also been associated with coronary artery disease and the development of cancers.[249]

Folic acid intake significantly reduces HCys levels and the re-methylation of HCys is both folate and vitamin B12 dependent.[256,257] Their deficiency results in elevated HCys and low methionine.[249] Studies have shown that rats fed diets low in methionine have lower SAM, higher SAH and suppressed DNA methylation. These rats develop hepatocellular carcinoma, raising interest for HCys as a potential tumour marker for the future. Indeed, it has also been long recognised that cancer patients are folic acid deficient.[258] Studies investigating the relationship between SAM, SAH and HCys levels, relative to diet in humans, show that SAM and HCys are higher in males than females, correlating positively between SAM and folate intake and negatively between HCys levels and folate intake.[259]

There is increasing evidence to suggest that a breakdown in SAM and SAH homeostasis can cause liver disease through the development of fatty liver and increased vulnerability to oxidative stress. Downregulation of SAM reduces the metabolic substrates for glutathione synthesis.[253] A deficit in the formation of glutathione, a potent antioxidant, leads to vulnerability of the liver to oxidative stress from drugs.[260, 261] Lipopolysaccharide challenges in mice lower hepatic GSH and induces liver injury. Exogenous GSH can in turn increase SAM and suppress LPS-induced liver injury.[262] Patients with alcoholic liver disease have markedly reduced GSH levels, thought to be as a result of nutritional deficiencies.[263]

Deletion of *MAT1A, BMHT and CβS* genes leads to reduced SAM and the development of fatty liver disease, and hepatocellular carcinoma in mice.[264] Patients with cirrhosis have been shown to have lower SAM, because of reduced activity of MAT1A, potentially leaving them more vulnerable to oxidative stress through downregulation of GSH.[265] Collectively, these studies highlight the relationship between SAM and SAH dysregulation and liver disease.

The measurement of SAM and SAH

Measuring SAM and SAH has been previously difficult due to time consuming methods to measure them both at such low concentrations. SAM and SAH are detectable by HPLC combined with ultraviolet detection in human tissue, RBCs and lymphocytes.[266] However, in plasma, concentrations are within the nanomolar range and therefore even more sensitive laboratory techniques are required. Measuring SAM and SAH in plasma is more representative of their metabolism in the liver than measurement in RBCs.[244] Previously, a more sensitive method was established based on the principle that SAM and SAH could be converted into their 6-etheno fluorescent derivatives which can be detectable in the nanomolar range.[267] However, this method was still laborious. A faster HPLC method was developed which separated neutralised SAM and SAH extracts in trichoroacetate, using isocratic elution and coulometric electrochemical detection.[268] This method could only measure 20 samples at a time. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) allows for the measurement of SAM, SAH and HCys in both plasma and cerebrospinal fluid (CSF) with high selectivity, sensitivity and sample throughput using stable-isotope-labelled internal standards.[269]

Table 1.17.2: Average blood concentrations of SAM, SAH and HCys in RBCs using an HPLC. These values are consistent with previously published values.[259]

Measured compounds in RBC	Men	Women
SAM (µmol/L)	2.4	1.69
SAH (µmol/L)	0.38	0.38
SAM:SAH ratio	7.84	4.9
HCys (µmol/L)	11.2	9.3

In another study, mean plasma SAM was 60 nmol/l (0.06 µmol/L)(± 19 SD) and SAH was 24.4 nmol/l (0.024 µmol/L)(± 8 SD).[269] SAM and SAH have also been measured as a surrogate marker for renal allograft rejection. Using a LC-MS/MS method, plasma SAM increased from 100nM (0.1 µmol/L) in healthy volunteers to 210 nmol/L (0.21 µmol/L) in patients with early allograft rejection whereas SAH rose from 20 nmol/L (0.02 µmol/L) to 200nM (0.2 µmol/L). This meant that the SAM:SAH ratio fell when comparing the plasma of healthy volunteers to patients (3.5 to 1.7).[270]

The Methionine Cycle and TPMT

The relevance of SAM to TPMT in the context of thiopurines has been previously investigated. In a model cell line for ALL, cells that were incubated with both MP and SAM showed less cytotoxicity, higher MeMP and lower TGN levels, than cells incubated with MP alone.[271] 10-50 µmol/L SAM was able to delay the cytotoxic effects of MP by stabilising TPMT, thereby increasing the methylation of MP to MeMP and reducing the amount of MP available to form TGNs.

Other studies have measured concentrations of SAM, SAH, methionine, and HCys in association with increasing concentrations of MP and methylmercaptopurine riboside (MeMPR), another purine antimetabolite.[272] MeMPR is metabolised to MeTIMP by ADK in an ATP-dependent process, a process which also inhibits de novo purine synthesis. The hypothesis is that this process depletes the ATP available for the conversion of methionine to SAM. Concentrations of SAM and the SAM:SAH ratio fall whereas SAH, HCys and methionine all increase.

The γ- glutamyl Cycle

Paracetamol is the classic example of a drug which reduces glutathione resulting in hepatotoxicity. Thiopurines have also been shown to cause hepatotoxicity by reducing glutathione (see Section 1.11). The Methionine Cycle results in the formation of glutathione and therefore potential disruption to the cycle by abnormal thiopurine metabolism is a potential cause of thiopurine hepatotoxicity. An understanding of the γ- glutamyl cycle is therefore important.

Glutathione donates (or reduces) electrons to many molecules and in so doing is a critically important anti-oxidant. It is made up of 3 amino acids: glutamate, cysteine and glycine (Figure 1.17.3). Glutamate and cysteine share a unique bond between the carboxyl group of glutamate and the nitrogen of cysteine, catalysed by γ-glutamyl cysteine synthetase (γGCS). Glutathione synthetase then catalyses the addition of glycine to γ-glutamyl cysteine (γGC) to form glutathione.

Pyroglutamic acid (PG), otherwise known as oxyproline, is an intermediate in the γ-glutamyl Cycle and accumulates in rare conditions associated with glutathione synthetase or 5-oxoprolinase deficiency but also following paracetamol overdoses. Since γGCS is negatively influenced by glutathione, depletion of glutathione stores increases the activity of γGCS and the formation of γGC.[273] High γGC concentrations result in its conversion to oxyproline by γ-glutamyl cyclotransferase (γGCT). This has been shown in rats fed paracetamol, who develop gross excretion of oxyproline in urine.[274, 275] Cysteine is a rate-limiting amino acid in the γ-glutamyl Cycle.[276, 277] Methionine and NAC, both cysteine precursors, have been shown to be protective in patients and animal models of acquired 5-oxoprolinemia and 5-oxoprolinuria, due to the restoration of cysteine and hence glutathione stores. Whilst PG is a relatively stable compound *in vivo* and therefore possible to measure, cysteine and glutathione are unstable and less measurable by LC-MS.[278]

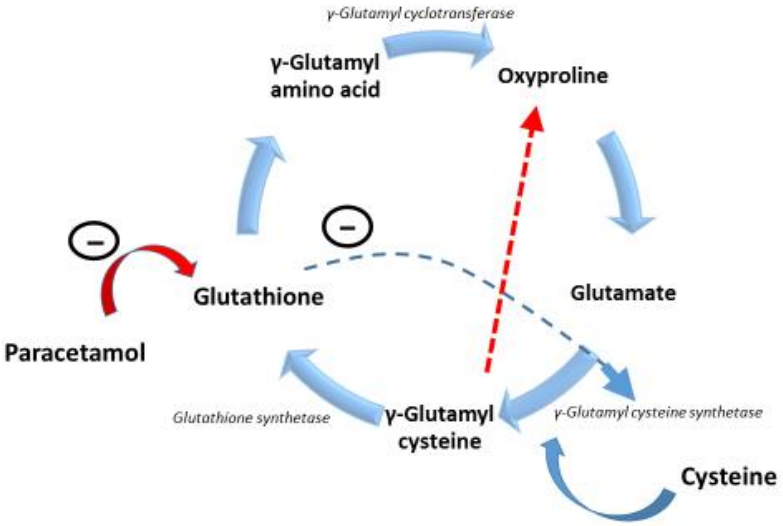


Figure 1.17.3: The γ- glutamyl Cycle. Glutamate in converted to glutathione after binding to cysteine and glycine in a reaction catalysed by 2 enzymes: γ-glutamyl cysteine synthetase (γGCS) and glutathione synthetase (GS). Paracetamol irreversibly reacts with glutathione reducing its antioxidant abilities but also leading to the accumulation of γ-glutamyl cysteine (γGC) which is then converted into oxyproline by β-Glutamyl cyclotransferase. The γ-glutamyl Cycle is therefore cysteine-dependent.

1.18 Aims of thesis

This thesis aims to determine the mechanisms which contribute towards liver injury in patients with IBD and how we might better screen for them in our patients. To achieve this, the causes of liver disease in a cohort of IBD patients were first analysed. As thiopurines are the commonest cause of hepatotoxicity, a search for the compounds by which thiopurines might cause hepatotoxicity, was performed using a combination of candidate gene analyses and a targeted measurement of specific compounds using mass spectrometry. Lastly, MRE was assessed as a screening tool to diagnose NRH. Each chapter of this thesis addresses these aims, which are as follows:

Chapter 3 – A cohort study of abnormal LFTs in IBD: Causes of abnormal LFTs were analysed retrospectively in our cohort of IBD patients to determine the most prevalent cause of hepatotoxicity and the long-term effects on patients.

Chapter 4 – **A candidate gene analysis**: A GWAS performed in our laboratory had revealed several candidate genes that predispose to hepatotoxicity in hypermethylators. These studies compared 15 patients with hypermethylation and hepatotoxicity against 64 patients with hypermethylation alone and found that SNPs for IL-15 (rs10519613 and rs17007695) were significantly (P=0.005 and P=0.007 respectively, Fisher's exact test) associated with the development of hepatotoxicity in hypermethylators. Likewise, MTHFR SNPs (rs17367504 and rs17375901) were also associated with hepatotoxicity (P=0.003, Fisher's exact test). Lastly, two other polymorphisms are potentially contributory. These were ADK rs946185, an enzyme responsible for the conversion of TG riboside to TGMP, and AOX rs55754655, which was protective against hepatotoxicity, and which has been shown previously by *Smith et al* to be protective against other adverse drug reactions by increasing the breakdown of AZA and MP to TUA.[205] A candidate gene analysis was performed to investigate these SNPs on 4 groups of patients:

- Patients who break down thiopurines normally - "Normomethylators"
- Patients who hypermethylate - "Hypermethylators"
- Patients who hypermethylate and develop hepatotoxicity – "Hypermethylators with hepatotoxicity"
- Patients who do not hypermethylate but developed hepatotoxicity regardless – "Hepatotoxicity only"

The hypothesis is that genetic variants are responsible for the difference in thiopurine metabolism between these 4 phenotypes.

Chapter 5 – The relationship between smoking and thiopurine metabolism: The formation of formaldehyde, through the demethylation of MeMP, may also result in hepatotoxicity. Formaldehyde is not a stable compound and therefore is difficult to measure precisely. However, the measurement of metabolite concentrations in smokers and non-smokers on thiopurines acts as a surrogate marker of demethylation, since smoking upregulates CYP450 enzymes. The hypothesis is that smokers are prone to hepatotoxicity from thiopurines through formaldehyde release, as a result of upregulated demethylation. The aim was to first establish that there are indeed differences in metabolite concentrations between smokers and non-smokers and second, that there are differences in the rates of hepatotoxicity. Thirdly, phenotypic differences (hypermethylation and hepatotoxicity) were compared between patients according to CYP1A2 variants.

Chapter 6 – Measurement of SAM by mass spectrometry in patients on thiopurines: Another aim of the thesis was to determine a biomarker which could differentiate between abnormal metabolisers of thiopurines. Studies has shown that SAM concentrations vary between healthy individuals and patients with liver disease. The hypothesis is that hepatotoxicity occurs as a result of reduced glutathione due to alterations of SAM, SAH, and other compounds of the Methionine Cycle and that the concentration of these compounds would vary significantly between normomethylators, hypermethylators and hypermethylators with hepatotoxicity.

Chapter 7 – The use of MRE to diagnose NRH: The final aim of the thesis was to assess MRE as a screening tool for NRH, a condition associated with IBD patients on thiopurines. Ultrasound, fibroscan, and MRI all lack sensitivity and liver biopsies are invasive. Concerns about NRH commonly lead to the withdraw of thiopurines thereby risking a relapse in IBD. If MRE could diagnose NRH with high sensitivity, then the withdraw of thiopurines would only be needed if necessary.

Chapter 2: Methods

2.1 Recruitment to studies

Cohort Study on abnormal LFTs in IBD (Chapter 3)

This was a retrospective analysis of IBD patients attending clinic with either current or previous abnormal LFTs. Patients with abnormal LFTs were analysed for IBD-type, the duration of abnormal LFTs, and the severity of the abnormal LFTs according to how high the ALT, the bilirubin and the INR were. Based on the information collected, the cause for liver dysfunction was determined by the author, although in most cases the medical notes had established a diagnosis.

A Candidate gene analysis of thiopurine hypermethylation and hepatotoxicity (Chapter 4)

Patients on thiopurines were recruited as they came to the IBD clinic. A blood sample was obtained at clinic in an ethylenediaminetetracetic acid (EDTA) blood tube and then stored at -70°C. Samples were also collected where patients had previously agreed to be part of the IBD Genetics study and where their blood had been taken for clinically relevant reasons. For each patient, data on IBD-type, ethnicity, smoking status, drug therapy and dosing, TPMT and weight were collected for analysis.

The relationship between smoking and thiopurine metabolism (Chapter 5)

Patients attending the IBD clinic were questioned as to whether they had ever smoked on thiopurines. Using this data, a cohort of smokers and non-smokers with a history of thiopurine use was created. These cohorts were retrospectively analysed for thiopurine metabolite levels and comparisons between the two cohorts made. For each patient, data on IBD-type, ethnicity, amount smoked, drug therapy and dosing, TPMT and weight were collected for analysis. For each patient recruited, a blood sample was obtained in an EDTA blood tube and then stored at -70°C for a candidate gene analysis.

Measurement of SAM by mass spectrometry in patients on thiopurines (Chapter 6)

IBD patients were recruited who had real-time thiopurine hepatotoxicity or thiopurine hypermethylation alongside patients who had normal thiopurine metabolite profiles. LFTs and thiopurine metabolites were repeated at the time that bloods were taken in a heparinised blood tube for the measurement of SAM, SAH, HCys, and PG. During this time-period, patients continued thiopurine treatment at the current dose unless they were feeling unwell. Blood samples were also taken from 5 healthy volunteers as a comparison.

2.2 DNA extraction

Two of the studies (Chapters 4 and 5) required DNA extraction as part of a candidate gene analysis. DNA was extracted from EDTA whole blood using a QIAamp DNA Mini Kit (Qiagen Ltd, Crawley UK). 200 µl of blood was pipetted into a 2 mL microfuge tube. Approximately, 20 µl of protease enzyme and 200 µl of lysis (AL) buffer were added to each tube. The mixture was vortexed and incubated at 56°C for 10 min. 200 µL of 100% ethanol was added and the whole mixture transferred to a 2 ml collection tube within a spin column. The mixture was centrifuged at 8000 g for 1 min. DNA was absorbed onto a silica-gel QIAamp membrane. AW1 buffer was added, the mixture then centrifuged again at 8000 g for 1 min into a new 2 mL microfuge tube. 500 µl of AW2 buffer was added, the sample centrifuged at 12,000 g for 3 min and 100 µl of elution buffer added to the spin column and the solution incubated at 56°C for 1 min. 50 µL of tris-EDTA (TE buffer) was added to the extracted DNA to inhibit DNAases and the sample stored at -20°C.

2.3 SNP selection for candidate gene analysis

SNPs were selected based on the Purine Laboratory's previous GWAS results which highlighted certain variants as being significantly different between hypermethylators and hypermethylators with hepatotoxicity (see Section 1.18: Aims of thesis and Table 2.3.1).[279] TaqMan probe sequences of the SNPs were acquired along with probe sequences for the common TPMT genotypes. Patients' extracted DNA then underwent Real Time assay and amplification (Section 2.4) followed by genotype validation and Sanger sequencing (Section 2.5).

Table 2.3.1: Candidate genes showing the chromosomal location of the SNP and its function based on that reported on the Pubmed SNP database.

Gene (cDNA base change)	SNP rs number	Chromosome	Function
MTHFR 1286 A>C	rs1801131	1	Missense
MTHFR 677 C>T	rs1801133	1	Missense
MTHFR 1654-80 G>A	rs17375901	1	Intron variant
MTHFR 359+160 A>G	rs17367504	1	Intron variant
AOX 3404 A>G	rs55754655	2	Missense
IL-15 *83 C>A	rs10519613	3	Transcript variant
IL-15 g.142709723T T>C	rs17007695	4	Unknown
CYP1A2*C 3860 G>A	rs2069514	15	Unknown
CYP1A2*F 163 C>A	rs762551	15	Intron variant
ADK 827-6202A A>G	rs946185	10	Intron variant
ABCB5 343 A>G	rs2301641	7	Missense
SLC 38A9 6899 A>G	rs6897117	5	Intron variant

Linkage disequilibrium (LD) is where 2 genes occur on the same chromosome. It is therefore possible for these genes to be inherited with each other if they are situated close together and if there is a small chance of genetic recombination between them. Therefore, SNPs may occur in LD with each other - these are known as haplotypes. This is important in association studies because a variant that is statistically significant for a given trait may not actually be the causative SNP but rather it might be tagging the true SNP through LD. The SNPs chosen for the candidate gene analyses have been assessed as unlikely to be in LD (Table 2.3.2). All patients were genotyped for the 3 commonest TPMT genotypes as highlighted in Table 2.3.3: TPMT*2, TPMT*3A and 3C. The TaqMan probe sequences used for TPMT genotyping are shown in Table 2.3.4.

Table 2.3.2: Linkage disequilibrium (LD), r^2 value obtained from the Ensembl database of SNPs which are on the same chromosome.

	rs1801131	rs1801133	rs17375901	rs17367504	rs2069514	rs762551
rs1801131	X	0.24	0.11	0.35	X	X
rs1801133	0.24	X	0.06	0.08	X	X
rs17375901	0.11	0.06	X	0.32	X	X
rs17367504	0.35	0.08	0.32	X	X	X
rs2069514	X	X	X	X	X	0.13
rs762551	X	X	X	X	0.13	X

r^2 (correlation coefficient) is a measure of the likelihood of the null hypothesis being rejected. The null hypothesis is this case is that the genes in question are in LD ($P < 0.05$). As all the correlations between the genes in the table are > 0.05, it can be assumed that there is a < 5% chance of LD occurring.

Table 2.3.3: Common TPMT genotypes.

Allele	dsSNP rs number at positive strand	Nucleotide changes	Amino acid change	Exon
TPMT*2	C>G rs1800462	238G>C	Ala80Pro	5
TPMT*3A	C>T rs1800460 T>C rs1142345	460G>A 719A>G	Ala154Thr Tyr240Cys	7 10
TPMT*3B	C>T rs1800460	460G>A	Ala154Thr	7
TPMT*3C	T>C rs1142345	719A>G	Tyr240Cys	10

Table 2.3.4: TaqMan probes used for TPMT genotyping.

Allele	Rs number	Assay ID	Probe sequence
TPMT*2	Rs1800462	C_12091552_30	CCAACTACACTGTGTCCCCGGTCTG(C/G)AAACCTGCAT AAAATACATTTA
TPMT*3B	Rs1800460	C_30634116_20	TCACCTGGATTGATGGCAACTAATG(T/C)TCCTCTATCCC AAATCATGTCAAAT
TPMT*3C	Rs1142345	C_19567_20	TCTCATTTACTTTTCTGTAAGTAGA(C/T)ATAACTTTTCAAA AAAGACAGTCAAT

The *3A mutation is confirmed by using the TaqMan probe which binds to the *3B mutation, confirming the difference between the *3A and *3C variants.

2.4 Real Time assay and amplification plots

Sequence variant analysis for the candidate genes were performed using TaqMan Allele Discrimination Real Time Polymerase Chain Reaction (PCR) assays (Thermo Fisher Scientific Ltd). The TaqMan assay contained 18 µmol/L of each primer and 4 µmol/L of probe in a 40x mix. The probes were stored at -20°C. A "PCR ready mix" was used containing PerfectCTaq PCR SuperMix, low ROX (Quanta Biosciences, Lutterworth, UK). This is a mixture containing ROX reference dye, a buffer, the four deoxynucleotides (dNTPs: dATP, dCTP, dGTP and dTTP), magnesium chloride ($MgCL_2$) and Taq polymerase. The probe and DNA were defrosted and vortexed. A master mix was made up and 9 µl (0.25 µl of probe if 40X and 0.5 µl if 20X, 3.75 µl of DNA free water and 5 µl of "PCR ready mix") pipetted into each of the wells of a 96 well plate using a single-channel repetitive positive displacement pipette. Using an eight-channel pipette, 1 µl of DNA was added to each well from a DNA stock plate. 2 blank wells were included in each plate to control for contamination. Plates were closed using optically clear lids ensuring the absence of finger prints marks which could affect the measurements. Plates were spun at 1500 g for 30 s to remove any bubbles which form at the bottom of the wells. They were then loaded onto a Stratagene Mx300SP RT PCR instrument (Agilent Technologies UK Ltd.Winnersh, UK) for fluorometric quantification using a 2-step PCR programme with denaturing at 95°C and annealing/extension at 60°C. The fluorescence from each well was measured, producing the amplification plots which were then interpreted to determine the genotype.

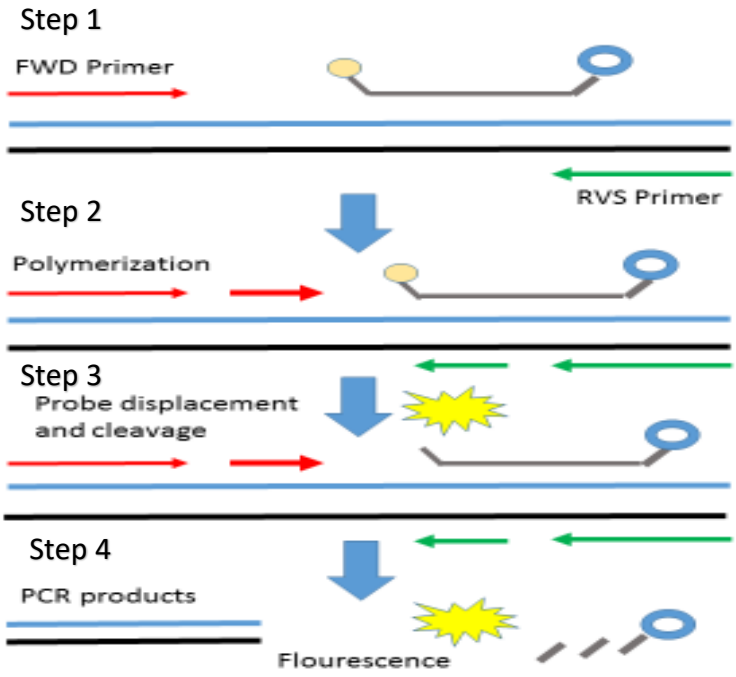


Figure 2.4.1: The four steps for the TaqMan probe assay. Step 1 is the denaturing and unwinding of double stranded DNA at a high temperature with subsequent binding of the forward (FWD) and reverse (RVS) primers to their complementary strands. Step 2 is the extension phase where Taq polymerase locates the primer and creates a complementary strand of DNA. The probe contains a fluorescent molecule at its 5'end known as the "reporter" whereas at the 3' end is the "quencher." Each TaqMan probe contains a probe which binds to the wild type allele and a probe that binds to the variant. For most probes, the reporter is Vic for the wild type probe and Fam for the variant. The probes lack a free hydroxyl group with two covalent bonds instead. Therefore, where they bind to DNA, they are not extended by DNA polymerase, but instead undergo exonuclease activity by Taq polymerase whereby the reported molecule is cleaved and released (step 3). In the absence of the quencher, the free reporter molecule is able to absorb light and omit fluorescence (step 4). It is this degree of fluorescence, proportional to the amount of probe bound to DNA, which is measured to create the amplification plot (Figures 2.4.2 – 2.4.4).

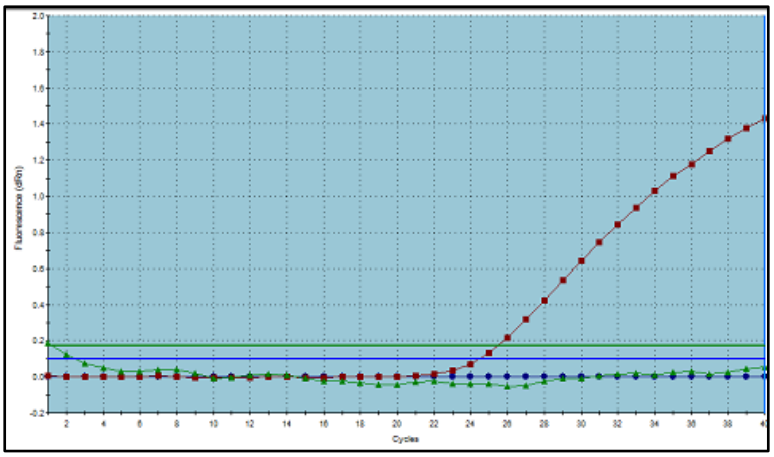


Figure 2.4.2: An amplification plot with a signal from Vic (red line) only. This suggests the presence of a wild type allele.

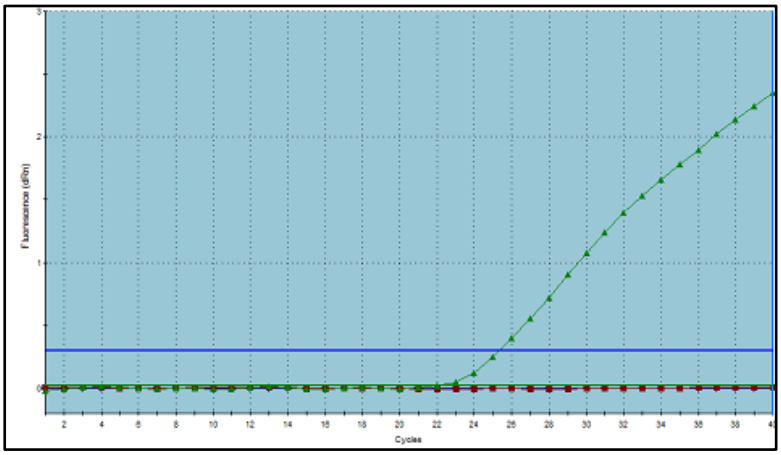


Figure 2.4.3: An amplification plot with a signal from Fam (green line) only. This suggests the presence of a variant allele only**.**

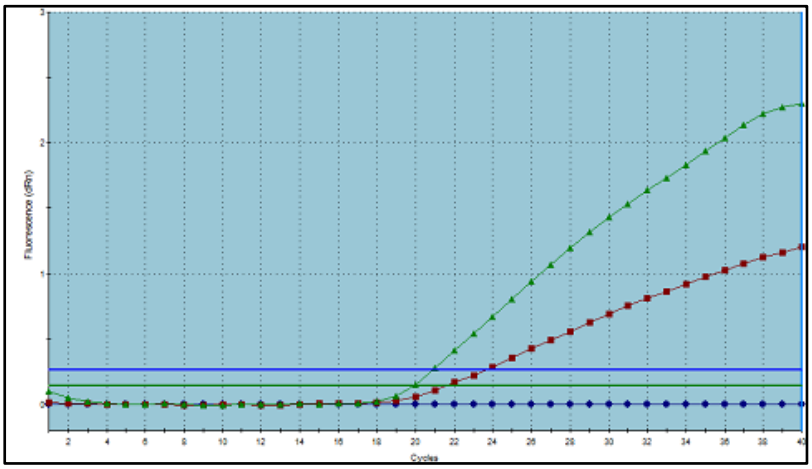


Figure 2.4.4: An amplification plot with Vic and Fam. This suggests the presence of a heterozygote mutation where both variants of the allele are present.

2.5 Genotyping validation by Sanger sequencing

To ensure that the TaqMan assays were calling the variants correctly, genotypes were confirmed by DNA sequencing of PCR products amplified to include the variant of interest.

PCR primers were created using the programme Primer 3 output. The sequence of the gene was downloaded from Ensembl (Table 2.5). Pseudogenes were taken into account when designing gene specific primers. The sequence context of the SNP was copied from the Pubmed SNP record and used to search within the gene sequence. A base sequence 70 bases both upstream and downstream of the SNP was bracketed to exclude as possible locations for PCR primers and a total of 400 bases used to then allow Primer 3 output to find the best primer pair for synthesis.

The lyophilised forward and reverse primers were spun down at 11,000 g for 5 s. Each primer was dissolved in TE buffer to reach a concentration of 50 pmol/L working stock solution and vortexed for 5 s. 50 µl was pipetted out into a microfuge tube for use. Primers were stored at -20°C until they were used.

For 6 primers (including forward and reverse), a stock solution was made up containing: 630 µl water, 40 µl deoxynucleotides (dNTPs), 50 µl dimethyl sulfoxide (DMSO), 60 µl $MgCL_2$, 200 µl of 5 x buffer and 5 µl of Taq DNA polymerase. 150 µl of stock solution was then aliquoted into 6 microfuge tubes. 1.5 µl of both a forward and reverse primers were added per gene. 50 µl of each 150 µl microfuge tube was then aliquoted into three 0.2mL 8-strip tubes. 1 µl of wild type DNA corresponding to the primer was pipetted into one tube, and 1 µl of homozygous variant DNA was added to a second tube. The third tube was left as a blank.

The samples then underwent PCR in a thermocycler (40 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, elongation 72°C for 30 s and a final extension at 70°C for 10 min). Whilst this was in process, the gel plate electrophoresis was prepared using 2% agarose gel, prepared as 2 g in 100 mL of Tris-acetate -EDTA buffer (TAE) with 1 µl per 50 mL of Gel Red dye as a DNA stain.

Once the PCR reaction had occurred, 12 µl of PCR reaction from each of the tubes, including blanks, were loaded onto gel and run in 1X TAE buffer.

Gel electrophoresis was run under a constant voltage at 100 volts for 25 min. The gel was visualised under UV (Figure 2.5.1).

Table 2.5: Forward (F) and reverse (R) primers for validation of the genotypes by Sanger sequencing.

SNP	SNP rs number	Primer	Length of PCR primer (bp)
MTHFR 1286 A>C	rs1801131	F:GGAGGAGTCAGGGGCAGAA R:GAGGGGAGGGCACAGGAT	19 18
MTHFR 677 C>T	rs1801133	F:GGACTCTCTCTGCCCAGTC R:TGGGAAGAACTCAGCGAACT	19 20
MTHFR 1654-80 G>A	rs17375901	F:CTGACTGATTGGGAGAGGGG R: GGTGCTGGGTGTTTGCTC	20 18
MTHFR 359+160 A>G	rs17367504	F:TGAGGGAGCTGTCAATCTCA R:TCAGTTGGCATTTCTAGAGTTCA	20 23
AOX 3404 A>G	rs55754655	F:TGATAGTTTTATTTAACACACAGGGT R:TGCCAAAATTGCAAGGGAAC	26 20
IL-15 *83C > A	rs10519613	F:TTTCATATCTCAAGACCTCACCAT R:AGTCTGAGAGAAGTTCATAGTCACT	24 25
CYP1A2*F 163C>A	rs762551	F: TGATGTGTGGAGGAGAGAGC R: GCTGAGGGTTGAGATGGAGA	20 20
ADK 827-6202A A>G	rs946185	F:ATTAAAGCAGGGATGTCA R:TCATAAACAACGTCACAAAGCA	22 22
ABCB5 343 A>G	rs2301641	F:TCTGTTTCTGTGCTTCTTTCCT R:TGCTTGTGTATCAGCCTCATG	22 21
SLC 38A9 C > T	rs6897117	F:CCTACTGCCCCTTCAAACAACC R:AGATAAACTCTTCACTTTGCCTTAGT	21 26

bp: Base pairs.

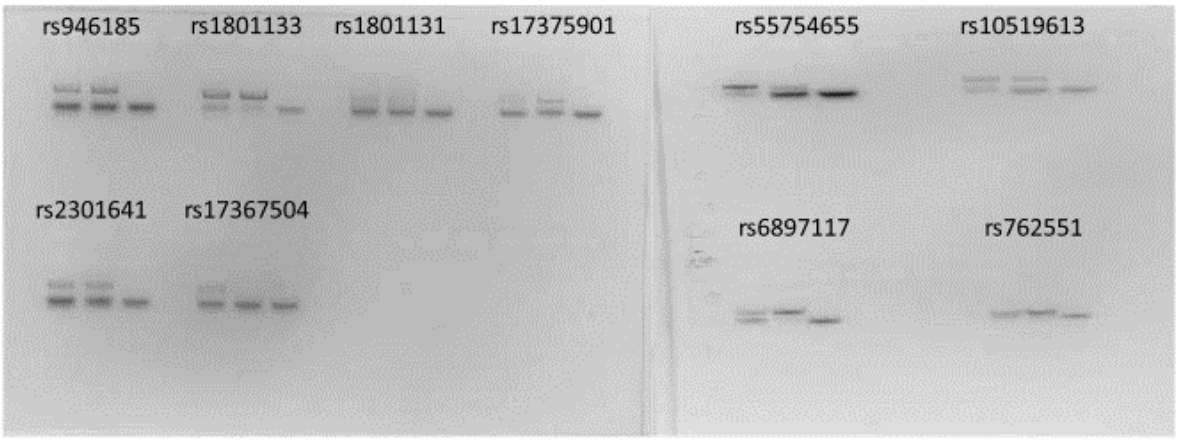


Figure 2.5.1: Gel electrophoresis following PCR amplification, isolation and PCR of 10 SNPs. There are 3 lanes per SNP. Each lane represents wild type, homozygote variant and blanks (from right to left). Two bands are present, a fast running band corresponding to primer-dimers and a slower running band corresponding to the PCR fragment.

The next step was PCR product clean-up, to get rid of any un-incorporated primers and surplus deoxynucleotides (dNTPs). This prevents any interference with the sequencing. 5 µl of PCR product were pipetted into corresponding 0.2 mL 8-strip tubes according to a previously created gene sequencing chart. A stock solution was made up containing 25 µl ALP and 12.5 µl of exonuclease. 1.5 µl of the solution was pipetted into each of the strip tubes. The strip tubes were then placed in the thermocycler (digestion at 37°C for 15 min, followed by inactivation of the enzymes at 80°C for 15 min).

A stock solution was made up for the sequencing reaction, using 42 µl of 5 x buffer, 124 µl of water, and 22.4 µl of the Big Dye terminator mix. 7 µl of stock solution was pipetted into 24 wells of the 0.2 mL 8-strip tubes.

A solution of 5 pmol/µl of each primer was made up in TE buffer. 1 µl of 5 pmol/ µl primer was added to the corresponding tube for forward or reverse sequencing according to the gene sequencing chart.

The next step was cycle sequencing in the thermocycler (denaturation at 94°C for 1 min, followed by 28 cycles of annealing at 48°C for 15 s, and elongation at 60°C for 4 min).

Un-incorporated dye terminators were removed by binding sequencing products to 10 µl of AgenCourt beads and adding 52 µl of 85% ethanol. The samples were placed on a magnetic plate for 10 min, the

ethanol removed, and the sequencing products washed a further 3 times with 100 µl of 85% ethanol. All the ethanol was then removed and the tubes air dried for 10 min.

For elution of the sequencing products, 40 µl of 0.1 mM EDTA was added to each tube, the mixture vortexed and then incubated for 10 min. The tubes were then spun at 1500 g for 1 min. 20 µl from each tube was pipetted into a 96 well plate and loaded on an ABI 3130xl for sequencing.

These sequences were then verified according to the reference sequence in the Ensembl database so that the genotype could be established. Figures 2.5.2 and 2.5.3 illustrate an example for the gene MTHFR rs1801133 in 2 different DNA samples visualised using Mutation Surveyor. Example genotypes were confirmed by Sanger sequencing to ensure the variants were called correctly by the Real Time assay. This confirmed that what was being called as wild type for SLC38A9 rs6897117 was a homozygous variant and vice versa. This was the same for rs1801131 and rs17375904.

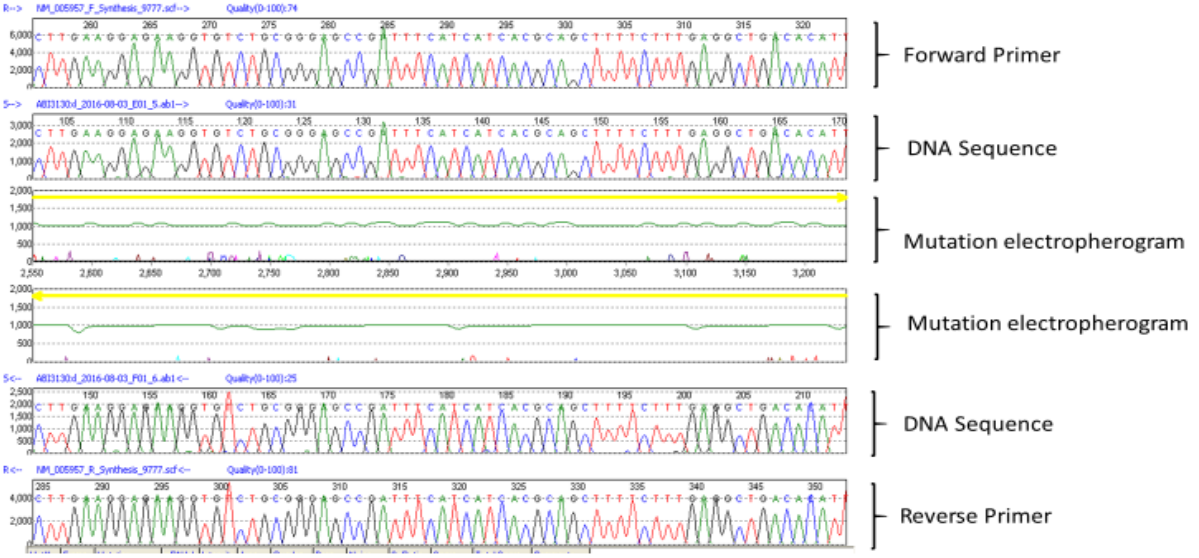


Figure 2.5.2: Chromatograph mutation tables from Mutation Surveyor. MTHFR rs1801133 forward and reverse primer sequencing shows complete concordance between the primers and DNA sequences confirming this individual to be have a wild type genotype.

Figure 2.5.3: The Chromatograph for MTHFR rs1801133 with discordance between both primers and their corresponding DNA sequences. Both sequences have a thymine base instead of a cytosine base at the SNP point confirming this individual to be a homozygous variant.

2.6 Measurement of SAM and SAH by HPLC

Neither SAM nor SAH had been measured before at GSTT. Initially, these compounds were measured using an HPLC method according to that used by Fullerton and Wise.[280] This was performed using 50 mM of NaH_2P0_4. 10 mM heptanesulfonic acid (Sigma Aldrich UK) as the mobile phase in 20% methanol buffered to pH 4.38 with phosphoric acid with a 25 cm Beckman ultrasphere column. However, although 1 µmol/L of SAM and SAH standards were detectable, the peaks could not be seen when spiked in plasma and RBCs due to interference from other compounds (Figures 2.6.1 to 2.6.3). These compounds were then more accurately measured using LC-MS/MS as discussed in the next section.

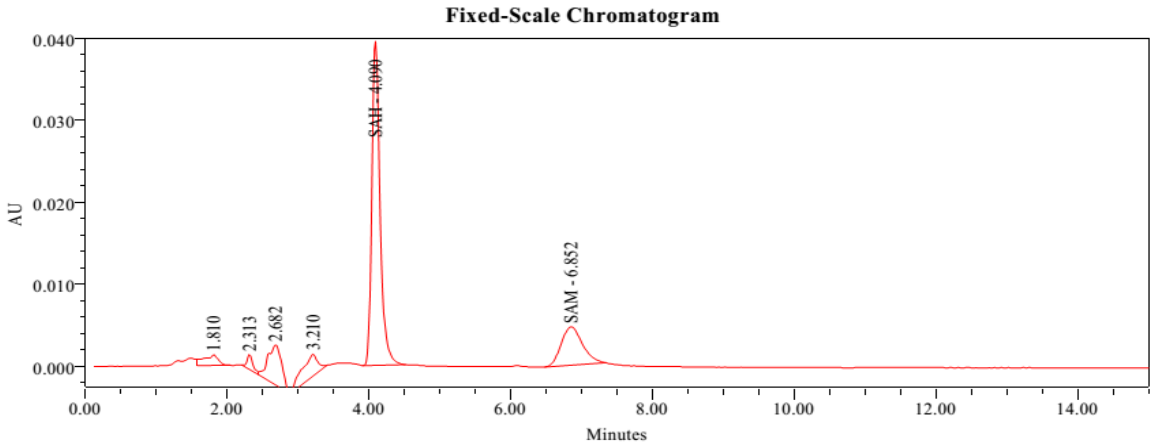


Figure 2.6.1: An HPLC chromatogram with 1 µmol/L of SAM and SAH standards. The concentration response curves are clearly visible for both compounds. AU: Absorbance Unit. The X axis is the retention time.

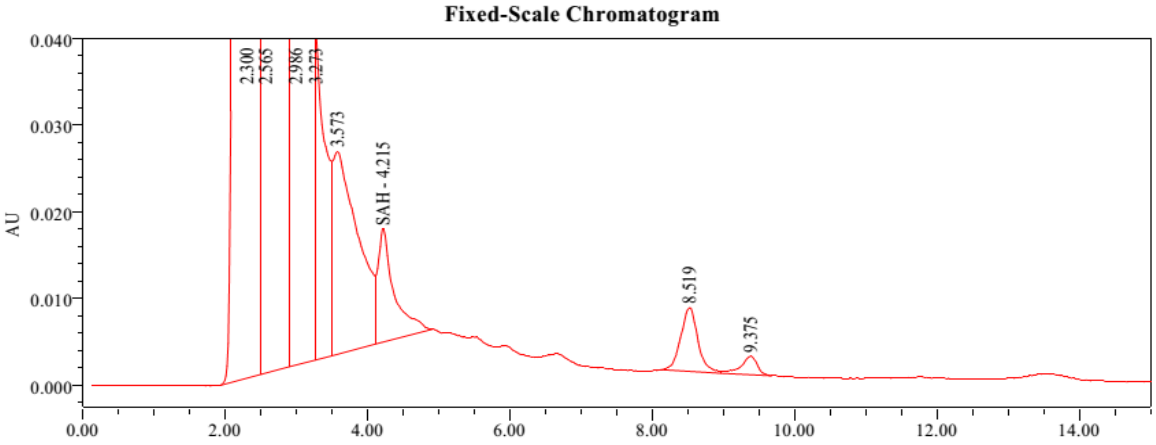


Figure 2.6.2: An HPLC chromatogram with 1 µmol/L of SAM and SAH spiked in RBC. The response curves for both compounds are less visible than in the previous chromatogram (Figure 2.7.1) due to interference from other compounds in RBC. The irregular baseline exposes the inaccuracy of the assay.

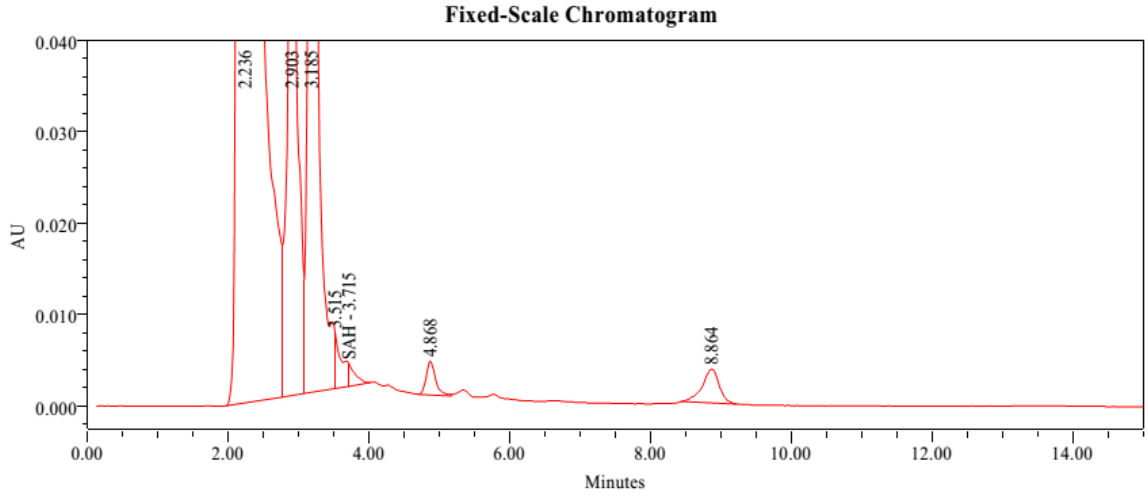


Figure 2.6.3: An HPLC chromatogram with 1 µM SAM and SAH spiked in plasma. Again, there is no separation of peaks for both compounds due to interference from other compounds in plasma.

2.7 Measurement of SAM, SAH, HCys, methionine, 5-MTHF, and PG using LC-MS

As the measurement of SAM and SAH using LC-MS had not been done before at GSTT, a completely new assay needed to be designed; the method by Klepacki et al was replicated.[270] Our assays used the LC-MS/MS API 6500 (AB Sciex, Warrington, UK). The following paragraphs detail how the assays were designed using deuterated standards and how the measurement of the specific compounds in our patients' samples was achieved.

There were 5 experiments performed to measure these compounds. The first experiment involved creating a standard curve of different concentrations of SAM and SAH on their own and when spiked in plasma. The second experiment involved creating a calibration assay, where SAM and SAH was measured alongside their deuterated standards and the assay's accuracy assessed. The third involved measuring SAM, SAH, HCys, methionine and PG in patient samples. The fourth involved measuring 5-MTHF in the patients' samples to assess the effects of 5-MTHF on the other compounds of the Methionine Cycle. This did not involve LC-MS/MS but used a more conventional assay used in routine clinical practice. The fifth experiment measured PG using LC-MS/MS with its deuterated standard, along with glutamine and glutamate.

1) Creating a standard curve

Based on previous reports, SAM and SAH are detectable in the range 5 to 500 nmol/L. A standard curve was created with 4 different concentrations of SAM and SAH at 5, 50, 200 and 500 nmol/L.

Our starting concentration of SAM was a stock solution of 1 mg/mL or 2.51 mmol/L (molecular weight (MW) 398.44 Da). The compound was obtained in solution with water from the Purine laboratory at where it is used as part of the TPMT assay. To obtain the calibrators required, 500 nmol/L and 50 nmol/L, the solution was diluted by 1/5000 and 1/50000 respectively in a volumetric flask.

Our starting concentration of SAH was 50 mmol/L obtained as a 10 mg powder (MW 384.4 Da) bought from Sigma Aldrich. 10 µL of this concentration was added to 50 mL water in a volumetric flask for a dilution of 1/5000. This gave a stock solution of 10 µmol/L. To obtain 500 nmol/L and 200 nmol/L, 50 µl was pipetted into 950 µl of water (1/1000 dilution) and 20 µl into 980 µl of water (1/2500 dilution) respectively to obtain the calibrators required.

The samples of different SAM and SAH concentrations were then aspirated through the LC-MS/MS with the MS tuned for multiple reaction monitoring (MRM) mode for transition m/z 384.41 Da and 398.4 Da - the product ions for SAH and SAM respectively (Figures 2.7.1 – 2.7.3).

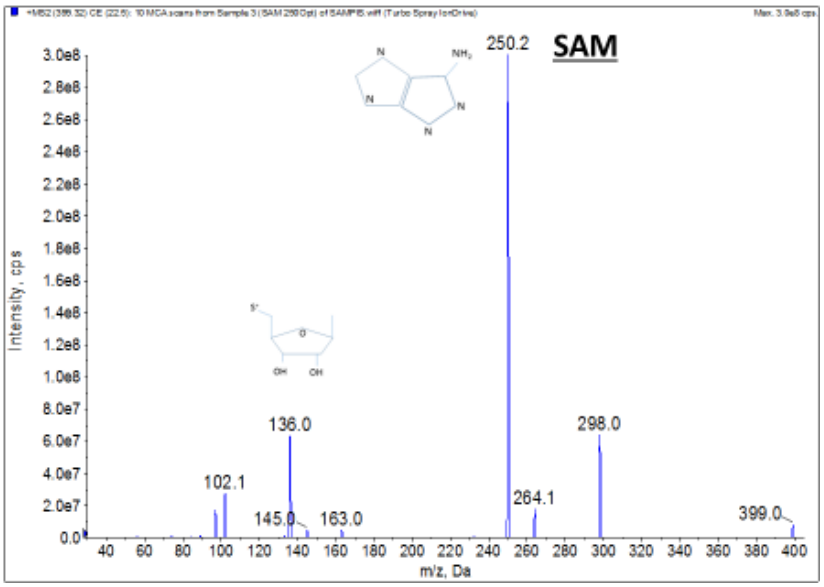


Figure 2.7.1: LC-MS/MS Product Ion Scan tuned in for SAM with the molecular weight of the first ion being 399 Da and the second ions being 250 Da and 136 Da.

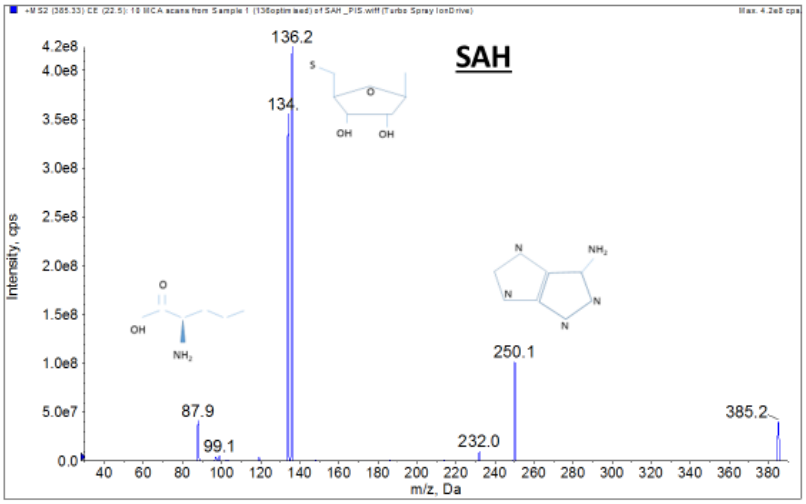


Figure 2.7.2: Product Ion Scan tuned in for SAH with the molecular weight of the first ion being 385 Da and the second ions being 136 Da and 88 Da.

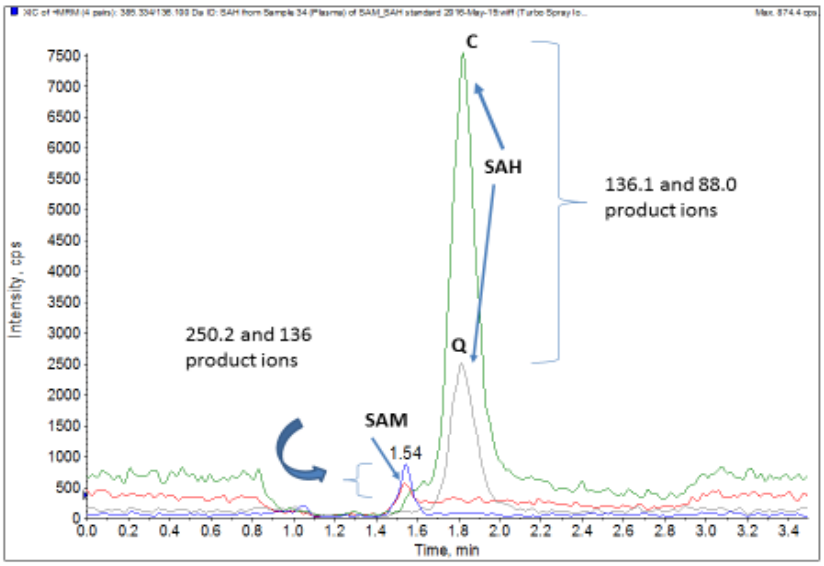
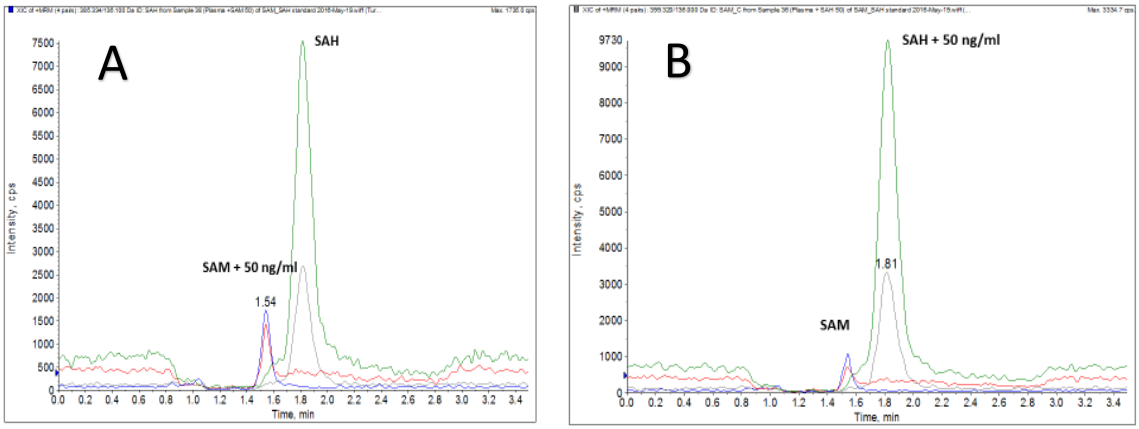


Figure 2.7.3: Chromatogram showing the measurement of both compounds SAM and SAH against their retention time, with the respective acquisitions for their product ions (C and Q for SAH) through LC-MS/MS.

To evaluate the accuracy of the method, in the absence of an internal standard, the measured concentration was compared to the predicted using two standards for both SAM and SAH at 5 and 50 nmol/L on their own and when spiked in 50 µL of plasma (Figure 2.7.4 and 2.7.5). This was achieved by tuning the LC-MS/MS first quadrupole (Q1) to select SAM at 399.32 Da and SAH at 385.33 Da whilst the 3rd quadrupole (Q3) was tuned to select SAM at 250.20 Da and SAH at 136.10 Da. Table 2.7.1 compares both the measured and predicted concentrations and the accuracy of the assay.



Figures 2.7.4 and 2.7.5: Ion chromatograms showing the measurement of SAM and SAH in 2 different plasma samples spiked with 50 nmol/L of SAM (A) or SAH (B), illustrating the difference in the concentration of compound detected.

Table 2.7.1: The predicted compared to the measured concentration of different standards (5 and 50 nmol/L) without and when spiked in plasma, but without a deuterated standard.

Concentration of analyte (ng/mL)	Predicted Concentration nmol/L		Measured Concentration nmol/L		Accuracy %	
	SAM	SAH	SAM	SAH	SAM	SAH
Water	0	0	0	0	100%	100%
SAM 5	5	0	7.94	0.149	159%	
SAM 50	50	0	49.7	1.29	99.4%	
SAH 5	0	5	0	4.64		92.8%
SAH 50	0	50	0	50		100%
Plasma	Unknown	Unknown	126	17.8		
Plasma + SAM 5	126+5 (131)	17.8	131	19.4	100%	109%
Plasma + SAH 5	126	17.8+5 (22.8)	126	23.4	100%	103%
Plasma + SAM 50	126+50 (176)	17.8	166	22	94.3%	124%
Plasma + SAH 50	126	17.8+50 (67.8)	137	44.7	109%	66.0%

The accuracy of the assay without a deuterated standard is very variable even when the compounds are measured without plasma.

2) The calibration assays

The inaccuracy of the assays above, highlighted the need for internal standards from which a calibration curve could be created and against which the true analyte concentration could be adjusted. Deuterated standards were therefore used to correct for variable recovery with respect to the measured concentrations of SAM and SAH by LC-MS/MS. Two calibration assays were therefore performed, the first to measure SAM and SAH in association with their deuterated standards and the second in a volunteer's blood sample. SAH-d4 was bought from Cambridge Bioscience, UK (MW 388.4 >99% purity) and SAM-d3 (S-adenosyl-L-methionine-d3 (s-methyl-d3) tetra (p-Toluenesulfonate) salt (>85% purity) from QMX laboratories Ltd, UK. These deuterated standards were made up as follows:

SAM-d3

1.84 mL of water was added to the 10 mg/mL of SAM-d3 (MW 1089.24 g) to get a concentration of 5 mmol/L. This was diluted by 1/100 by adding 4.9 mL water to 50 µL of 5 mmol/L SAM-d3 to get a concentration of 50 µmol/L.

SAH-d4

1.29 mL of water was added to the 1 mg/mL of SAH-d4 (MW 388.4) to achieve a concentration of 2 mmol/L. This was diluted by 1/100 by adding 4.9 mL of water to 50 µL of 2 mmol/L SAH-d4 to get a concentration of 20 µmol/L.

50 µL of each standard was mixed together and diluted 1/200 by diluting 10 µL in 2 mL water to give a stock solution of 100 nmol/L SAH-d4 and 250 nmol/L SAM-d3. These solutions were frozen at -70°C for future assays.

The first calibration assay was performed in a 96-deep well plate. Three of the wells were left blank, containing 50 µl of water only. Five wells contained varying concentrations of SAM (50, 125, 250, 1000 and 5000 nmol/L) and varying concentrations of SAH (10, 25, 50, 200 and 1000 nmol/L). 50 µl of the deuterated standards were added to each well along with 200 µl of acetonitrile (Table 2.7.2). The assay was then measured using LC-MS/MS. The standard curves for predicted versus measured concentrations of SAM and SAH are shown in Figure 2.7.6 and are linear reflecting the accuracy of the assay. Coefficient of Variance (CV) reflects the precision and repeatability of the assay by comparing replicated measurements of the compounds (Table 2.7.3). The intraassay CVs for SAM are similar to that described in other studies , although the CV for SAH is higher.[269]

Table 2.7.2: Compares the predicted concentration of SAM and SAH compared to what was measured using the deuterated standards.

	Predicted Concentration nmol/L		Measured Concentration nmol/L		Accuracy %	
Concentration of analyte (nmol/L)	SAM	SAH	SAM	SAH	SAM	SAH
SAM 50 SAH 10	50	10	51.7	12.5	103%	125%
SAM 125 SAH 25	125	25	125	31	100%	124%
SAM 250 SAH 50	250	50	250	56.8	100%	114%
SAM 1000 SAH 200	1000	200	1030	228	103%	114%
SAM 5000 SAH 1000	5000	1000	4970	1090	99.4%	109%

The accuracy of the assay had improved after calibration using the deuterated standards.

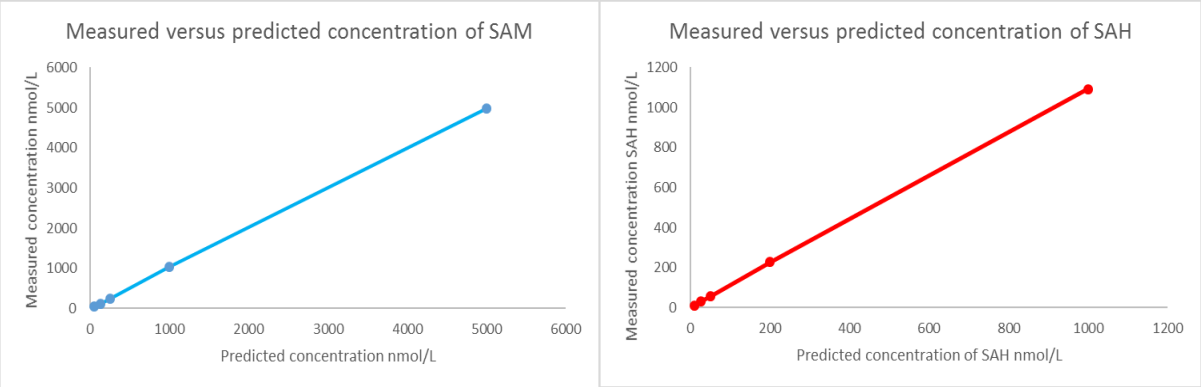


Figure 2.7.6: Standard calibration curves for measured versus predicted SAM and SAH using deuterated standards according to results in Table 2.7.2.

Table 2.7.3: Compares two replicate assays using deuterated standard to determine the intraassay coefficient variance (CV).

Concentration of analyte (nmol/L)	Replicate determination assays nmol/L		Mean nmol/L		Coefficient Variance (%)	
	SAM	SAH	SAM	SAH	SAM	SAH
SAM 50 SAH 10	51.7, 53.1	12.5, 11.3	52.4	11.9	1.9	7.1
SAM 125 SAH 25	125, 131	31, 25.5	128	28.3	13.8	3.3
SAM 250 SAH 50	250, 248	56.8, 49.9	249	53.4	0.6	9.1
SAM 1000 SAH 200	1030, 997	228, 180	1013	204	2.3	16.6
SAM 5000 SAH 1000	4970, 4990	1090, 882	4980	986	0.3	14.9

Different concentrations of SAM and SAH were measured in two replicate assays in order to determine the intraassay coefficient variance (CV). The mean CV for SAM was 3.78% whilst the CV for SAH was 10.2% and is comparable to other studies.[269]

The second calibration assay was performed to measure the SAM and SAH in volunteer's blood spiked with varying concentrations of SAM and SAH to obtain data on % recovery. Discrepancies between the concentration of deuterated SAM-d3 and actual SAM measured lead to questioning over the purity of the 1 mg/mL SAM solution used. SAM as a compound in powder (Sigma Aldrich UK (A2408) as the free base of SAM attached to a p.touenesulfonate salt ($C_{15}H_{22}N_60_{55}$-XC7H80$_3$S purity ≥80%)) was therefore acquired and the accuracy of the assay improved (The MW of 398.44 Da was adjusted to 742.44 Da to reflect the p.touenesulfonate salt estimated at being 2 moles (MW 172)).

Table 2.7.4: The actual and expected concentrations of SAM and SAH in plasma, whole blood (WB) and RBC samples spiked with 2 different concentrations of deuterated SAM (1000 and 5000 nmol/L) and SAH (200 and 1000 nmol/L) standards to determine the recovery.

	Replicate determinations of actual SAM and SAH nmol/L		Expected nmol/L		Recovery %	
	SAM	SAH	SAM	SAH	SAM	SAH
Plasma + Water	Mean=107.6	Mean=18.4	NA	NA	NA	NA
Plasma + 1/11 SAM 1000 SAH 200	210, 193	34.9, 37	198.5	36.5	103.3	96.9
Plasma + 1/11 SAM 5000 SAH 1000	561, 553	102, 107	562.1	109.2	98.9	94.8
WB + Water	662	1052				
WB + 1/11 SAM 1000 SAH 200	721, 1050	758, 1220	752.4	1069	85.8	459.3
WB + 1/11 SAM 5000 SAH 1000	888, 1050	995, 1250	1116	1142	61.6	108.4
RBC + Water	1120	520				
RBC + 1/11 SAM 1000 SAH 200	1250, 1320	508, 570	1211	538.2	181.5	104.5
RBC + 1/11 SAM 5000 SAH 1000	1400, 1610	496, 522	1574	610.9	84.7	-12.1

The mean recovery (the % measured of the spiked SAM and SAH in the samples (collected conc/starting conc*100)) was 102.6% for SAM and 146% for SAH but the variation in recovery was large. None of the blanks in any of the assays measured any peaks suggesting there was no cross contamination.

Once the accuracy of the assay had been established, SAM and SAH calibrator standards were then made up ready to measure alongside the SAM and SAH within the patients' samples already collected. 25 mg of SAM ($C_{15}H_{22}N_60_{55}$-XC7H80$_3$S) was dissolved in 25 mL of water (1 mg/mL or 1.34 mmol/L) and diluted to half by dissolving 5 mL of the solution in 5 mL of hydrochloric acid (HCL) giving a concentration of 0.5 mg/mL (0.674 mmol/L). To obtain a top calibrator standard for SAM of 5000nmol/L, 370µl of this solution was added to a conical flask containing 50 mL of water (7.4 µl/mL of 0.67 mmol/L of SAM or 5000 nmol/L). 5 mg of SAH (MW 384.41) was dissolved in 10 mL of HCL to give a concentration of 0.5 mg/mL (1.3 mmol/L). To reach a top calibrator standard for SAH of 1000 nmol/L, 38.5 µL of this solution was added to the 50 mL of water containing the 370.92 µl of SAM giving a top calibrator standard stock mixture of 5000 nmol/L SAM and 1000 nmol/L of SAH (0.77 µl/mL of 1.3 mmol/L of SAH or 1000 nmol/L). This was diluted by a fifth to obtain SAM 1000 nmol/L and SAH 200 nmol/L and so on to achieve calibrator standards of: SAM 5000 nmol/L - SAH 1000 nmol/L, SAM 1000 nmol/L - SAH 200 nmol/L, SAM 250 nmol/L-SAH 50 nmol/L, SAM 125 nmol/L-SAH 25 nmol/L, and SAM 50 nmol/L-SAH 10 nmol/L. Deuterated standards were prepared as above to give a working solution of 250 nmol/L SAM-d3 and 100 nmol/L of SAH-d4.

A blood sample obtained from a volunteer in a heparinised container was immediately spun down and separated into plasma, buffy coat and RBCs. RBCs were washed twice with 0.9% saline. WB, plasma and RBCs were used for analysis straight away.

Using a deep 96 well plate, 50 µL of plasma, WB and RBCs were pipetted into separate wells both on their own (6 wells each for plasma and WB, 2 wells for RBCs) and spiked with the varying concentrations of standards. Each concentration of standard was also pipetted into 2 wells each. Blanks containing 50 µL of water were analysed for comparison. 50 µL of deuterated standards were added to each well along with 200 µL of acetonitrile. After use of the standards and calibrators, the stock was frozen at -70°C for later use.

3) Measurement of SAM, SAH, HCys, methionine and PG in the patient samples

Both patients and volunteers had venous blood samples taken in heparinized tubes. All samples were then processed within 1 hour of having been taken. One microfuge tube was reserved for WB whilst another was centrifuged at 25,000 g for 30 s and separated into separate microfuge tube of RBCs, buffy coat and plasma. RBCs were washed with 0.9% saline and centrifuged at 25,000 g for 30 s, twice. 200 µL RBCs were pipetted into microfuge tubes. All samples were stored at -70°C until analysis.

Plasma, WB and RBCs were analysed using the LC-MS/MS assay. Samples were thawed, vortexed and centrifuged at 1500 g for 4 min. 50 µL of each sample was pipetted into separate microfuge tubes along with 50 µL of the previously prepared deuterated internal standards and then dissolved in 200 µL of acetonitrile. SAM and SAH calibrator standards at 50 nmol/L SAM – 10 nmol/L SAH, 125 nmol/L SAM – 25 nmol/L SAH, 250 nmol/L SAM – 50 nmol/L SAH, 1000 nmol/L SAM -200 nmol/L SAH, 5000 nmol/L SAM -1000 nmol/L SAH with 50 µL of deuterated internal standards dissolved in 200 µL of acetonitrile were also analysed. Blank wells contained water only. Samples and calibrators were vortexed and centrifuged again at 20800 g for 4 mins. 150 µl from each were then pipetted into deep 96 well plates for LC-MS/MS analysis.

Calibration curves for the measurement of PG were created using concentrations of 150 µL of 1/10 and 1/40 of 10 mmol/L PG (1000 µmol/L and 250 µmol/L) in water. Initially, the PG measured in the samples was calibrated against SAM-d3 as the deuterated standard. This analysis would later be repeated once the standard for PG had been acquired.

The MS was run in multiple monitoring mode. The first quadrupole was set to select the protonated molecular ion ($M+H^+$) of each compound, SAM (m/z = 399.0), SAM –d3 (m/z =402), SAH (m/z = 385.1) and SAH-d4 (m/z 389.3). The ion spray voltage was 5250 V, declustering potential 50 volts and collision energy 22.5 volts. The 2nd quadrupole was used as the collision chamber and the 3rd quadrupole was set to select the product ions of SAM (m/z = 250.2 and m/z 136.2), SAM-d3 (m/z 250.2 and m/z 136.2), SAH (m/z 136.1 and m/z 88) and SAH-d4 (m/z 136.1).

Chromatography was performed on a 5 cm Chirobiotic T column using acetonitrile with 0.05% formic acid flowing as the mobile phase. Samples were injected from a 96-deep well plate using an autosampler. Each cycle time was 0.74 s per ion with a total of 284 cycles. Figure 2.7.7 combines all the chromatograms used to measure SAM, SAH and PG within our samples.

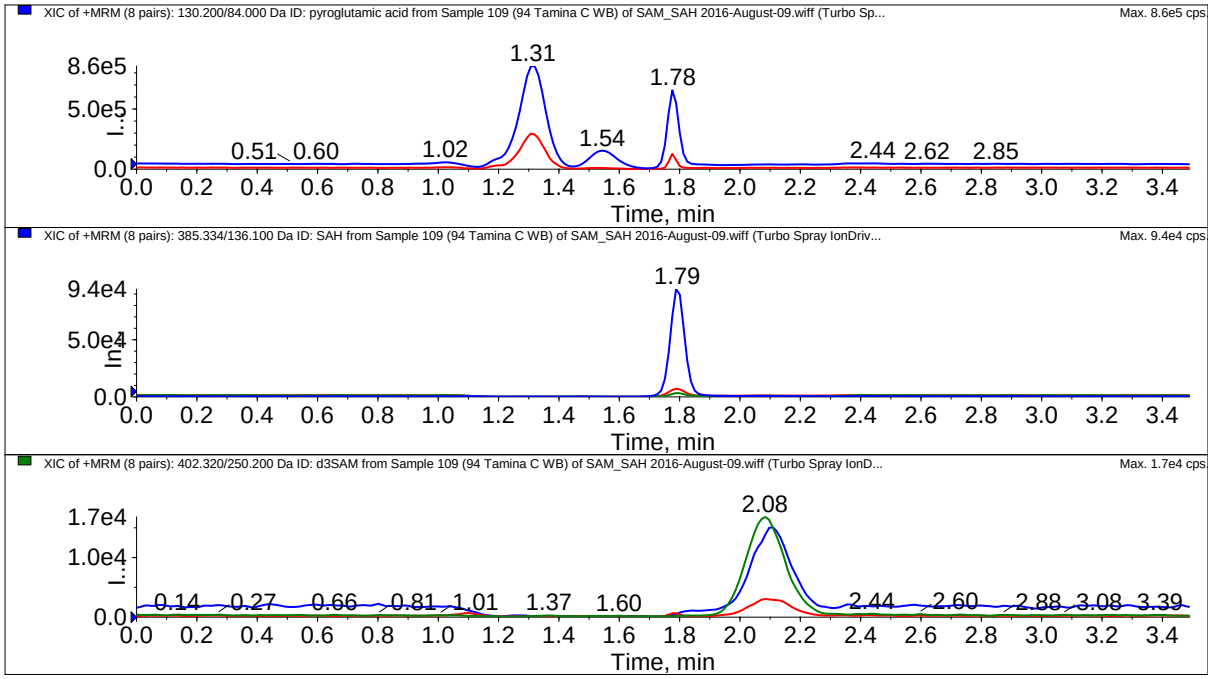


Figure 2.7.7: The three chromatograms for PG, SAH and SAM with deuterated standards for SAH and SAM only. The 1st chromatogram shows acquisitions set for the measurement of PG, eluting from the column at 1.31 min followed soon after by other ions, possibly Glu and Gln. The 2nd and 3rd chromatograms measure SAH and SAM respectively along with their deuterated standards (with 4 acquisitions seen (2 ions for each)).

A separate assay was performed on the API 5000 (Applied Biosystems, Warrington, UK) using a 3.3 cm Supelcosil column to measure total HCys according to the method of Magera et al.[281] 5 µL of each thawed, vortexed and centrifuged (as above) plasma sample was added to a working solution of 10 µL of 100 µmol/L D,L – HCys – 3,3,3',3',4,4,4',4' –d_8 and 0.3 mol/L d_4 methionine (Cambridge Isotope Laboratory). Reduction of disulfides was accomplished by the addition of 10 µL of D,L –dithiothreitol (from Sigma Aldrich, UK) which was allowed to react for 15 min at room temperature. The precipitation of proteins was achieved by the addition of 150 µl of 0.01% formic acid and 0.05% trifluoroacetic acid in acetonitrile. The solution was centrifuged at 20800 g for 4 min. 150 µL from each solution was pipetted into a deep 96 well plate for analysis by LC-MS/MS performed in MRM mode in which total HCys and HCys-d_4 were detected through the transition from the precursor to the product ion (m/z 136 to m/z 90 and 140 to m/z 94) respectively.

4) 5-MTHF measurement

Previous studies have demonstrated that folate levels are a strong predictor of SAM and HCys concentrations. 5-MTHF is the predominant form of folate in the body constituting around 80-90% of total folate.[282] 5-MTHF can be measured in either plasma or RBCs. Plasma concentrations of <7.9nmol/L (<3 µg/L) usually indicate folate insufficiency. Plasma 5-MTHF was measured for each patient and control within the cohort using HPLC as described in the method by Sobczynska-Malefora et al.[283] The plasma samples used were identical samples to those used for the SAM and SAH measurements but after 2 freeze-thaw cycles. 5-MTHF was correlated with SAM and SAH differences between the different patient groups.

The accuracy of 5-MTHF concentrations was compared against "home-made" quality controls (QCs) previously prepared from pooled plasma and checked against the calibration curve produced using three reference samples with certified 5-MTHF concentrations (Standard Reference Material 1955, National Institute of Standards & Technology, USA).[283] The ± 2SD from the mean is used as the acceptable criteria for QCs in the assay (Table 2.7.5).

Table 2.7.5: The expected concentrations (QCs) for mean MTHF in plasma assayed in two different sample on two different days

	Mean 5-MTHF in plasma sample (nmol/L)	Mean 5-MTHF in plasma sample 2 (nmol/L)
Day 1	57.3	21.28
Day 2	59.59	21.9

The results in Table 2.7.5 are within 2 standard deviations of each other (48.9 -62.7 nmol/L and 18.2 - 24.1 nmol/L respectively) and therefore not significantly different on different days.

5) Analysis of PG using its deuterated standard

In the previous analysis, PG was measured without its deuterated standard. Instead a callibration curve was produced against the measurement of deuterated SAM. However, there was a degree of imprecision between replicate data points (Figure 2.7.8). Deuterated PG was therefore acquired resulting a more linear calibration curve and a more accurate measurement of the compound (Figure 2.7.9 and Table 2.7.6).

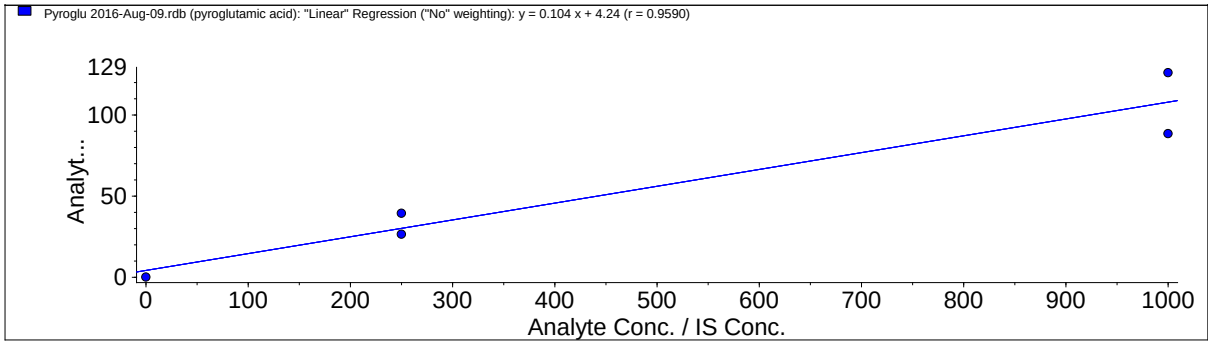


Figure 2.7.8: The measurement of PG without the deuterated standard but instead calibrating it against SAM-d3. There is a degree of imprecision seen between data points.

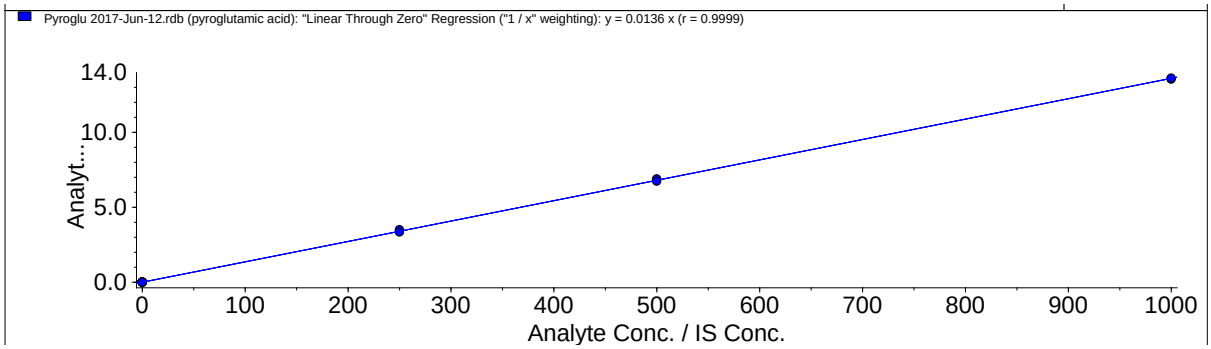


Figure 2.7.9: Standard curve between the PG calibrator standards and the PG deuterated standard. The curve is exactly linear and therefore the assay is far more accurate than that used in Figure 2.7.8.

Table 2.7.6: Measured concentrations and CV of the PG standard (250 and 1000 ng/mL PG).

Measured concentration for PG 250 ng/mL	255.5 ng/mL
CV for PG 250 ng/mL	0.83
Accuracy for measurement of PG 250 ng/mL	102.2%
Measured concentration for PG 1000 ng/mL	1002.5 ng/mL
CV for PG 1000 ng/mL	1.058015134
Accuracy for measurement of PG 1000 ng/mL	100.25%

Glutamine, an abundant metabolite in blood, spontaneously cyclizes to PG due to the electrospray ion source in LC-MS analysis.[284] It was therefore important to measure the amount of PG that was produced solely as a product of the SAM/SAH/HCys/Methionine Cycle rather than from the breakdown of glutamine within the MS chamber. To confirm good recovery of PG, 0.01 g of the deuterated standard, L-2-Pyrrolidinone-d5-5-carboxylic acid (MW 134.15 g, QMX laboratories Ltd, UK) was dissolved in 1 mL water (74.5 mmol/L). The opportunity was taken to measure glutamate and glutamine, but without their deuterated standards. 100 µL of 10 mmol/L glutamate, 100 µL of 33 mmol/L glutamine and 20 µL of 74.5 mmol/L L-2-Pyrrolidinone-d5-5-carboxylic acid were mixed with 1930 µL of water. Of this stock mixture, 100 µL was mixed with 20 mL methanol. 150 µL of this mixture was then pipetted into microfuge tubes containing 10 µL of each sample (98 samples of plasma, RBC and WB). These microfuge tubes were then vortexed and centrifuged for 5 min and pipetted alongside the standards into 96 well plates for LC-MS/MS analysis.

2.8 Screening for NRH using MRE

All patients underwent MRI and MRE sequences on a 3T system (Biograph mMr, Siemens Healthineers). Out of the 32 sequences performed, 4 were used for quantitative analysis. Healthy volunteers were also recruited as controls; comparisons between patients and these volunteers were made. The volunteers did not undergo primovist sequences. The 4 quantitative sequences were:

1) T1 mapping – This is a technique to measure liver T1 relaxation times using multiple T1 weighted images. Relaxation times are a measure of the spin magnetization component in the direction of the magnetic field in the magnetic resonance scanner after being disturbed from its equilibrium state.
2) R2* mapping- Iron shortens T1, T2, and $T2^*$ relaxation times, darkening images. The reciprocals of T2 and $T2^*$, known as R2 and $R2^*$, are directly proportional to iron.
3) Apparent diffusion coefficient (ADC) mapping – This is a measure of the magnitude of diffusion of water molecules within any tissue using MRI. Because different tissues contain different amounts of water, impedance varies, and is presented differently on diffusion weighted imaging (DWI).
4) MRE- was based on a prototype gradient-echo sequence synchronised with a mechanical vibrating transducer sited over the liver (Figure 2.8.1). Viscoelastic parametric maps including shear wave velocity and attenuation are generated offline using dedicated in-house software. MRE elastography has already been proven to be able to differentiate between mild and severe fibrosis in other liver disease cohorts (Figure 2.8.2).

Figure 2.8.1: A patient with the vibrating transducer situated over the liver, with a tube attached, through which the acoustic waves are passed from an acoustic generator. The transducer omits shear waves through the liver parenchyma, allowing software to then determine the viscoelastic properties of certain tissues.

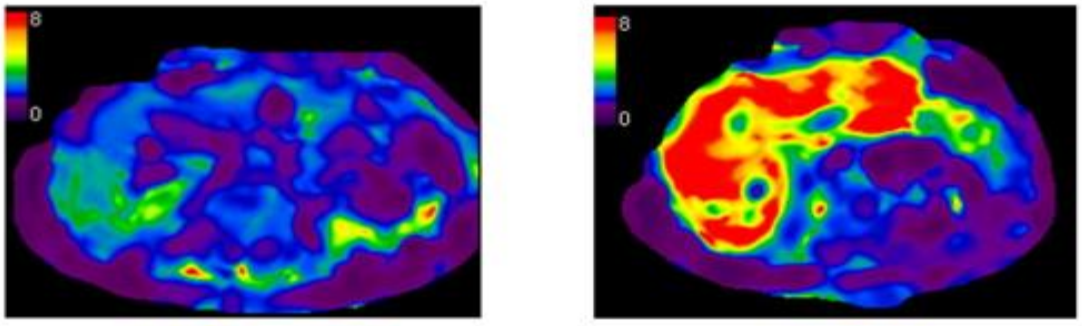


Figure 2.8.2: Two post-processed MRE images of liver with 2 grades of fibrosis (Taken from a publication by Griffin et al).[285] Red depicts liver stiffness or fibrosis, whereas blue depicts the absence of stiffness or fibrosis.

Scans of patients were first analysed across other sequences for nodules which might be regenerative. The levels set for analysis were based on the level at which MRE were performed. A region of interest (ROI) was demarcated around the liver at that level on all 4 sequences, avoiding areas prone to movement artefact from the heart. 3 smaller areas (5 cm^2) were chosen from the liver avoiding structures which were not hepatocytes, such as vasculature. These were located on axial images in the top right, middle left and bottom (Figure 2.8.3). On the ADC sequences, because this sequence did not involve breath holding and hence the liver not as stretched, 3 smaller areas of 2.5cm^2 were compared, but in the same regions as on the other sequences. Quantitative data was then obtained from these ROI which were: mean, SD and range of pixel density. MRE liver sequences were post-processed for velocity (stiffness), attenuation (viscosity -proportion of energy as heat) and non-linearity (deviation of displacement of signal or disorganisation).

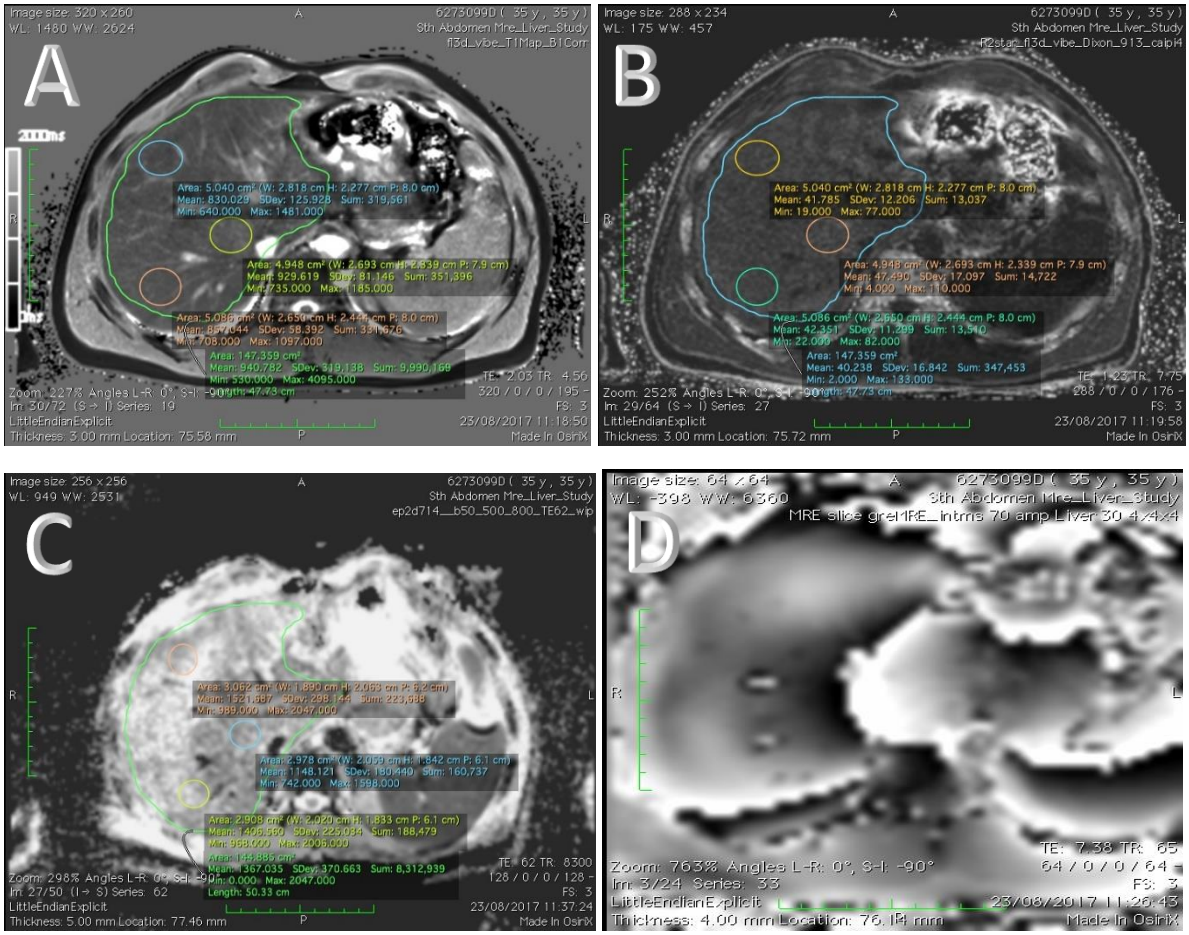


Figure 2.8.3: Sequences used to identify NRH: T_1-weighted mapping (A), $R2^*$ mapping (B), ADC mapping (C) and MRE (D). All sequences were performed on a 3T system (Biograph mMR, Siemens Halthineers) and then analysed at level 3 slice of the MRE sequence. ROI's were drawn using commercial software (Syngo, Siemens Healthineers) of 5 cm^2 in 3 regions of the liver except for the ADC sequence where the ROI was 2.5 cm^2. ROIs were also drawn around the whole liver for all sequences. The software then gave the mean pixel intensity, SD and range.

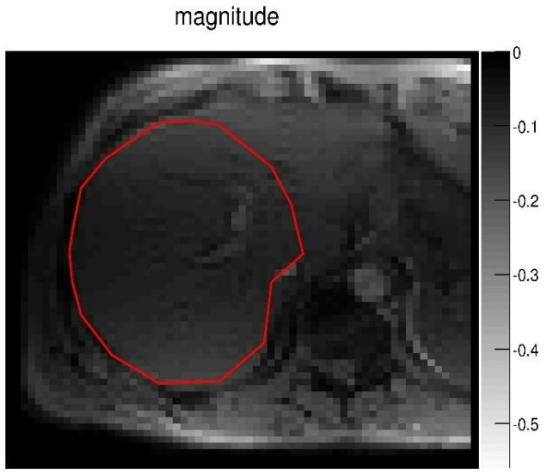


Figure 2.8.4: ROI drawn over the whole liver on the MRE sequence post processing generated on in-house software. Viscoelastic parametric maps measuring shear wave velocity (C, m/s) and attenuation (α, mm^{-1}) were then calculated based on the pixel densities.

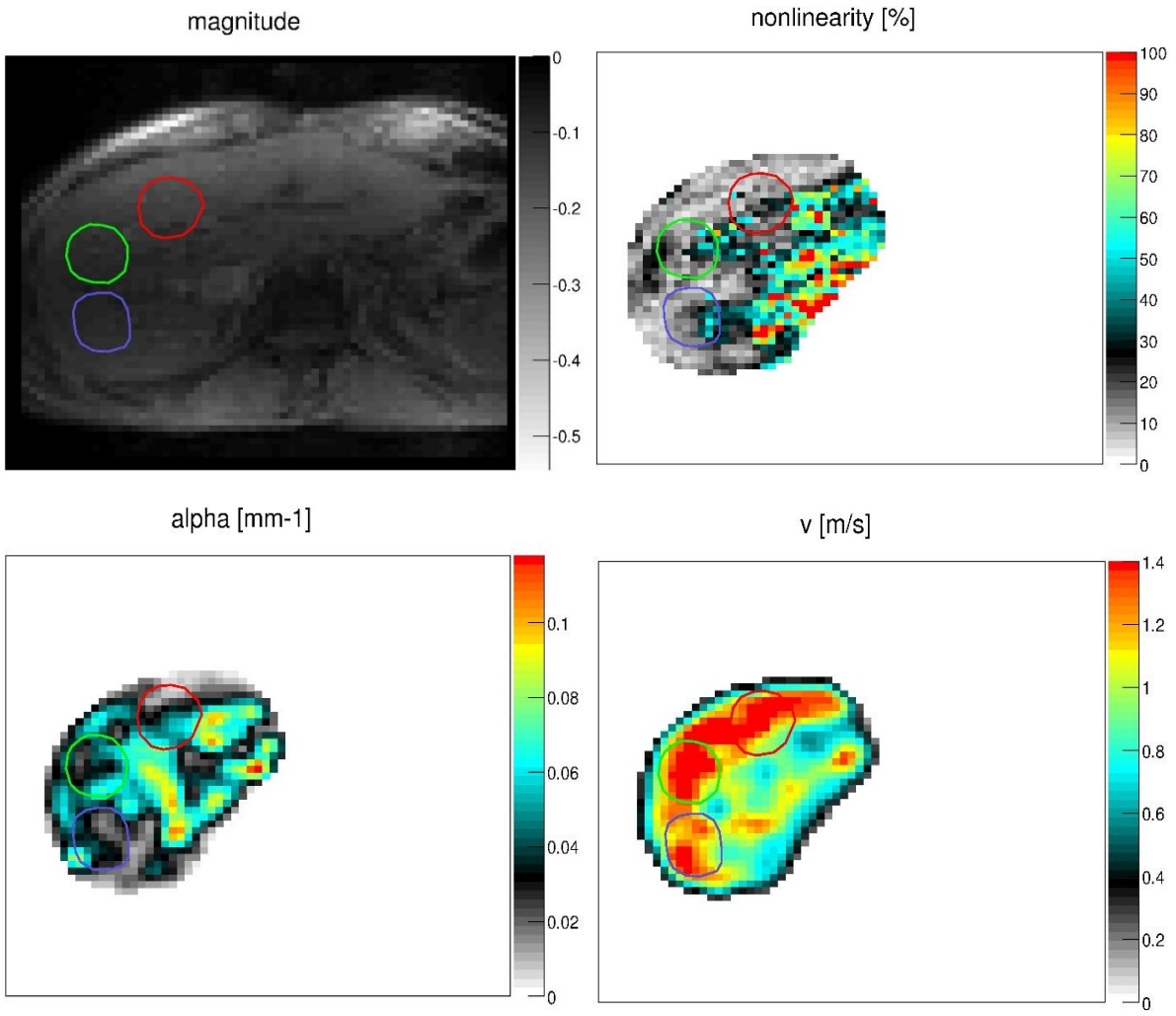


Figure 2.8.5: MRE magnitude image and parametric elastography maps with 3 ROIs analysed for non-linearity, shear wave attenuation (alpha) and velocity (v).

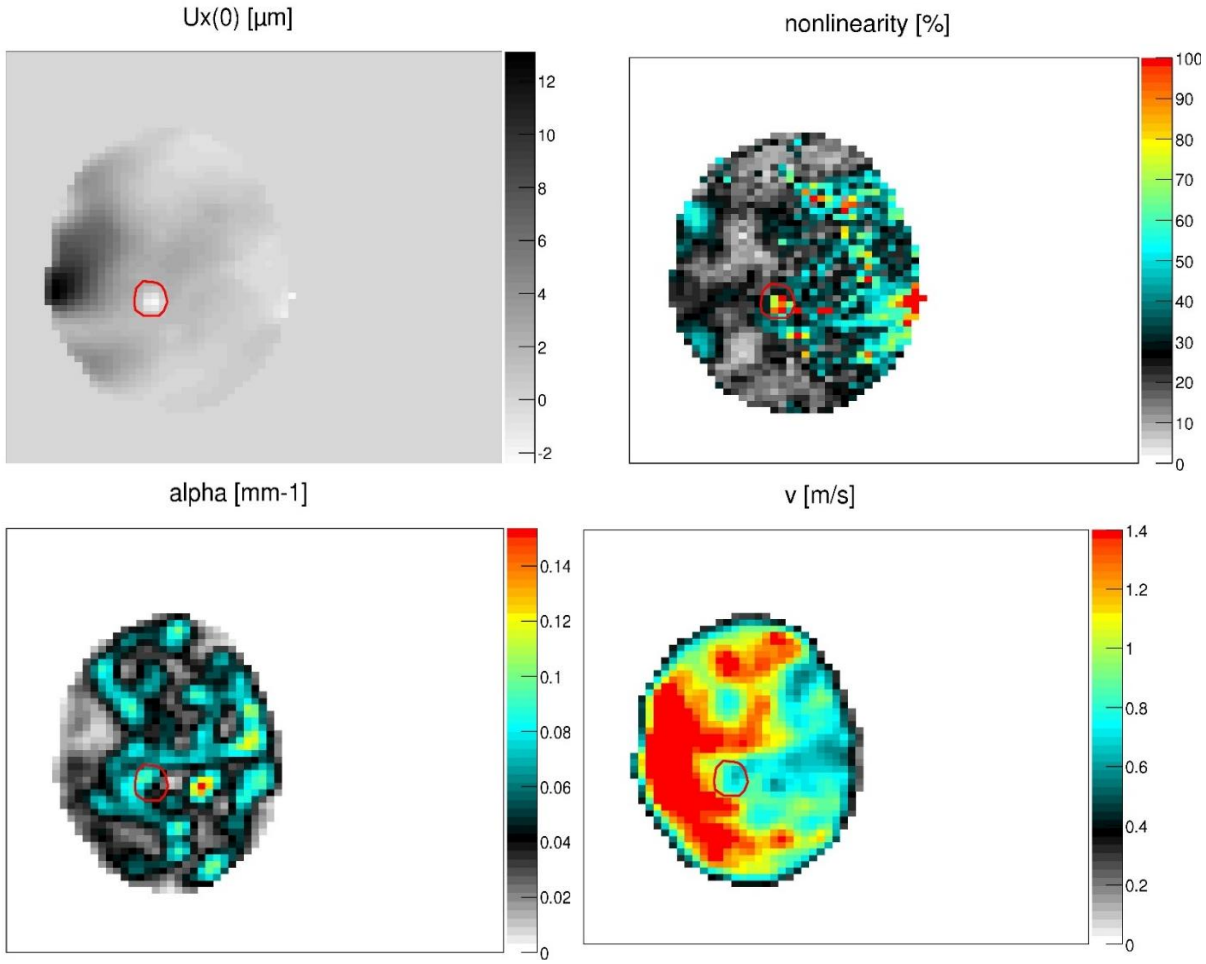


Figure 2.8.6: MRE magnitude image and parametric elastography maps of 1 ROI over a nodule analysed for non-linearity, shear wave attenuation (alpha) and velocity (v).

2.9 Inclusion criteria (definitions) and exclusion criteria

The inclusion and exclusion criteria were different between the studies. Apart from in the Screening of NRH using MRE study, all patients had proven IBD. For the candidate gene analyses, only patients on either AZA or MP were included. Patients with another cause for raised ALT, other than thiopurines, were excluded. Patients who had only ever had metabolite measurements whilst on LDTA were excluded.

The definition of hypermethylation fitted with that of other studies: an MeMP:TGN ratio > 11 on two occasions. The definition was added to by including any patient who had had an MeMP:TGN ratio > 20 on one occasion. This was because some patients were switched to LDTA after the first metabolite profile was measured but were clearly hypermethylators to begin with. Similarly, some patients were clearly hypermethylators if they had an MeMP:TGN ratio > 11 with an MeMP >5000 pmol/L on one occasion but were then switched to LDTA. Other studies have defined hypermethylators as those patients still hypermethylating after 12 weeks of therapy. However, hypermethylation at any timepoint was included after starting, because the clinic reviews patients' metabolites after 4-6 weeks of starting thiopurines and alter the therapy accordingly.

The traditional definition for hepatotoxicity is defined by an ALT ≥ 3 X ULN (males > 200 IU/L and females >175 IU/L). However, hepatotoxicity is identified early after starting therapy and therapy changed before the ALT can increase that high. Most of the patients diagnosed with thiopurine-induced hepatotoxicity never have an ALT ≥3 X ULN and yet are clearly a cohort worth studying. A lower threshold for ALT was chosen of above the ULN (>56 IU/L).

Patients recruited to the NRH study had to have been biopsied within the last year and have had evidence of NRH histologically with minimal or no fibrosis. Patients who were pregnant or who had other contraindications to MRI, including an allergy to contrast agents used, were excluded.

2.10 Power calculations

The number of patients required to be recruited was based on the power calculation as shown in Table 2.10.

Table 2.10: Using the MAF of genes within the general population from previously published studies and Pubmed, it was possible to predict the sample size required to determine significant genotypic differences ($P < 0.05$) between population groups at a power of 80%.

Gene (cDNA base change)	SNP rs number	Global MAF	MAF needed in cases	Sample size needed
MTHFR 1286 A>C	rs1801131	0.31	0.62 (x2)	79
MTHFR 677 C>T	rs1801133	0.33	0.66 (x2)	81
MTHFR 1654-80 G>A	rs17375901	0.03	0.15 (x5)	208
MTHFR 359+160 A>G	rs17367504	0.13	0.39 (x3)	93
AOX 3404 A>G	rs55754655	0.16	0.32 (x2)	246
IL-15 *83C > A	rs10519613	0.075	0.344 (x3)	201
IL-15 g.142709723T T>C	rs17007695	0.16	0.48 (x4)	64
ADK 827-6202A A>G	rs946185	0.344	0.68 (x2)	79
ABCB5 343 A>G	rs2301641	0.341	0.68 (x2)	79
SLC 38A9 6899 A>G	rs6897117	0.30	0.60 (x2)	84
CYP1A2*C 3860G>A	rs2069514	0.2	0.6 (x3)	45
CYP1A2*F 163C>A	rs762551	0.37	0.12 (÷3)	91

http://statpages.org/proppowr.html

2.11 Statistical analyses

All data was analysed using SPSS and presented as 3 significant figures or to 1 decimal place unless otherwise appropriate. Continuous variables were assessed for normal distribution and presented as mean ± standard deviation (SD) or median ± interquartile range (IQR); significance was assessed using independent and paired t-tests. Most of the catergorical data was analysed using a 2 x 2 web based contingency table, essentially a two-sided Fisher's exact test (http://graphpad.com/quickcalcs/contingency1.cfm). The SD for the MRE parameters is expressed as root mean square (RMS). Quantitative analysis of MRE sequences was analysed using an independent samples t-test for parametric data and a Mann-Whitney U-test for non-parametric data. Genotypes were tested for departure from Hardy-Weinberg equilibrium and the significance of genotypic differences compared to controls, corrected for multiple testing (Bonferroni correction). Models are presented as both univariate and multivariate analysis; in multivariate analysis, outcomes for thiopurine hepatotoxicity were adjusted for other variables. Correlation between parametric variables were assessed for collinearity and goodness of fit (R^2) using Pearson's test. Correlations were assigned either perfect positive or negatively linear (+1 or -1), strong positive or negatively linear (+0.7 or -0.7), moderate positive or negatively linear (+0.5 or -0.5), weak positive or negatively linear (+0.3 or -0.3) or no linearity (0). For each factor considered to be of important association, the odds ratio (OR) with its 95% confidence interval (CI) are presented. Receiver operator characteristic (ROC) curves for plasma SAM and RBC PG were created using SPSS to predict abnormal metabolism of thiopurines using randomly selected thresholds. From these thresholds, the relative sensitivities and specificities of the compounds at predicting abnormal thiopurines metabolism were determined.

Chapter 3: Cohort study of abnormal LFTs in IBD

3.1 Introduction

One third of IBD patients will have abnormal LFTs at some point during their disease which may or may not herald liver failure.[39] This concern could result in patients having their drugs stopped whilst investigations are performed. Whilst this approach could be entirely appropriate, it risks a relapse in their IBD. Investigating abnormal LFTs often involves a panel of tests (autoantibodies, virology and metal storage tests) which in a resource-stretched National Health Service, may not be an efficient way of managing patients unless the diagnostic yield is suspected to be high. The aim of this study was to scope out the causes of abnormal LFTs in IBD to establish a strategy to manage patients more effectively.

3.2 Patient recruitment and summary of methods

This study was a retrospective analysis of patients who attended the IBD clinic at GSTT between September 2014 and June 2016. Patients were included in the study if they had abnormal LFTs (defined as an ALT>56 IU/L at Guy's and St Thomas' NHS Foundation Trust) at any time since their IBD was diagnosed. Patients with either an elevated ALP or GGT on its own, without elevation of ALT, were not included. The reason for this was because elevation of ALP on its own can be due to bone diseases such as osteopenia or osteoporosis and is therefore not as specific to liver disease as ALT; and GGT is not routinely measured at GSTT. All patients recruited were assigned a cause based on which definition they best fitted (Table 3.2) and analysed as per Figure 3.2.

The pattern of abnormal LFTs was defined by comparing the ALT to the ALP: if the ratio of ALT:ALP was > 1, this was described as hepatocellular whereas if the ratio of ALT:ALP was < 1, this was defined as cholestatic. For suspected drug induced liver injury (DILI), abnormal LFTs were classified according to the FDA definition of hepatocellular and cholestatic injury (Table 1.5.1): the R ratio is (ALT/ULN÷ALP/ULN). An $R \geq 5$ was defined as hepatocellular whereas an $R \leq 2$ was cholestatic; an R of between 2 and 5 was mixed.

Table 3.2: Causes of abnormal LFTs to which patients in this cohort study were assigned.

Cause	Definition
Thiopurines	A temporal relationship between the abnormalities of LFTs and the start of thiopurine treatment, followed by normalisation of LFTs after reducing or stopping thiopurines
Flare of IBD	A temporal relationship between abnormal LFTs and a flare in the disease along with normalisation of LFTs where disease activity improved
PSC	Known PSC or PSC later diagnosed based on either MRCP or ERCP
Fatty liver	Radiology suggestive of a fatty liver or if the patient had already had a diagnosis of NAFLD
Drug-related (not thiopurines)	A temporal relationship between abnormal LFTs and the start of drug treatment (not thiopurines) and a normalisation of LFTs after change in drug treatment
Other	Where the cause of abnormal LFTs was found to be due to a specific cause such as alcohol, portal vein thrombosis or choledocholithiasis
Unknown	Where the cause of the abnormal LFTs did not fit any of the above categories and remains undiagnosed

Patients in the cohort study were analysed for the following:
• Age • Gender • Weight • Time between the first raised ALT and the maximum ALT • Total number of blood tests where LFTs were abnormal until both ALT and ALP normalised • The ALP, bilirubin and GGT at highest ALT • Previously diagnosed liver disease according to results from liver screens and ultrasounds • The length of time it took for LFTs to normalise (months) • The character of LFT abnormality was classified according to definitions: "Limited" - resolving completely within a short period "Chronic" - abnormal over a long period due to a known chronic liver disease "Flickering" - LFTs fluctuate between normal and abnormal "Persistent" – abnormal LFTs applied to DILI only as discussed in Section 1.5 • IBD-type and behaviour (CD, UC or IBD-U) according to Montreal classification • Disease activity (active disease was defined as: Harvey Bradshaw Index (HBI) or Simple Clinical Colitis Activity Index (SCCAI) ≥1, CRP≥5, endoscopically active disease, or a faecal calprotectin ≥ 50 µg/g) • Previous surgery

Figure 3.2: The variables the patients were analysed for.

3.3 Results

329 IBD patients with abnormal LFTs were analysed (51.4% male) from September 1996 through to July 2016. 61.4% had CD, 3.3% had IBD-U and 35.5% had UC. The location and behaviour of IBD is shown in Table 3.3.1. The mean age of the patients with abnormal LFTs was 39.6 years (SD 13.8). Median ALT at its highest was 110 IU/L (IQR 94.0, range 56.0 to 1980 IU/L). Table 3.3.2 compares abnormal LFTs with gender. The median number of times the ALT was abnormal for any patient was on 2 occasions (IQR 3.0, range 1.0 to 75.0). The median time it took for ALT to become normal from its highest point was 1 month (IQR 3.5, range 0.5 to 120 months) (Figure 3.3.1).

Of the whole cohort, four patients died: one due to biliary sepsis from choledocholithiasis, another from PSC with cholangiocarcinoma, one due to metastatic breast cancer and one patient with leukaemia and associated Graft-versus-Host-Disease.

ALT was overall more abnormal than ALP (median ALT:ALP was 1.1). Of those patients who had a cholestatic picture (ALT: ALP < 1), n=32), 10 had PSC, 8 were due to thiopurines, 7 were due to disease flares, 1 fatty Liver, 1 antibiotic-related, 1 unknown, 1 EBV, 1 choledocholithiasis, 1 MTX-related, and 1 post-operative (Figure 3.3.2).

97% of hepatocellular liver injuries were grade 1 according to the Koller classification whereas cholestatic liver injuries were split evenly between Grade 1 and 2 (Table 3.3.3).

Table 3.3.1: Shows the location and behaviour of inflammation in the cohort. There were 8 patients with IBD-U.

Crohn's Disease (N= 206)	%
Ileal disease (L1)	22
Colonic disease (L2)	23
Ileocolonic disease (L3)	54
Pan enteric (L4)	1

Crohn's Disease (N= 206)	%
Montreal B1 (non-stricturing, non-penetrating)	66
Montreal B2 (stricturing)	14
Montreal B3 (penetrating)	20
Perianal disease	17
Previous surgery	31

Ulcerative colitis (N= 115)	%
Proctitis	8
Proctosigmoiditis	17
Left sided colitis	20
Pancolitis	55

Table 3.3.2: LFTs of the cohort relative to the ALT at its highest, along with the ALT:ALP ratio.

	ALT (IU/L)	ALP (IU/L)	Bilirubin (µmol/L)	ALT:ALP
Mean	152	148	14.3	1.4
Males	144	143	14.2	1.4
Females	161	152	14.4	1.5
Median	110	93	9	1.1
Range	56-1980	21-1120	2-196	0.2-11.6
SD	160	145	21.6	1.2

The ULN for ALT was 56 IU/L and for ALP was 129 IU/L. ALT:ALP > 1 is defined as hepatocellular, ALT:ALP < 1 is defined as cholestatic.

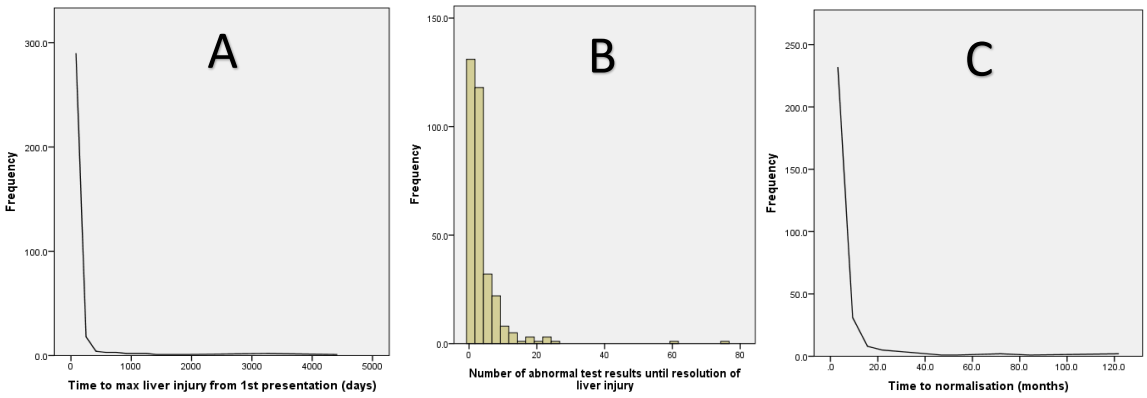


Figure 3.3.1: Analysis of our cohort of IBD patients with abnormal LFTs (n=329) according to the following: A) time to highest ALT from first presentation (days), B) the number of ALTs checked until complete resolution of liver injury (most had one or two tests, but some had over 20 tests), and C) the number of months to ALT normalisation from its highest point.

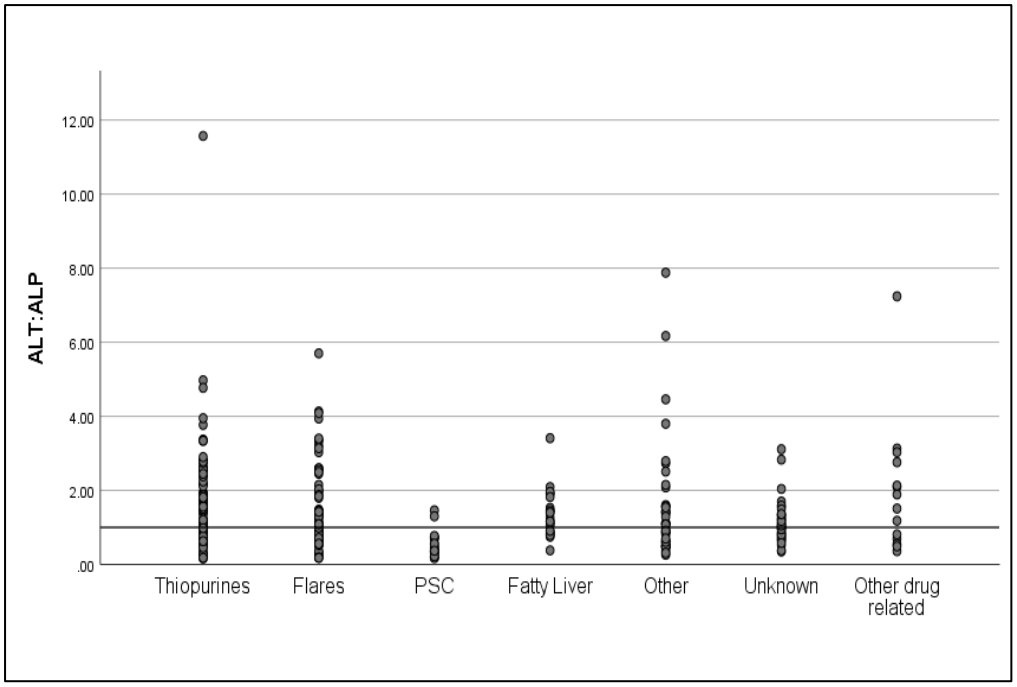


Figure 3.3.2: The ALT:ALP ratios amongst different causes of abnormal LFTs in IBD. Most causes have an ALT:ALP > 1 other than PSC where the ratio was mostly < 1.

Table 3.3.3: Grades of liver injury as used by Koller et al.[80]

Liver injury grade as used by Koller et al[80]	N (%)
Grade 1 hepatocellular (ALT <3 x ULN)	286/294 (97%)
Grade 2 hepatocellular (ALT >3 x ULN)	8/294 (2.7%)
Hepatocellular injury (ALT > 2 ULN)	23/294 (7.8%)
Grade 1 cholestatic (ALP < 2.5 x ULN)	16/32 (50%)
Grade 2 cholestatic (ALP >2.5 x ULN)	16/32 (50%)

Figure 3.3.3 categorises the 329 patients with abnormal LFTs into 7 causes of liver injury as assigned by the author. Table 3.3.4 compares the patients assigned to those groups by weight, highest ALT, resolution of liver injury and activity of the underlying IBD.

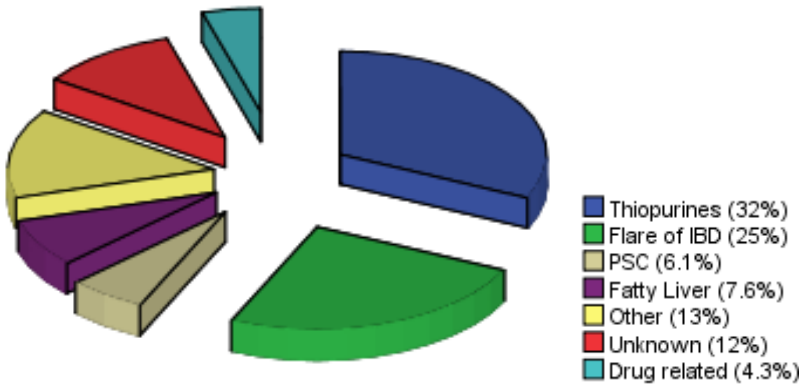


Figure 3.3.3: The percentage of patients in the 7 causes for liver injury.

Table 3.3.4: Mean weight, median highest ALT, ALT:ALP, % LFTs that settled, pattern of liver injury, % that had active disease and median time to normalisation from highest ALT in the liver injury groups.

Cause	N	Mean weight (kg)(SD)	Median Highest ALT IU/L (IQR)	Settled (%)	Median ALT:ALP (IQR)	R	Active disease (%)	Median months to normalisation (IQR)
Thiopurines	105	78.5 (18.6)	112 (105)	92.4	1.4 (1.4)	3.71	51.1	1.0 (2.1)
Flare	82	71.0 (18.2)	106 (61)	90.2	1.1 (1.1)	NA	100	1.0 (1.5)
PSC	20	76.7 (11.2)	83 (83)	50.0	0.4 (1.0)	NA	60.0	3.0 (7.4)
Fatty liver	25	94.2 (16.7)	100 (36)	80.0	1.1 (0.8)	NA	48.0	9.0 (14.5)
Other	43	70.0 (18.4)	127 (142)	95.7	1.0 (1.0)	NA	43.5	2.0 (4.5)
Unknown	40	74.3 (17.3)	75 (70)	86.1	1.1 (0.6)	NA	38.9	3.0 (10.0)
Drug related	14	77.8 (11.9)	151 (191)	93.3	2.1 (2.2)	4.55	73.3	1.0 (4.5)

ALT:ALP > 1 is defined as hepatocellular, ALT:ALP < 1 is defined as cholestatic. R is relevant to suspected drug induced liver injury only and is defined as (ALT/ULN÷ALP/ULN). An R ≥ 5 is hepatocellular whereas an R ≤ 2 is cholestatic. An R of between 2 and 5 is mixed. "Drug-related" excludes thiopurines.

88% of ALTs settled to normal within the study time-period with the remainder having ongoing ALT elevation. Abnormal ALT was felt to be "limited" in 70% of cases (normalising over a median time-period of 1 month, IQR 2.5, range 0.5 to 16.0), with 17% being "chronic" due to a known chronic liver disease (normalising over a median time period of 9.5 months, IQR 14.3, range 0.5 to 84.0). A further 11% had "flickering" abnormal ALT (normalising over a median time period of 11 months, IQR 20.0, range 1.0 to 120.1).

Patients with thiopurine hepatotoxicity were significantly heavier than patients with flares ($P < 0.01$, independent samples t-test). Both were equal for time to normalisation. Patients with fatty liver were significantly heavier when compared to all other causes (mean 94.2 kg versus 76.2 kg, $P < 0.01$, independent samples t-test). The ALT:ALP was 0.4 (cholestatic) in patients with PSC versus 1.1 (hepatocellular) in patients with Fatty Liver.

Thiopurine hepatotoxicity

105 patients had thiopurine hepatotoxicity. Of these, 56% had CD and 39% had UC. 82 (78%) patients had hepatotoxicity due to AZA. The mean dose of AZA was 128 mg (1.6 mg/kg). 3 patients had hepatotoxicity on LDTA, mean dose 25 mg with 100 mg allopurinol. The remainder had hepatotoxicity on MP at a mean dose of 79.2 mg (1.1 mg/kg). Methylation status was known in 88 patients (84%) of whom 39 (44%) were normomethylators and the remainder hypermethylators (66%). 51% were male, mean age 40.8 (SD 12.6). Only 1 had underlying NAFLD prior to commencing thiopurines. 92% of LFTs settled after either stopping thiopurines, reducing the dose or switching to LDTA. The remainder (8%) had some degree of persistent LFT abnormalities. A liver screen (panel of blood tests to screen for chronic liver diseases) was performed in 16% and an ultrasound in 23%. Of the patients in whom an ultrasound was performed, 54% had a fatty liver. 53% had active disease at the time of liver injury. The median highest ALT was 112 IU/L; the median bilirubin was 10.2 µmol/L (IQR 9.1, range 3.2-103). Only 3 patients had a bilirubin > ULN (34 µmol/L), one of whom had underlying Gilbert's syndrome as well as alcoholic liver disease. None had markers of liver failure (defined as having either encephalopathy or INR ≥ 3). The median time to normalisation of LFTs was 1 month. Abnormal LFTs were of a mixed pattern according to the FDA definition (the R ratio = 3.7) although the ALT:ALP of 1.4 defined this as hepatocellular. 21% were on concomitant mesalazines and 11% on biologics.

Of the 105 patients with thiopurine hepatotoxicity, 54 were switched to LDTA of whom 4 continued to have hepatotoxicity. LDTA was therefore effective in 93% of patients with hepatotoxicity. Table 3.3.5 shows the success rates according to methylation status. 34 of the 105 patients had thiopurines stopped, all of whom had a resolution in hepatotoxicity. A further 6 patients had their dose reduced, 5 of whom had successful resolution of hepatotoxicity. Of the remainder, 10 patients' abnormal LFTs resolved themselves whilst 1 patient was switched to MP with a resolution in hepatotoxicity.

Table 3.3.5: The relative success of switching to LDTA depending on methylation status

Allopurinol switch (N=54)	Unknown methylator status (N=7)	Hypermethylators (N=36)	Normomethylators (N=11)
Successful	6 (86%)	35 (97%)	9 (82%)
Unsuccessful	1	1	2

Flare-related

82 patients (24%) had flare-related abnormal LFTs; 41% male with only 1 having previously diagnosed liver disease (NAFLD). A liver screen was performed in 13% with an ultrasound performed in 24%; 6% of the latter had a fatty liver on ultrasound. 72% of patients had CD and 28% had UC. Of those patients with flare-related disease, 15% had ileal disease, 22% colonic and 60% ileocolonic - not significantly different from all other causes of abnormal LFTs. 55% had non-stricturing, non-penetrating disease (B1). 14% had stricturing (B2) whilst 31% had penetrating disease (B3) - this was significantly different (P =0.03, Chi-squared test) from all other causes of abnormal LFTs, where 71% had non-stricturing, non-penetrating disease (B1). 12% of patients with flare-related abnormal LFTs had perianal disease, 32% had had previous surgery – both not significantly different from other groups. The median highest ALT for this group was 106 IU/L. The median bilirubin at maximum ALT was 7.2 µmol/L (IQR 9.1). Only 1 patient had a bilirubin > ULN - this was felt to be due to an anastomotic bleed. The median time to normalisation was 1 month. Abnormal LFTs were overall hepatocellular (ALT:ALP of 1.1). The mean weight was 71kg.

PSC

There were 20 cases of PSC (65% male, mean age of 38); 15 had UC, 3 had CD, 2 had IBD-U. Of the patients with UC, all except 1 had pancolitis. Of the 3 with CD, all had non-stricturing, non-penetrating disease (B1); no patients had perianal disease. 60% of all patients with PSC had active IBD and 15% had had previous surgery. Median highest ALT was 83.1 IU/L. At the highest ALT, bilirubin was only raised in 1 patient at 35.2 µmol/L although there were patients in this group who at some point were jaundiced. The ALT:ALP was 0.4 suggesting this as being cholestatic.

2 patients developed colorectal cancer or high-grade dysplasia requiring colectomy. 2 patients developed a cholangiocarcinoma. 1 patient had cirrhosis and a dominant stricture. 16 patients had no complications from their PSC, one of whom only had small-duct PSC. However, although they had no complications, 4 were felt to have progressive disease. Of the 18 patients without cholangiocarcinoma, 2 patients were on vedolizumab, 2 patients were on AZA with 5-ASA, 2 were on AZA alone, 8 were on just 5-ASA and 4 were on no medications.

Drug-related (non-thiopurines)

Of the 14 cases of non-thiopurine DILI, 9 were caused by MTX, 2 by mesalazines and 3 by antibiotics. There were no cases of anti-TNF induced DILI. Bilirubin was not raised in any patients. The R ratio was 4.5 suggesting this being a mixed pattern although the ALT:ALP ratio was 2.1 suggesting it to be overall hepatocellular; all of the patients had CD.

Of the 9 related to MTX, all stopped MTX except 1 who reduced the dose and all settled except for 2 who had persistent pattern of abnormal LFTs, both of whom had stopped the drug.

Of the 2 with mesalazine-induced liver injury, the highest ALT was 543 IU/L and by the FDA definition was hepatocellular. Bilirubin was normal in all cases. 1 patient continued on mesalazines with ongoing abnormal LFTs and a biopsy suggestive of drug-induced cholestasis.

Of the 3 with antibiotic-induced liver injury, the highest ALT was 270 IU/L and the pattern cholestatic. Bilirubin was normal in all cases.

Other Causes

The "Other" causes are divided into 13 main diagnoses for liver disease as shown in Table 3.3.6.

Table 3.3.6: The most frequent causes for abnormal LFTs in patients with IBD and how likely they are to settle.

Cause	N (%)
Thiopurines	105(32%)
Flare of IBD	82 (25%)
Cause unknown	40 (12%)
Fatty Liver Disease	25 (8%)
PSC	20 (6%)
Other drug-related	14 (4%)
Post-operative	8
Biliary stone disease	6
Alcoholic liver disease	4
Autoimmune hepatitis	3
Viral hepatitis (2 x Hep C,2 x CMV and 1 x EBV)	7
Portal Vein Thrombosis	2
Haemochromatosis/iron overload	2
Total Parenteral Nutrition	2
IgG4 pancreatitis	1
Sepsis	2
Ischaemic hepatitis	2
Liver metastases	1
HELLP Syndrome/Pre-eclampsia	2

Hep C: Hepatitis C; CMV: Cytomegalovirus; EBV: Epstein Barr Virus; HELLP: Haemolysis, Elevated liver enzymes and Low platelets.

3.4 Discussion

This study represents one of the largest cohorts of IBD patients with abnormal LFTs ever analysed. Although the study does not measure the incidence of abnormal LFTs within our IBD community, an incidence of 30%, as suggested by other studies, seems reasonable.[39]

Abnormal LFTs were predominantly hepatocellular. Where they were cholestatic, the diagnosis was mostly PSC. Therefore, the first point to make is that where LFTs are cholestatic, an MRCP should be requested to rule out PSC.

A third of abnormal LFTs were due to thiopurines and another third due to flares of disease. In both, LFTs settled within a few months. The remainder were a mixture of different causes, although biliary disease from stones and PSC featured strongly.

Two thirds of the patients with abnormal LFTs in the whole cohort had CD although nearly all those patients with PSC, had UC; the reason for this is unclear. Thiopurines were no more likely to cause hepatotoxicity in patients with CD than UC. It is likely that as thiopurines are first-line treatment for patients with CD, more CD patients with thiopurine hepatotoxicity were collected. Patients with flares of disease and abnormal LFTs were more likely to have CD than UC.

We have one of the largest UK cohorts of patients on biologics and there were no cases of IFX causing liver injury, despite it being a recognised cause of liver injury in around 2-6% of patients.[80]

MTX was the cause of hepatocellular injury in 8 patients. Most patients' LFTs normalised after stopping MTX.

17% of IBD patients had an underlying chronic liver disease where LFTs continued to be chronically abnormal. This suggests that where LFTs continue to be abnormal beyond a few months, it is advisable to perform a liver screen to exclude an underlying chronic liver disease.

Mortality due to liver injury in IBD is low; bilirubin was rarely elevated, and no patients were coagulopathic or encephalopathic (both hallmarks of liver failure). In the 4 deaths, the outcome would have remained unchanged even if a more aggressive approach to the management of the abnormal LFTs had been taken. The severity of hepatocellular liver injury was only grade 2 in 2.7% of cases. This all suggests that there is time for the clinician to determine the likely cause of the abnormality, rather

than stop treatment where it might be otherwise providing benefit to the patient. Regarding how often to check abnormal LFTs to make sure they are not worsening, although there is no direct evidence, I would advise doing this monthly. Differentials are set out in the decision tree below (Figure 3.4) which serves as a guide to help diagnose IBD patients presenting with abnormal LFTs.

There are a few other interesting points raised by these results. Patients with thiopurine hepatotoxicity were heavier than the rest of the cohort and more likely to have a fatty liver on ultrasound. This suggests that a fatty liver may predispose to thiopurine hepatotoxicity. Patients with a fatty liver may need alerting to the increased risk they have for hepatotoxicity and may need closer monitoring. Males were no more likely to get thiopurine hepatotoxicity than females as suggested in other studies (see Section 1.11). Thiopurine hepatotoxicity was predominantly hepatocellular. Just under two thirds of patients had hepatotoxicity due to hypermethylation. LDTA was successful in over 95% of cases at treating hepatotoxicity due to hypermethylation and in over 85% of hepatotoxicity with normomethylation. Ongoing hepatotoxicity was seen in less than 8% of patients on LDTA but ongoing monitoring in patients switched to LDTA is needed. 34 (32%) patients who had their thiopurine stopped could have been switched to LDTA with successful resolution of hepatotoxicity. 10 of these patients had unknown methylation status whilst 17 were normomethylators and 7 were hypermethylators. This suggests that clinicians are more likely to stop thiopurines in patients who are normomethylators rather than hypermethylators, despite our findings that switching to LDTA seems successful for both.

Flares of disease accounted for a third of patients with abnormal LFTs and were significantly more likely to have CD. These patients were more likely to have stricturing or penetrating disease. The mechanism by which flares might cause hepatotoxicity is unclear and needs further research. Inflammation may be tracking transmurally across from small bowel to the liver, hence why this is more commonly seen in penetrating disease. Another mechanism may be that inflammatory cells are migrating via the portal circulation, similar to how PSC is thought to occur, although one would expect, as with PSC, for this to cause a more cholestatic liver injury.[43]

The cohort in this study had a larger percentage of patients with ileocolonic (B3) disease than that of other cohorts in other studies.[5] Similarly, there are more patients with extensive colitis (E3) than in other studies.[286] This suggests that these phenotypes may be more prone to developing liver disease due to the extensive location of the disease.

There are several limitations to this study. Firstly, the incidence of the different causes of hepatotoxicity were dependant on which patients came to the IBD clinic during the study period. Analysis of patients attending the IBD clinic over 2 years was designed to capture all patients within our IBD cohort who had abnormal LFTs and therefore represent a true reflection of the prevalence of the different causes of liver abnormalities in patients affected by IBD. However, patients who may have had liver failure and died, would not have been recorded whereas only the less acute patients would have been seen in outpatients. Secondly, liver disease in remission, such as PBC, IAC and even some PSC patients would not have been part of the cohort, although their inclusion would have been valuable. Thirdly, the retrospective nature of the study made it very difficult to standardise what was analysed. Liver screens and ultrasounds were not performed in all patients and blood tests were carried out at both infrequent and variable time points. A patient with thiopurine hepatotoxicity might have had an elevated ALT, told to stop the drug and then not seen in clinic for 6 months, hence making the true normalisation time inaccurate. Fourthly, not recruiting patients with an isolated raised ALP or GGT may have led to the under-representation of some cholestatic conditions, although a database has been collected of all PSC patients at GSTT and all of them were recruited. GGT, alongside ALP, is not routinely measured at GSTT and therefore it would not have been certain that a raised ALP was from the liver. Lastly, although the final diagnosis, or the group that the patient fitted into, was clear in most cases, there was in fact only one author making the decision on this (BW). Ideally, the diagnosis would have been made by one or more independent clinicians to avoid any bias.

It is difficult to make comparisons with other studies because the methods and definitions for liver injury vary. Other studies have not divided patients with liver injury up according to whether they are hepatocellular or cholestatic. However, there is the suggestion from other studies that BMI and a fatty liver are associated with an increased risk of liver injury from all causes.[80] Bastida et al prospectively monitored 161 patients commenced on thiopurines for hepatotoxicity, of whom 10% (16 patients, after a median 85 days, range 2-951 days) developed abnormal LFTs greater than twice the ULN.[287] The author's protocol was to perform a complete liver screen on all patients with hepatotoxicity and then reduce the dose. If LFTs failed to normalise, then the thiopurine was stopped. Of the 16 patients with hepatotoxicity, 14 had a hepatocellular pattern to their LFTs, whilst 2 had a cholestatic pattern. Treatment withdrawal occurred in 5 patients, with 2 having repeated hepatotoxicity when thiopurine treatment was re-attempted. Of the 11 patients where LFTs normalised after dose reduction, all were able to have their dose increased without further hepatotoxicity. Patients who had abnormal LFTs, but less than twice the ULN, had spontaneous resolution of LFTs without changing treatment. Patients with very abnormal LFTs were more likely to

have complete treatment withdrawal. Multivariate analysis concluded that whilst concomitant steroids were a risk factor for thiopurine hepatotoxicity, anti-TNF agents were protective. Although our study is larger, we do share similar findings especially the hepatocellular nature of the toxicity. Our study was not able to compare steroid or anti-TNF therapy use with patients who did not get thiopurine hepatotoxicity.

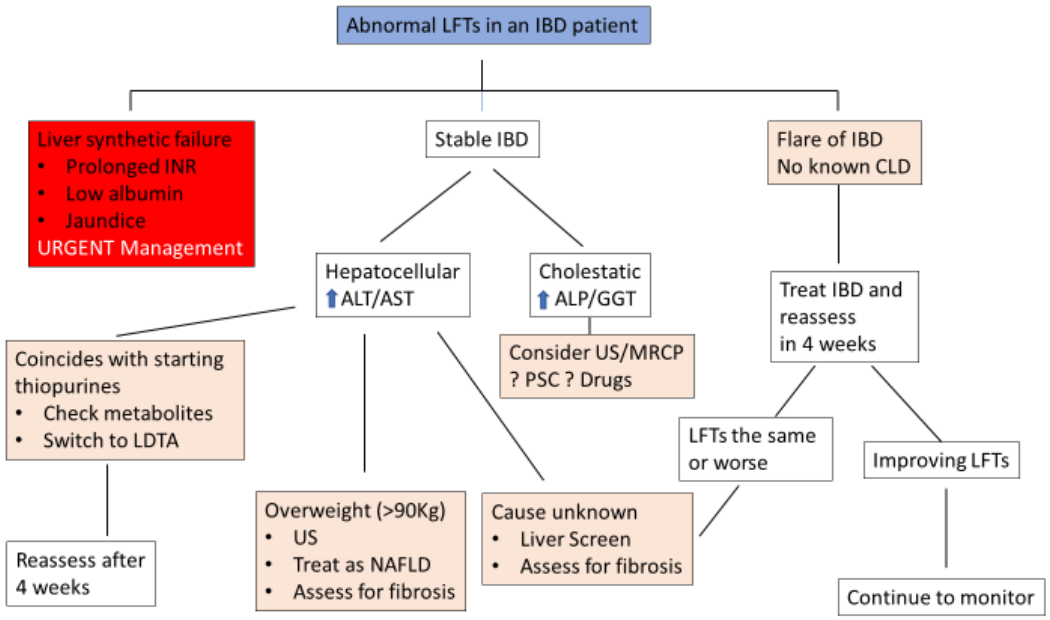


Figure 3.4: A decision tree to guide clinicians on how to investigate abnormal LFTs in IBD patients. It assumes that a history and examination has taken place and that common causes of abnormal LFTs, such as alcohol, have been excluded. Where there are signs of liver failure, these patients should undergo intensive investigation and management. However, the investigation of abnormal LFTs, in the absence of liver failure, should undergo a systematic approach. When the diagnosis is confirmed, patients should be assessed for fibrosis by either a FIB-4 score, a fibroscan or liver biopsy. INR: International normalised ratio; CLD: Chronic liver disease; US: Ultrasound; PSC, Primary sclerosing cholangitis; MRCP: Magnetic resonance cholangiopancreatography; LDTA: Low dose thiopurine with allopurinol; NAFLD: Non-alcoholic fatty liver disease.

The decision tree in Figure 3.4 is a suggestion as to how patients could be investigated strategically based on the findings from our study where flares were a common cause of abnormal LFTs, whilst cholestatic LFTs were suggestive of PSC. However, when patients are in disease remission and LFTs are hepatocellular and coincide with starting thiopurines, their metabolite profile should be checked. If hypermethylating, thiopurines are the likely cause and it is advised to switch to LDTA. Thiopurines could still be the cause if the patient is normomethylating, although it is worth considering other causes for liver disease such as NAFLD. If the cause is likely to be thiopurines, even in the absence of hypermethylation, hepatotoxicity is still likely to resolve by switching to LDTA.

In summary, flares of disease and thiopurines are common causes of abnormal LFTs in what appears to be a unique relationship between IBD and liver disease. Further research must target the mechanisms that underlie this relationship and identify those patients most at risk of liver injury.

Summary of study findings:

- Two thirds of IBD patients with abnormal LFTs had CD and a third had UC - therefore, abnormal LFTs are more common in patients with CD
- Inflammation is mostly hepatocellular, except in biliary diseases such as PSC or choledocholithiaisis, where inflammation is cholestatic
- One third of abnormal LFTs are due to thiopurines, one third due to disease flares and one third due to other causes (PSC accounts for 7% of other causes)
- Flares of disease are a common cause of abnormal LFTs, affecting patients with CD more than patients with UC (71% versus 28%)
- Thiopurine hepatotoxicity was more common in heavier patients
- Two thirds of hepatotoxicity from thiopurines occurs with hypermethylation, the remainder third in the absence of hypermethylation
- >85% of thiopurine hepatotoxicity corrects by switching to low dose thiopurine with allopurinol
- Abnormal LFTs account for low mortality in IBD. There is, therefore, the time to investigate patients using a strategic approach

Chapter 4: A Candidate gene analysis of thiopurine hypermethylation and hepatotoxicity

4.1 Introduction

The cohort study (Chapter 3) found that a common cause of hepatotoxicity in IBD patients is thiopurines. Although IBD patients stop thiopurines due to a range of side effects, hepatotoxicity could be avoidable if the genes which are associated with it were found. A GWAS performed by our laboratory identified several genes which were more prevalent in patients who were hypermethylators and in patients who developed thiopurine hepatotoxicity.[279]

The aim of this study was to explore these genes as part of a candidate gene analysis (see Chapter 2.3 for the candidate gene selection). The hypothesis was that genetic variants are responsible for the differences in thiopurine metabolism, particularly hypermethylation and hepatotoxicity.

4.2 Patient recruitment and summary of methods

Patients were recruited as they came to the IBD clinic but also from a database of smokers on thiopurines (Chapter 5). Patients were divided into 5 groups according to their metabolite profiles as shown in Table 4.2. Patients who were on tioguanine or LDTA were excluded. Patients who had abnormal LFTs due to another known cause were also excluded. Patients were consented to be part of the study according to the Pharmacogenetics of AZA studies (Research and Ethics committee reference 12/YH/0172, RJ112/N179 and LREC 06/Q0707/84). A blood sample was obtained in an EDTA blood tube and then stored at -70°C. When ready to analyse, the samples were thawed and the DNA was extracted. Realtime TaqMan PCR was then performed and genotypes validated using Sanger sequencing as per the methods outlined in Section 2.2 – 2.5. Statistical analysis was performed using Fisher's exact tests for categorical variables and an independent samples t-test for parametric variables as outlined in Section 2.11. Allelic variances were assessed using a 2 x 2 web based contingency table (http://graphpad.com/quickcalcs/contingency1.cfm. The ORs were calculated between different phenotypes using the website www.medcalc.org/calc/odds_ratio.php. Multivariate analyses were performed using SPSS.

Table 4.2: Thiopurine metabolite profiles and how they were defined.

Groups	Definition
Normomethylators	Patients with ALT < 56 IU/L and a MeMP:TGN < 11
Hypermethylators only	Patients with ALT < 56 IU/L but MeMP:TGN > 20
Hypermethylators with Hepatotoxicity	Patients with ALT >56 IU/L and MeMP:TGN > 20 and a temporal relationship of this abnormality to the start of thiopurine
Hepatotoxicity only	Patients with ALT >56 IU/L and MeMP:TGN < 11 and a temporal relationship of LFT abnormality to the start of thiopurine
Hepatotoxicity, unknown methylation status	Patients with ALT <56 IU/L but where metabolites were not checked at the time of hepatotoxicity

4.3 The cohort

266 patients were enrolled in the study of whom 134 were females, 65.4% had CD, 31.6% had UC. Of the remainder, 2.6% had orofacial granulomatosis and 0.4% had IBD-U. The mean age at starting thiopurines was 34 years old (SD 11.1, median 32, range 15-79 years). The date of starting thiopurines was known in 99% of cases and the median time interval between diagnosis and starting thiopurines was 32.5 months (IQR 108, range 0 to 520). 85% of the cohort were Caucasian, 8% (n=21) Asian, 5% (n=14) Black, 1% (n=3) Jewish, and 1% (n=2) describing themselves as mixed race. 82% were on AZA, the remainder were on MP. The mean weight-based dosing (WBD) for AZA was 1.8 mg/kg (SD 0.5, median 1.9, range 0.1 to 3.8), whilst that of MP was 0.9 mg/kg (SD 0.3, median 1.0, range 0.3 to 1.9).

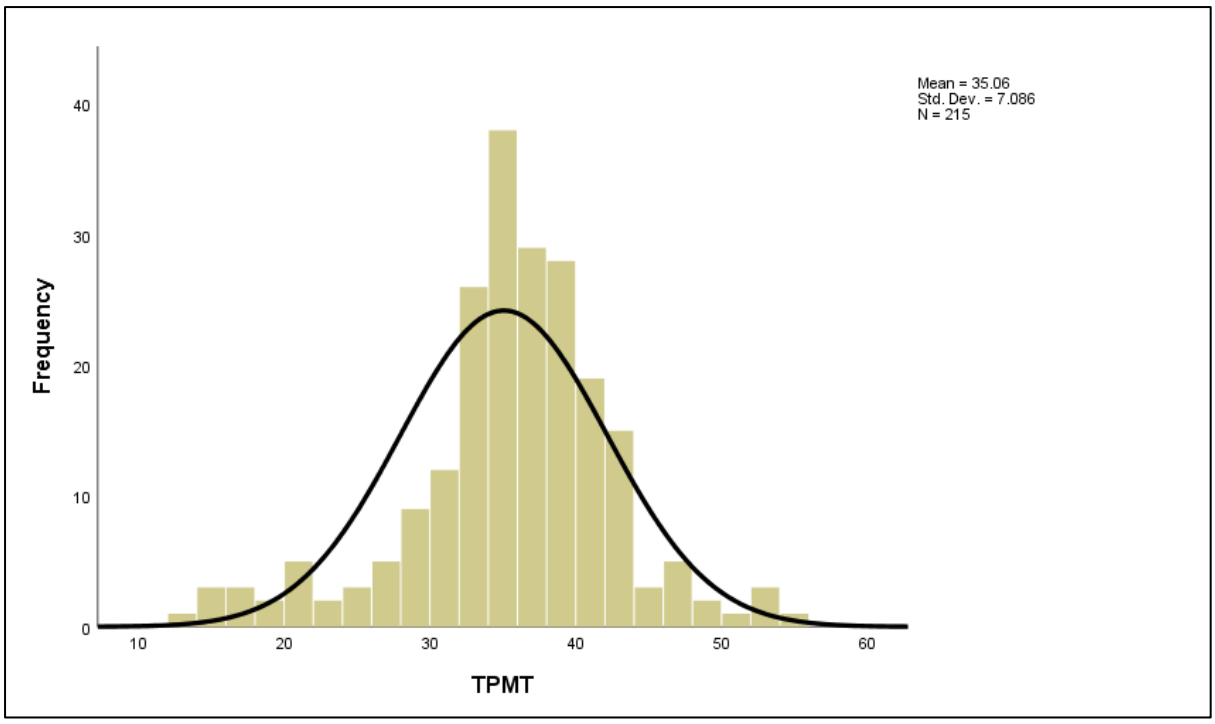


Figure 4.3.1: The distribution of TPMT (pmol/h/mgHb) within the whole cohort.

TPMT activity was known for 215 patients, and known for a further 12 patients but measured as mU/L. The mean TPMT, as measured in pmol/h/mgHb, was 35.1 (SD 7.1, range 13 to 54); TPMT activity (as measured in pmol/h/mgHb) was normally distributed (Figure 4.3.1). In total, there were 19 patients in the carrier range (TPMT 11-25 pmol/h/mgHb) (Table 4.3).

11 patients with TPMT measured as mU/L were in the wild type range whilst one patient was in the carrier range. Therefore, in the whole cohort, there were a total of 20 TPMT carrier phenotypes.

Of the patients with CD, 51/174 (29%) had hepatotoxicity compared to 37/84 (44%) with UC. Patients on MP were significantly more likely to be hypermethylators and to develop hepatotoxicity than patients on AZA (P <0.01, Fisher's exact test). Table 4.3 and Figure 4.3.2 show how the cohort was distributed according to the 5 phenotypic groups.

Table 4.3: The frequency of the 5 phenotypes within the cohort and their corresponding TPMT range.

Phenotype	N	%	TPMT 11- 25	TPMT ≤10
Normomethylators	113	42.5%	10	0
Hypermethylators only	61	22.9%	2	0
Hypermethylators with hepatotoxicity	36	13.5%	0	0
Hepatotoxicity only	31	11.7%	4	0
Hepatotoxicity, unknown methylation status	25	9.4%	3	0

TPMT measured as pmol/h/mgHb.

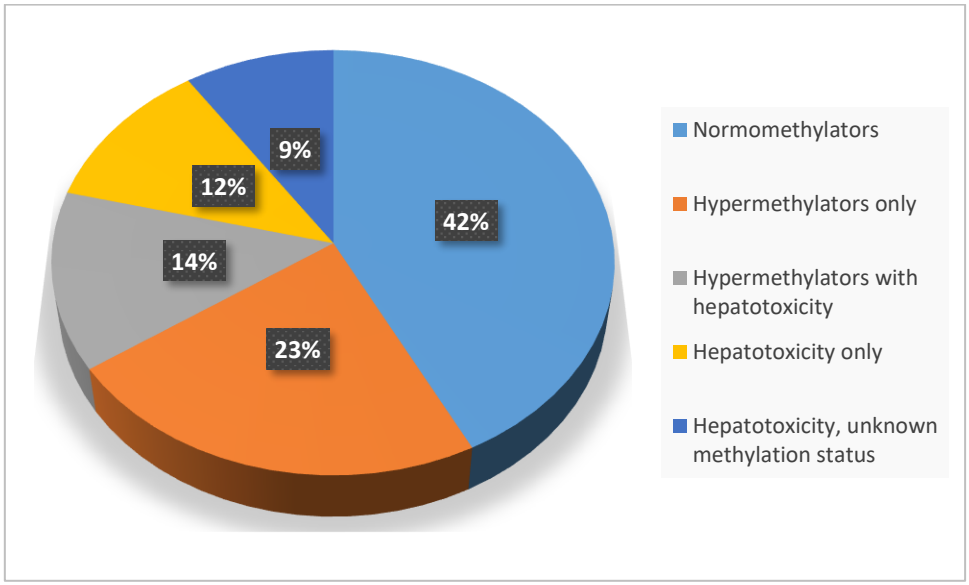


Figure 4.3.2: The distribution of the patients according to the 5 main phenotypes for thiopurines metabolism (N=266).

4.4 TPMT genotyping of the cohort

All patients had Real Time PCR performed for the 3 common TPMT genotypes: TPMT*3A (c.460G>A;p.Ala154Thr and c.719A>G;p.Tyr240Cys), TPMT*3C (c.719A>G; p.Tyr240Cys) and TPMT *2 (c.238G>C; p.Ala80Pro). The following tables and figures compare the prevalence of individual TPMT variants with TPMT activity (Tables 4.4.2 and 4.4.3); the concordance between TPMT genotype and phenotype was 97%. Table 4.4.4 compares TPMT variants according to ethnicity. Of the 17 TPMT genetic variants, 8 were normomethylators, 4 were hepatotoxicity of unknown methylation status, and 5 were hepatotoxicity only. As expected for patients with low TPMT activity, no TPMT variants were hypermethylators only (Table 4.4.6).

Table 4.4.1: The incidence of the TPMT genotypes and allele frequencies within the cohort.

N=266	TPMT*3A	TPMT*3C	TPMT*2
Wild type	254	262	265
Heterozygote	12	4	1
Homozygous variant	0	0	0
MAF in cohort	0.02 (2%)	0.032 (3%)	0.0019 (0.2%)

Table 4.4.2: Correlation between TPMT genotype and TPMT phenotype.

TPMT Genotype	N	Mean TPMT	Range TPMT (pmol/h/mgHb)
Wild type	249	36.2	16-54
Heterozygotes	17	19.0	13-28
Homozygous variant	0		

Although TPMT activity was only known for 227 patients.

Table 4.4.3: Correlates TPMT activity according to individual TPMT mutations.

TPMT genotype	N	%	Mean (range) activity (pmol/h/mgHb) (SD)
*1/*1	249	93.6%	36.3 (16-54) (5.6)
*1/*3C	4	1.5%	16 (13-21) (4)
*1/*2	1	0.4%	19
*1/*3A	12	4.5%	19.4 (15-28) (3.7)

Table 4.4.4: The frequency of variant TPMT genotypes by ethnicity.

Mutations	Caucasians	Black	Asians
TPMT*2	1	0	0
TPMT*3A	12	0	0
TPMT*3C	1	1	2

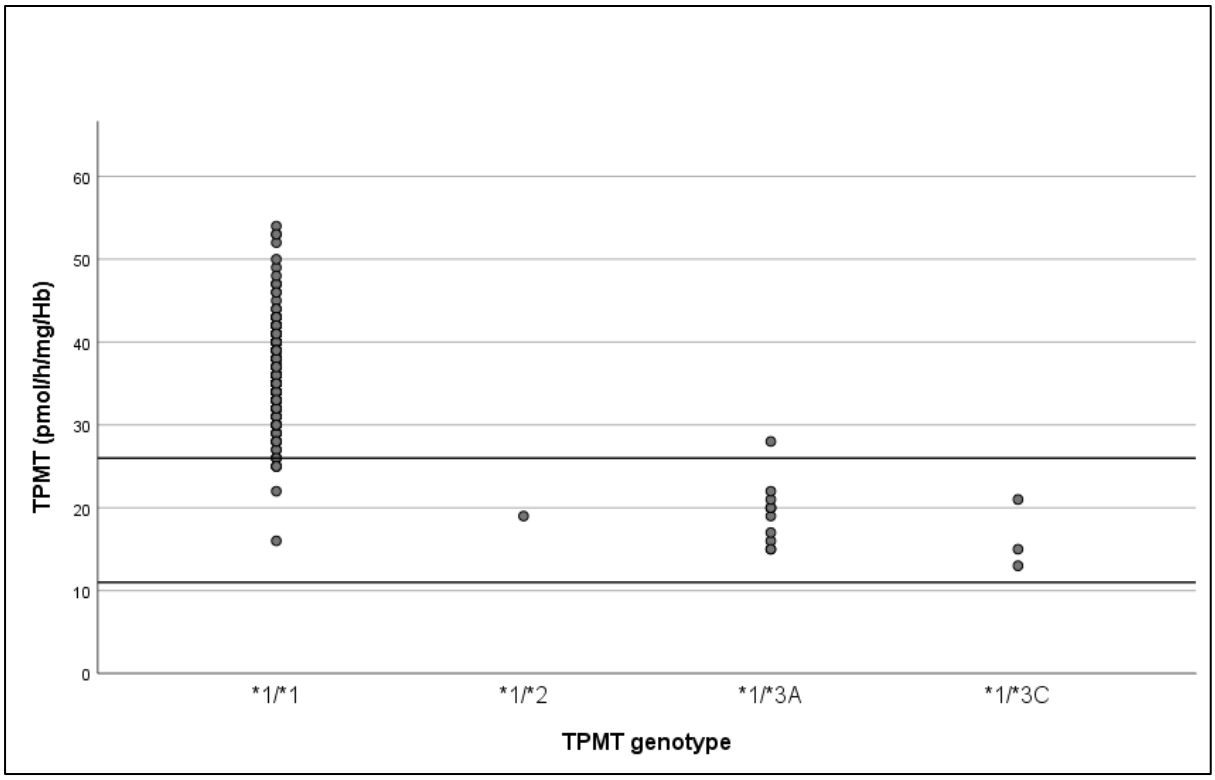


Figure 4.4.1: The distribution of TPMT activity (as measured in pmol/h/mg/Hb) among 215 patients in relation to their individual TPMT genotypes. The area between the lines depicts the range of TPMT activity that defines intermediate TPMT activity and separates individuals with very low TPMT activity from normal activity.

Table 4.4.5: Compares genotype with phenotype for TPMT.

	WT Phenotype	Carrier Phenotype	HV Phenotype
WT Genotype	205	5	0
Carrier Genotype	2	15	0
HV Genotype	0	0	0

In the cohort there was a 97% concordance between genotype and phenotype (or just 75% if analysing just the carrier genotype). Although 266 patients were recruited, TPMT was only known for 227 patients (215 with TPMT activity measured as pmol/h/mgHb (19 carriers phenotypically, 196 wild-types phenotypically) and 12 as mU/L (1 carrier phenotypically, 11 wild-types). WT: Wild-type; HV: Homozygous variant.

Table 4.4.6: The number of TPMT variants in methylator and hepatoxicity groups

Phenotype	N	TPMT variants
Normomethylators	113	8
Hypermethylators only	61	0
Hyperpermethylators with hepatotoxicity	36	0
Hepatotoxicity only	31	5
Hepatotoxicity, unknown methylation status	25	4
Total	266	17

Given the fact that 4 patients with hepatotoxicity of unknown methylation status were heterozygous for a TPMT variant and therefore could not have been hypermethylators, they were added to the hepatotoxicity only phenotype, making 35 patients in this group.

4.5 Comparisons between the four main groups for thiopurine metabolism

Variables were compared across all groups with the number of patients in the hepatotoxicity only group corrected to 35 following TPMT genotyping. Table 4.5.1-4.5.4 analyses the characteristics of the 4 main groups according to age, weight, dosing, LFTs, TPMT and metabolite profiles, separated according to parametric and non-parametric data.

The normomethylators

Table 4.5.1: Characteristics of the normomethylator group.

	N	Min	Max	Mean	SD
Age	113	15	60	32.9	10.3
Weight (Kg)	59	45	112	70.8	15.0
WBD for AZA (mg/Kg)	100	0.81	3.75	1.83	0.47
WBD for MP (mg/Kg)	10	0.38	1.30	0.86	0.27
TPMT (pmol/h/mgHb)	91	15	54	34	6.9

	N	Min	Max	Median	IQR
Highest MeMP (pmol/L)	110	126	5595	1136	1707
Ratio	110	0	18	3.7	6

The hypermethylators with hepatotoxicity group

Table 4.5.2: Characteristics of the hypermethylator with hepatotoxicity group

	N	Min	Max	Mean	SD
Age	36	19	64	38.8	12.2
TPMT (pmol/h/mgHb)	30	28	46	36.6	4.0
Weight (kg)	36	44.1	140	79.6	21.5
WBD (mg/kg) AZA	20	0.58	2.66	1.92	0.60
WBD (mg/kg) MP	16	0.51	1.92	1.04	0.37

	N	Min	Max	Median	IQR
Bilirubin (µmol/L)	35	5	42	11	11
ALT (IU/L)	36	56	334	89	75
ALP (IU/L)	36	31	517	61	52
MeMP at highest ALT (pmol/L)	36	2505	31500	9958	7284
Ratio	36	11	79	33	28

When comparing ALT:ULN in comparison ALP:ULN, the median was 3.65 (minimum 1.42, max 10.9, IQR 3.23). This suggests that on average ALT rose four times higher than ALP in this group of patients.

There was no correlation between the MeMP and highest ALT (Figure 4.5.1) in the hypermethylation with hepatotoxicity group. This would suggest that the degree of hepatotoxicity is independent of the degree of hypermethylation.

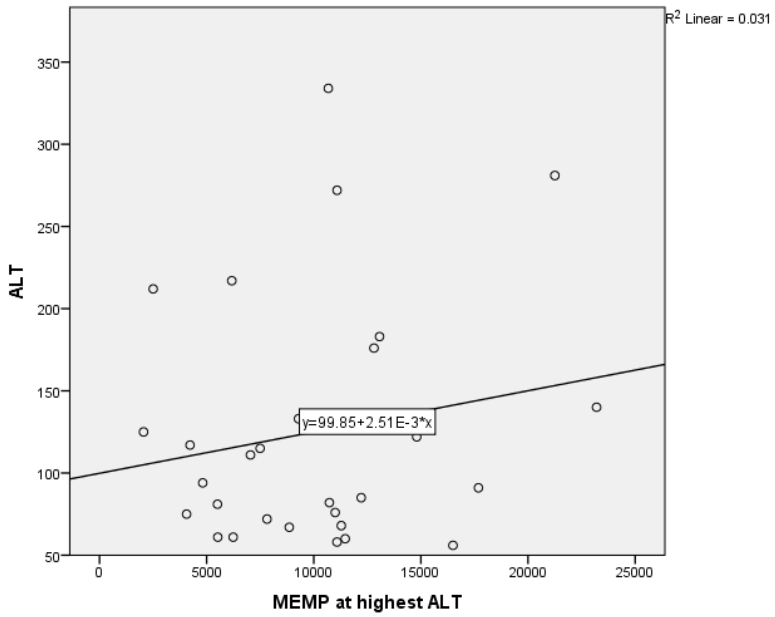


Figure 4.5.1: The correlation (r^2 = 0.031) between the highest MeMP and ALT was non-linear.

The median time between either the drug starting, or the nearest dose change, and hepatotoxicity was 92 days (range 16 to 2332, IQR 318). The median time to ALT settling from its highest point was 67 days (range 7 to 497, IQR 102) (Figure 4.5.2).

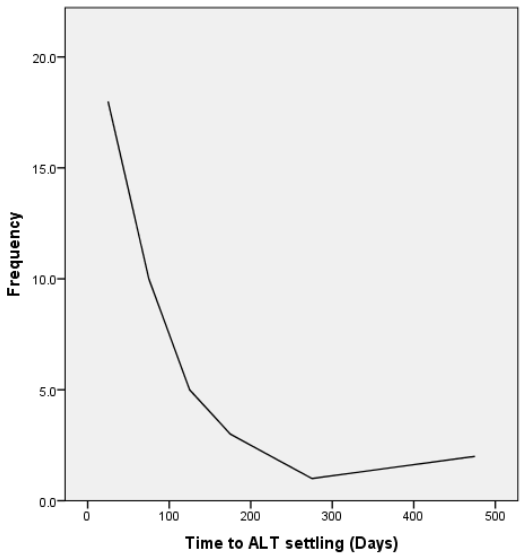


Figure 4.5.2: The mean time to ALT settling in the hypermethylation with hepatotoxicity group.

The hepatotoxicity only group

Table 4.5.3: Characteristics of the hepatotoxicity only group.

	N	Min	Max	Mean	SD
Age	35	19	79	35.9	11.3
TPMT (pmol/h/mgHb)	31	13	34	31	8.8
Weight (Kg)	34	51.5	109	76.5	13.9
WBD (mg/kg) AZA	26	0.1	2.4	1.3	0.7
WBD (mg/kg) MP	8	0.3	1.0	0.8	0.2

	N	Min	Max	Median	IQR
Bilirubin (µmol/L)	35	3	28	7	5
ALT (IU/L)	35	60	439	136	116
ALP (IU/L)	35	38	650	109	123
MeMP at highest ALT (pmol/L)	21	107	4211	626	1244
Ratio	27	0	13	6	8

When comparing the ALT:ULN and the ALP:ULN, the median was 2.7 (range 0.6 - 9.3, IQR 2.5). This suggests that on average ALT rose 3 times higher than ALP in this group of patients. The median time between either the drug starting or the nearest dose change and hepatotoxicity, was 42 days (range 4.0 to 1242, IQR 68.0). The median time to ALT settling from the highest point was 29 days (range 1.1 to 616, IQR 63.2).

The hypermethylators only

Table 4.5.4: Characteristics of the hypermethylators only group.

	N	Min	Max	Mean	SD
Age	61	15	66	30.3	10.4
Weight (Kg)	59	45	112	70.7	15.0
WBD for AZA (mg/Kg)	49	1.04	2.12	2.1	0.4
WBD for MP (mg/Kg)	10	0.64	1.18	1.0	0.2
TPMT (pmol/h/mgHb)	47	25	53	38.0	6.1

	N	Min	Max	Median	IQR
Highest MeMP (pmol/L)	60	2410	21302	6751	4090
Ratio	60	12	104	24.3	13

The differences between hypermethylators only (n=61) and hypermethylators with hepatotoxicity (n=36)

When comparing these two groups, patients in the hypermethylator with hepatotoxicity group were significantly older (mean age 39 versus 30) and had significantly higher MeMP (median MeMP 9958 (IQR 7284, range 2505-31500) versus 6751 pmol/L (IQR 4090, range 2410-18892)). Interestingly, this was irrespective of the WBD which was significantly higher in the hypermethylator only group (1.9 versus 1.5 mg/kg). However, this may be accounted for by the significant differences in mean weight with the hypermethylator only group being 71 Kg compared to 80 Kg in the hypermethylators with hepatotoxicity group (P = 0.01, independent samples t-test). TPMT was not significantly different between both groups. The box plots in Figure 4.5.3 illustrate the range of the differences between the groups.

Table 4.5.5: Hypermethylators with hepatotoxicity group compared to hypermethylators only.

Mean	Hypermethylators with hepatotoxicity (n=36)(SD)	Hypermethylators only (n= 61)(SD)	P-value
Age	38.8 (12.2)	30.3 (10.4)	**0.001**‡
CD (%) UC (%)	N= 23 (63.9%) N= 13 (36.1%)	N=38 (62.2%) N= 20 (32.8%)	1.00*
Gender (Males) (%)	17 (47.2%)	23 (37.7%)	0.38*
Smokers (%)	5 (13.9%)	10 (16.4%)	1.00*
TPMT (pmol/h/mgHb)	36.6 (4.0)	37.8 (6.1)	0.29‡
Weight (Kg)	79.6 (21.5)	70.8 (15.0)	**0.02**‡
WBD (mg/Kg) AZA	1.91 (0.6) (n=20)	2.12 (0.42) (n=49)	0.17‡
WBD (mg/Kg) MP	1.05 (0.4) (n=16)	0.96 (0.2) (n=10)	0.43‡
Log10 Highest MeMP (pmol/L)	3.96 (0.3)	3.82 (0.2)	**0.006**‡
MeMP:TGN	35.0 (18.1)	30.6 (18.4)	0.26‡

‡Independent samples t-test; *Two-sided Fisher's exact test.

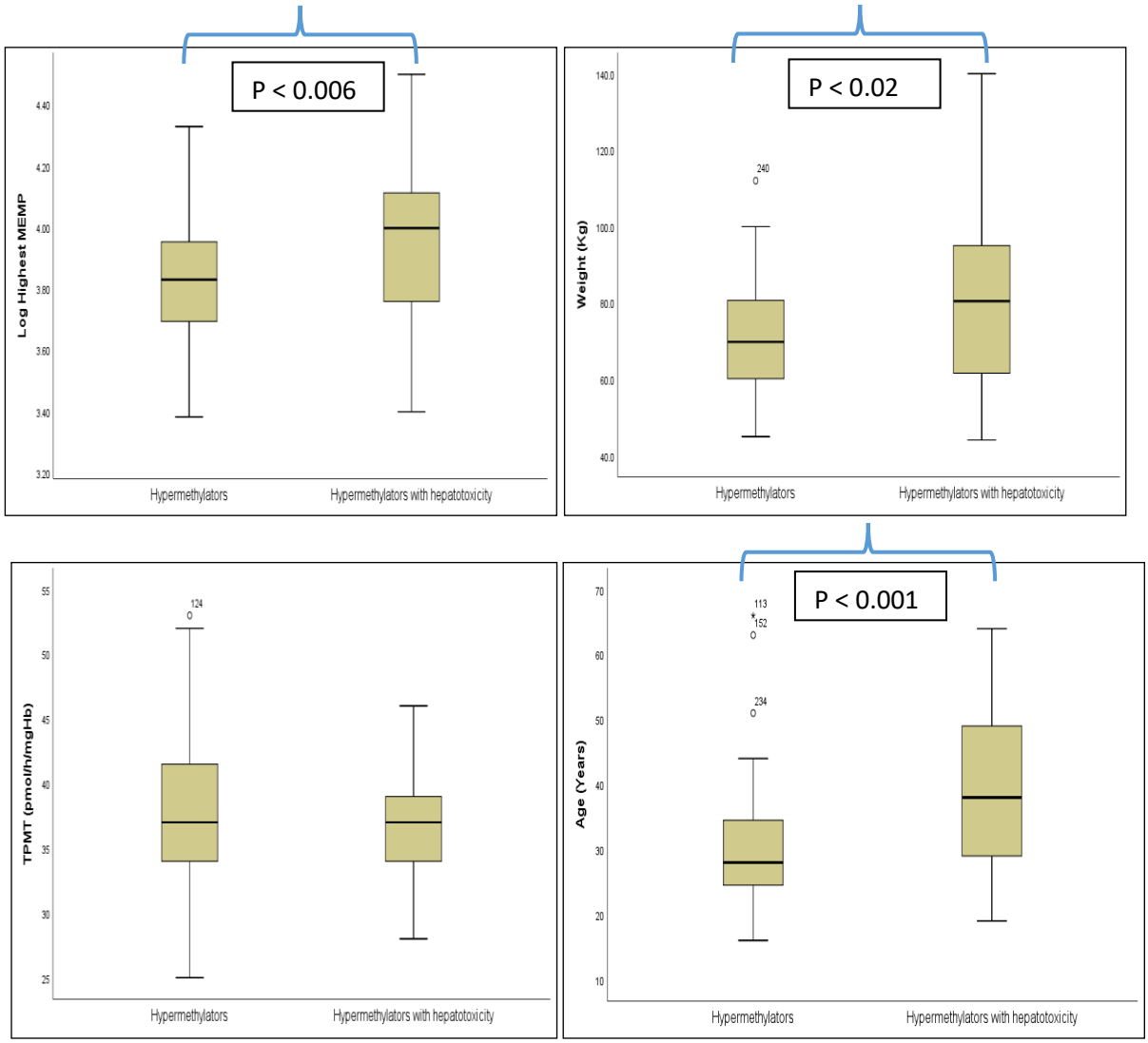


Figure 4.5.3: Box plots showing the differences between hypermethylators only and hypermethylators with hepatotoxicity when compared for highest MeMP (pmol/L), weight (Kg), TPMT (pmol/h/mgHb) and age.

<u>Comparing hepatotoxicity arising from hypermethylation versus without hypermethylation</u>

The hypermethylators with hepatotoxicity and hepatotoxicity only groups were compared for differences (Table 4.5.6). Both were hepatocellular reactions. There were no significant differences in the ALT:ALP ratios between the 2 groups. However, ALP was significantly higher in the hepatotoxicity only group whilst bilirubin was significantly lower. WBD for AZA and TPMT were significantly lower in the hepatotoxicity only group. The time between starting thiopurines and hepatotoxicity and the time taken for ALT to normalise after stopping the drug were significantly longer in the hypermethylators with hepatotoxicity group

Table 4.5.6: Hypermethylators with hepatotoxicity group compared to patients in the hepatotoxicity only group.

Mean	Hypermethylation with hepatotoxicity (SD) (n=36)	Hepatotoxics only (SD) (n=35)	P-value
Age	38.8 (12.2)	35.9 (11.2)	0.3‡
CD (%) UC (%)	N= 23 (63.9%) N= 13 (36.1%)	N=19 (54.3%) N= 13 (37.1)	0.8*
Gender (Males)	17 (47.2%)	22 (62.9%)	0.2*
Smokers	5 (13.9%)	6 (17.1%)	0.8*
Log10 ALT (IU/L)	2.03 (0.2)	2.14 (0.2)	0.06‡
TPMT (pmol/h/mgHb)	36.6 (4.0)	31 (8.8)	**0.01**‡
Log10 Bilirubin (µmol/L)	1.09 (0.2)	0.90 (0.2)	**0.001‡**
Log10 ALP (IU/L)	1.83 (0.2)	2.07 (0.3)	**<0.001**‡
Weight (kg)	79.6 (21.5)	76.7 (13.9)	0.5‡
WBD (mg/kg) AZA	1.91 (0.6) (n=20)	1.31 (0.7) (n=26)	**0.004**‡
WBD (mg/kg) MP	1.05 (0.4) (n=16)	0.78 (0.2) (n=8)	0.07‡
Log 10 Time between dose and Hepatotoxicity (days)	2.14 (0.6)	1.68 (0.6)	**0.002‡**
Time to ALT settling (days)	1.76 (0.5)	1.50 (0.6)	**0.036‡**

‡Independent samples t-test; *Two-sided Fisher's exact test.

Comparing normomethylators versus all hypermethylators (with or without hepatotoxicity)

After comparing normomethylators versus all hypermethylators, females were 3.6 times more likely (OR 3.64, CI 2.0 to 6.6) to be hypermethylators than males, whilst non-smokers were 9.8 times more likely (OR 9.9, CI 4.6 to 21) to be hypermethylators than smokers (Table 4.5.7). TPMT and WBD for AZA were also significantly higher in hypermethylators.

Table 4.5.7: Normomethylator group compared to all hypermethylators.

Mean	Normomethylators (n=113)	All hypermethylators (n= 97)	P-value
Age	32.9 (10.3)	33.4 (11.8)	0.6‡
Crohn's UC	N=85 (75.2%) N=27 (23.9%)	N=38 (62.2%) N= 20 (32.8%)	0.2*
Gender (Males)	60 (53.1%)	23 (37.7%)	**0.001***
Smokers	60 (53.1%)	10 (16.4%)	**0.001***
TPMT (pmol/h/mgHb)	33.9 (6.9)	37.1 (5.4)	**0.001**‡
Weight	69.6 (15.6)	74.1 (18.1)	0.05‡
WBD (mg/Kg) AZA	1.83(0.5) (n=100)	2.12 (0.4) (n=49)	**0.001**‡
WBD (mg/Kg) MP	0.86 (0.3) (n=10)	0.96 (0.2) (n=10)	0.4‡

‡Independent samples t-test; *Two-sided Fisher's exact test.

Comparing normomethylators versus all hepatotoxicity patients

When comparing normomethylators with all hepatotoxicity patients, UC patients were more than twice as likely to develop hepatotoxicity from thiopurines than patients with CD (OR 2.3, 95% CI 1.3 to 4.3) (Table 4.5.8). Non-smokers were 6.3 times likely (OR 6.3, CI 3.2 to 12) to develop hepatotoxicity than smokers.

Table 4.5.8: Normomethylator group compared to all hepatotoxics.

Mean	Normomethylators (n=113)	All hepatotoxics (n=92)	P-value
Age at starting thiopurine	32.9 (10.3)	37.8 (12.0)	**0.002**‡
Crohn's UC	n=85 (75.2%) n=27 (23.9%)	n= 51 (55.4%) n= 37 (40.2%)	**0.009***
Gender (Males)	60 (53.1%)	49 (53.3%)	1.00*
Smokers	60 (53.1%)	14 (15.2%)	**<0.001***
TPMT (pmol/h/mgHb)	33.9 (6.9)	35.0 (7.49)	0.4‡
Weight	69.6 (15.6)	76.8 (17.4)	**0.003**‡
WBD (mg/Kg) AZA	1.83 (0.5) (n=100)	1.57 (0.658) (n=61)	**0.01**‡
WBD (mg/Kg) MP	0.86 (0.3) (n=10)	0.98 (0.345) (n=26)	0.4‡
Log10 Highest MeMP (pmol/8x10^8 RBC)	3.01 (0.4)	3.52 (0.65)	**<0.001**‡
Log10 MeMP:TGN	0.54 (0.4)	1.04 (0.66)	**<0.001**‡

‡Independent samples t-test; *Two-sided Fisher's exact test.

Hepatotoxicity versus non hepatotoxicity in univariate analysis

When comparing non-hepatotoxics with all hepatotoxicity patients, UC patients were significantly more likely to develop hepatotoxicity from thiopurines than patients with CD (OR 1.9, 95% CI 1.1 to 3.3) (Table 4.5.9). Smokers were significantly less likely (OR 0.3, CI 0.1 to 0.5) to develop hepatotoxicity than non-smokers. Age, weight, MP treatment, AZA WBD, MeMP and the MeMP:TGN ratio were all significantly associated with hepatotoxicity. 33 (79%) out of the 42 patients with hepatotoxicity who were switched to LDTA, responded with normalisation of LFTs (Table 4.5.10).

Table 4.5.9: Non-hepatotoxics compared to all hepatotoxics.

Mean	Non-hepatotoxics (n=174)	All hepatotoxics (n=92)	P-value
Age at starting thiopurine	32.9 (10.3)	37.8 (12.0)	**<0.001**‡
Crohn's UC	n= 123 (70.7%) n= 47 (27.0%)	n= 51 (55.4%) n= 37 (40.2%)	**0.02***
Gender (Males)	83 (47.7%)	49 (53.3%)	0.4*
Smokers	70 (40.2%)	14 (15.2%)	**<0.001***
TPMT (pmol/h/mgHb)	35.2 (6.9)	35.0 (7.5)	0.7‡
Weight	70.0 (15.3)	76.8 (17.4)	**0.002**‡
AZA MP	n= 153 (87.9%) n= 21 (12.1%)	n=66 (71.7%) n=26 (28.3%)	**0.002***
WBD (mg/Kg) AZA	1.92 (0.5) (n=149)	1.57 (0.7) (n=61)	**<0.001**‡
WBD (mg/Kg) MP	0.908 (0.2) (n=20)	0.98 (0.3) (n=26)	0.5‡
Log10 Highest MeMP (pmol/8x10^8 RBC)	3.29 (0.5)	3.52 (0.7)	**0.006**‡
Log10 MeMP:TGN	0.86 (0.6)	1.04 (0.7)	**0.029**‡

‡Independent samples t-test; *Two-sided Fisher's exact test.

Table 4.5.10: Management of both types of hepatotoxicity and the relative success of that management at normalising LFTs.

Successful Management	Hepatotoxics only (n=35)	Hypermethylators with hepatotoxicity (n=36)	Methylation status unknown (n=21)
Switching to LDTA	7/10	18/22	8/10
Stop	12/12	5/5	10/10
Reduce dose	3/3	3/3	1/1
Settled	9/9	5/5	0/0
Switch to MP	1/1	0/0	0/0
Split dose	0/0	1/1	0/0

70% of the hepatoxicity only group responded to LDTA compared to 82% of the hypermethylators with hepatotoxicity. Of note, 14 patients' LFTs settled without any change to their management.

4.6 Logistical regression

Both continuous and discrete variables were analysed using multiple logistical regression modelling to assess for a significant relationship relating to hepatotoxicity. Patients with IBD-U (N=9) and certain variables, such as TPMT and highest MeMP, were excluded if low numbers of patients or missing data resulted in a significant reduction in the size of the model. Non-parametric data such as MeMP and overlap data, such as weight-based dosing, were also not analysed. MP, non-smoking, age and weight were all significantly associated with hepatotoxicity whereas IBD type was not (Table 4.6.1).

Table 4.6.1: Covariate analyses for hepatotoxicity (n= 84) as the dependent variable compared to non-hepatotoxics (n=165) for gender, IBD Type (CD), thiopurine type (MP), smoking, age and weight (Kg).

Variable	Odds Ratio (OR) for hepatotoxicity	P-value
IBD Type (CD)	0.7	0.34
Thiopurine type (AZA)	0.3	**<0.01**
Smoker	0.3	**<0.01**
Age (years)	1.0	**0.011**
Weight (Kg)	1.0	**0.046**

4.7 Candidate gene analysis results

Table 4.7.1 shows the MAF in the cohort for all 12 of the polymorphisms investigated. TPMT genotypes were analysed according to the four phenotypes (Tables 4.7.2 and 4.7.3). There were significantly more TPMT heterozygotes amongst the normomethylators compared to the hypermethylators (Table 4.7.2), but no significant differences for TPMT genotypes between all hepatotoxics and all non-hepatotoxics (Table 4.7.3).

Table 4.7.1: The MAF of all SNPs investigated.

SNP	MAF in cohort
MTHFR 1286 A>C	0.30
MTHFR 677 C>T	0.32
MTHFR 1654-80 G>A	0.06
MTHFR 359+160 A>G	0.13
AOX 3404 A>G	0.15
IL-15 *83C > A	0.10
IL-15 g.142709723T T>C	0.06
CYP1A2*C 3860 G>A	0.04
CYP1A2*F 163C>A	0.27
ADK 827-6202A A>G	0.37
ABCB5 343 A>G	0.35
SLC 38A9 C > T	0.28

Table 4.7.2: Compares TPMT genotypes in normomethylators versus all hypermethylators (with and without hepatotoxicity).

Genotype	Allele	Normomethylators (n=113)	All hypermethylators (n=97)	Significance in RM (P-value*)	Significance in DM (P-value*)
TPMT*2 (exon 5) c.238G>C; p.Ala80Pro	GG (%) GC (%) CC (%)	113 (100) 0 0	97 (100) 0 0	1.00	1.00
TPMT*3A (exon 10) c.460G>A ; p.Ala154Thr c.719A>G; p.Tyr240Cys	GG (%) GA (%) AA (%)	106 (94) 7 (6) 0	97 (100) 0 0	1.00	0.016
TPMT*3C (exon 10) c.719A>G; p.Tyr240Cys	AA (%) AG (%) GG (%)	112 (99) 1 (1) 0	97 (100) 0 0	1.00	1.00
All TPMT	WT (%) HZ (%) HV (%)	105 (93) 8 (7) 0	97 (100) 0 0	1.00	0.008

DM: dominant model; RM: recessive model. *Two-tailed Fisher's exact test.

Table 4.7.3: Compares TPMT genotypes in hepatotoxics versus non-hepatotoxics

SNP	Genotype	Hepatotoxics (n=92)	Non-hepatotoxics (n=174)	Significance in RM (P-value*)	Significance in DM (P-value*)
TPMT*2 c.238G>C; p.Ala80Pro	GG (%) GC (%) CC (%)	91 (99) 1 (1) 0	174 (100) 0 (0) 0	1.00	0.35
TPMT*3A c.460G>A ; p.Ala154Thr	GG (%) GA (%) AA (%)	87 (95) 5 (5) 0	167 (96) 7 (4) 0	1.00	0.76
TPMT*3C c.719A>G; p.Tyr240Cys	AA (%) AG (%) GG (%)	89 (97) 3 (3) 0	173 (99) 1 (1) 0	1.00	0.12
All TPMT	WT (%) HZ (%) HV (%)	83 (90) 9 (10) 0	166 (95) 8 (5) 0	1.00	0.12

*Two-tailed Fisher's exact test.

The frequency of the SNP variants were compared between the different phenotypes for thiopurine metabolism. TPMT heterozygotes were excluded from the analysis to standardise against TPMT variants.

There were significantly more CYP1A2*F variants in the hypermethylator with hepatoxicity group when tested for significance in a dominant model, compared to hypermethylators (P=0.03, Fisher's exact test) (Table 4.7.4). The MTHFR 359+160 variant was significantly more prevalent in the hepatotoxicity group compared to the non-hepatotoxicity groups when tested for significance in a dominant model (P = 0.03, Fisher's exact test) (Table 4.7.5). There were no differences when comparing the variants between normomethylators and hypermethylators and between hypermethylators with hepatotoxicity and normomethylators.

Table 4.7.4: Compares genotypes in hypermethylators with hepatotoxicity versus hypermethylators only.

SNP	Genotype	Hypermethylators only (n=61)	Hypermethylators with hepatotoxicity (n=36)	Significance in RM (P-value*)	Significance in DM (P-value*)
MTHFR 1286 A>C	AA (%) AC (%) CC (%)	30 (49) 26 (43) 5 (8)	18 (50) 18 (50) 0	0.15	1.00
MTHFR 677 C>T	CC (%) CT (%) TT (%)	30 (49) 23 (38) 8 (13)	14 (39) 20 (56) 2 (5)	0.31	0.40
MTHFR 1654-80 G>A	GG (%) GA (%) AA (%)	56 (92) 5 (8) 0 (0)	31 (86) 5 (14) 0	1.00	0.49
MTHFR 359+160 A>G	AA (%) AG (%) GG (%)	49 (80) 11 (18) 1 (2)	27 (87) 9 (29) 0	1.00	0.61
AOX 3404 A>G	AA (%) AG (%) GG (%)	50 (82) 9 (15) 2 (3)	24 (77) 12 (23) 0	0.52	0.13
IL-15 *83C>A	CC (%) CA (%) AA (%)	54 (89) 7 (11) 0	26 (84) 10 (16) 0	1.00	0.05
IL-15 g.142709723T T>C	TT (%) TC (%) CC (%)	54 (89) 7 (11) 0	33 (92) 3 (8) 0	1.00	0.74
CYP1A2*C 4745879 G>A	GG (%) GA (%) AA (%)	59 (97) 1 (1.5) 1 (1.5)	32 (89) 4 (11) 0	1.00	0.19
CYP1A2*F 163C>A	CC (%) CA (%) AA (%)	35 (57) 23 (38) 3 (5)	12 (33) 19 (53) 5 (14)	0.14	**0.03**
ADK 827-6202A A>G	AA (%) AG (%) GG (%)	24 (39) 30 (49) 7 (12)	17 (47) 16 (44) 3 (9)	0.74	0.52
ABCB5 343 A>G	AA (%) AG (%) GG (%)	23 (38) 32 (52) 6 (10)	17 (55) 14 (39) 5 (16)	0.53	0.40
SLC 38A9 C>T	CC (%) CT (%) TT (%)	31 (51) 25 (41) 5 (8)	17 (47) 15 (42) 4 (11)	0.72	0.83

*Two-tailed Fisher's exact test.

Table 4.7.5: Compares genotypes in non-hepatotoxicity versus all hepatotoxicity groups (with and without hypermethylation).

SNP	Genotype	Non-hepatotoxics (n=166)	All hepatotoxics (n=84)	Significance in RM (P-value*)	Significance in DM (P-value*)
MTHFR 1286 A>C	AA (%) AC (%) CC (%)	80 (48) 76 (46) 10 (6)	37 (44) 40 (48) 7 (8)	0.60	0.59
MTHFR 677 C>T	CC (%) CT (%) TT (%)	76 (46) 74 (45) 16 (9)	38 (45) 38 (45) 8 (10)	1.00	1.00
MTHFR 1654-80 G>A	GG (%) GA (%) AA (%)	150 (90) 15 (9) 1 (1)	69 (82) 14 (17) 1 (1)	1.00	0.07
MTHFR 359+160 A>G	AA (%) AG (%) GG (%)	132 (80) 32 (19) 2 (1)	56 (67) 26 (31) 2 (2)	0.60	**0.03**
AOX 3404 A>G	AA (%) AG (%) GG (%)	123 (74) 38 (23) 5 (3)	59 (70) 25 (30) 0	0.17	0.55
IL-15 *83C > A	CC (%) CA (%) AA (%)	135 (81) 30 (18) 1 (1)	66 (79) 17 (20) 1 (1)	1.00	0.62
IL-15 g.142709723T T>C	TT (%) TC (%) CC (%)	144 (87) 22 (13) 0	76 (90) 8 (10) 0	1.00	0.54
CYP1A2*C 3860 G>A	GG (%) GA (%) AA (%)	156 (94) 7 (4) 3 (2)	77 (92) 6 (7) 1 (1)	1.00	0.60
CYP1A2*F 163C>A	CC (%) CA (%) AA (%)	87 (52) 64 (39) 15 (9)	41 (49) 34 (40) 9 (11)	0.66	0.60
ADK 827-6202A A>G	AA (%) AG (%) GG (%)	61 (37) 85 (51) 20 (12)	34 (40) 38 (45) 12 (15)	0.69	0.58
ABCB5 343 A>G	AA (%) AG (%) GG (%)	66(40) 81 (49) 18 (11)	39 (46) 32 (38) 13 (16)	0.32	0.35
SLC 38A9 C > T	CC (%) CT (%) TT (%)	92 (55) 64 (39) 10 (6)	42 (50) 34 (40) 8 (10)	0.31	0.42

*Two-tailed Fisher's exact test.

This candidate gene analysis suggested that certain polymorphisms were significantly different between certain phenotypic groups. The most significant ORs at predicting hepatotoxicity in hypermethylators were MTHFR 359+160 and CYP1A2*F variants (Table 4.7.6). The MTHFR 359+160 variant was the most significant at predicting hepatotoxicity compared to non-hepatotoxicity (Table 4.7.7).

Table 4.7.6: OR of one SNP in predisposing to hepatotoxicity when comparing hypermethylators only versus hypermethylators with hepatotoxicity.

Heterozygous variants in hypermethylators only versus hypermethylators with hepatotoxicity	OR	95% CI	P-value
MTHFR 1286 A>C	0.9	0.4-2.2	0.9
MTHFR 677 C>T	1.5	0.7-3.5	0.3
MTHFR 1654-80 G>A	1.8	0.5-6.7	0.4
MTHFR 359+160 A>G	1.9	1.1-3.5	**0.028**
CYP1A2*F 163C>A	2.7	1.1-6.4	**0.024**

Table 4.7.7: OR of one SNP in predisposing to hepatotoxicity when comparing hepatotoxicity (hypermethylators and normomethylators) versus non-hepatotoxicity.

Heterozygous variants in hepatotoxicity versus non-hepatotoxicity	OR	95% CI	P-value
MTHFR 1286 A>C	1.2	0.7-2.0	0.5
MTHFR 677 C>T	1.1	0.6-1.8	0.8
MTHFR 1654-80 G>A	2.0	0.9-4.4	0.07
MTHFR 359+160 A>G	1.9	1.1-3.5	**0.028**

4.8 Discussion

This study identifies some phenotypic and genotypic differences which might predispose patients to developing hepatotoxicity, either with hypermethylation or in the absence of hypermethylation.

The study suggests that half of patients developed hepatotoxicity due to hypermethylation (n=36) whilst half developed hepatotoxicity in the absence of hypermethylation (n=35). Both groups had a hepatocellular liver injury (ALT: ALP > 1) although the FDA definition of DILI using the R ratio (ALT:ULN/ALP:ULN) would have defined them both as mixed.[74] Where hepatotoxicity occurred with hypermethylation, there was only a weak positive correlation between the highest MeMP and the ALT (r^2=0.03). However, MeMP was significantly higher in those hypermethylators who developed hepatotoxicity, suggesting a positive correlation between hepatotoxicity in this group and the degree of hypermethylation. MeMP:TGN was also significantly higher in all patients who developed hepatotoxicity when compared to normomethylators (P <0.001, independent samples t-test) although this is likely to be accounted for by the hypermethylators within this analysis.

Patients with UC were nearly twice as likely as patients with CD to develop hepatotoxicity from thiopurines (44% versus 29%, P = 0.02, Fishers exact test). This is consistent with the findings in Chapter 3, where more patients with UC had thiopurine hepatotoxicity compared to patients with CD (35% versus 29%).

MP increases the risk of hepatotoxicity compared to AZA in both univariate and multivariate analysis. This finding is interesting for a number of reasons: Firstly, it runs contrary to the hypothesis that hepatotoxicity occurs through the depletion of GSH by GST from the conversion of AZA to MP (See Figure 1.11). Secondly, it does not explain why switching to MP in some patients with thiopurine hepatotoxicity in AZA, resolved hepatotoxicity (Table 4.5.10). Higher rates of hepatotoxicity related to MP have also been found in other studies but felt to be related to higher relative weight-based dosing.[288]

Although females appear to be more likely to hypermethylate, there were no gender differences in hepatotoxicity.

The median time to hepatotoxicity in the hypermethylators was 92 days, settling within 67 days, whereas in the hepatotoxicity only group, the median time to hepatotoxicity was 42 days, settling within 29 days. This, combined with finding that ALP was significantly higher in the hepatotoxicity only group, suggests that the underlying mechanisms of hepatotoxicity may be different between the two types with the hepatotoxicity group being more cholestatic. Age was also significantly different when comparing both groups, with hepatotoxicity being more likely to develop in hypermethylators

of an older age (30 versus 39 years). Contrary to what one might have predicted, WBD was higher in those patients who did not develop hepatotoxicity due to patients with hepatotoxicity being significantly heavier (mean 80 Kg versus 71 Kg for hypermethylators with hepatotoxicity compared to hypermethylators respectively). WBD was significantly higher in hypermethylators compared to normomethylators suggesting that hypermethylation is a weight-based, dose-dependent effect and that in some hypermethylators, a reduction in dose may be all that is necessary to restore a normal MeMP:TGN ratio. This finding is also in keeping with the same rationale behind dose-splitting which is based on the hypothesis that lowering the dose of thiopurines, reduces the concentration of MP to below the substrate affinity for TPMT, thereby reducing its methylating activity and the amount of MeMP produced.

Smoking was more prevalent in the normomethylator group suggesting a protective effect of smoking against hepatotoxicity and hypermethylation. Indeed, studies have demonstrated that smoking upregulates CYP450 enzymes which are known to reduce hypermethylation.[289] This may explain why MP was more effective at preventing post-operative recurrence in smokers.[87] This finding runs contrary to the hypothesis that hepatotoxicity results from the release of formaldehyde during demethylation, a process supposedly upregulated by smoking. However, normomethylators were recruited from a database of smokers on thiopurines (Chapter 5), hence why a large proportion (53%) were smokers; this will have biased the results. The finding is also contradicted by a study which has shown that smoking increases TPMT activity.[290]

TPMT was normally distributed throughout the cohort with both carrier and deficient phenotypes being appropriately prevalent (i.e 1/10 for heterozygotes, 1/300 for deficient phenotypes). The MAF for each TPMT genotype within our cohort was similar to that of the global MAF except for TPMT*C, representing the fact that our cohort contained a significant number of non-Caucasians. The concordance between genotype and phenotype was 97% (Table 4.4.5). The discrepancy may arise from other TPMT mutant alleles which were not tested for, such as TPMT *9.[290]

TPMT activity was significantly higher in hypermethylators compared to normomethylators (P = 0.001, independent samples t-test). Non-smokers had significantly higher TPMT activity compared to smokers (37.0 (SD 5.4) versus 34.8 pmol/h/mgHb (SD 5.6), P <0.01, independent samples t-test). There were no significant differences for TPMT genotypes or TPMT activity between the hepatotoxics compared to the non-hepatotoxics (P = 0.2, Fisher's exact test and 0.4, independent samples t-test respectively) although TPMT activity was higher in hepatotoxics compared to non-hepatotoxics in univariate analysis (P = 0.04, independent samples t-test). TPMT activity was significantly higher in hypermethylators with hepatotoxicity compared to hepatotoxics (P = 0.01, independent samples t-

test). This is likely to reflect the increased propensity for patients with a wild type TPMT to hypermethylate. Nevertheless, 8 TPMT heterozygotes developed hepatotoxicity, all in the absence of hypermethylation. In covariate analysis, MP use, non-smoking, age and weight were all significantly associated with hepatotoxicity when compared to non-hepatotoxicity whereas IBD-type was not.

LDTA was extremely successful at normalising abnormal LFTs (Table 4.5.10). This was not just the case for hypermethylators but also normomethylators with hepatotoxicity. This suggests that there are shared mechanisms for hepatotoxicity between the two types, which are independent of MeMP.

The prevalence of IL-15 polymorphisms varied between different phenotypic groups with the IL-15 *83C variant being twice as prevalent in hypermethylators with hepatotoxicity and IL-15 g.142709723T being four times less prevalent in all hepatotoxics compared to the global MAF. IL-15 variants have been shown to increase hepatotoxicity in mice exposed to paracetamol.[291] However, neither of these variants were able to distinguish between phenotypes in this candidate gene analysis.

It was hypothesised that CYP450 variants could be responsible for hepatotoxicity through the release of formaldehyde as part of the demethylation process of MeMP (Figure 4.8). In support of this hypothesis were two findings: Firstly, that variants for CYP450 1A2*C were four times less common in hepatotoxics than the global MAF (0.05 versus 0.2); the action of the 1A2*C variant is to reduce demethylation by decreasing CYP450 activity. Secondly, that CYP1A2*F variants predicted the hypermethylators with hepatotoxicity compared to the hypermethylators in a dominant model (P = 0.03, Fisher's exact test, OR 2.69, 95% CI 1.14 – 6.36). This suggests that hypermethylators who possess the variant for CYP1A2*F are twice as likely to develop hepatotoxicity. CYP1A2*F variants are associated with an ultra-rapid metaboliser phenotype and hence hypothesised to increase demethylation with the release of formaldehyde. This finding would explain why CYP1A2*F variants increase the risk of hepatotoxicity in patients where MeMP is high.

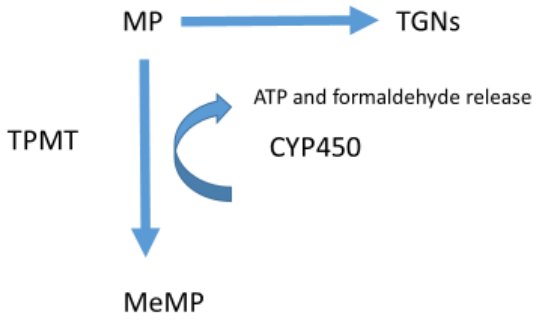


Figure 4.8: The demethylation of MeMP to MP by CYP450 enzymes.

Variants for MTHFR 359+160 were more common in all hepatotoxics compared to all non-hepatotoxics (P = 0.03, Fisher's exact test, OR 1.9, 95% CI 1.1 to 3.5), although not significantly different between hypermethylators and normomethylators, suggesting a role for the MTHR 359+260 variant in hepatotoxicity irrespective of hypermethylation. There were also more MTHFR 1286 homozygous variants in the hepatotoxicity only group compared to hypermethylators with hepatotoxicity although this was just short of significance (P = 0.05, Fisher's exact test). These findings support a role for the Methionine Cycle in thiopurine hepatotoxicity in the absence of hypermethylation. This could be because MTHFR variants reduce TPMT activity so hypermethylation does not occur and indeed, the influence of MTHFR variants on TPMT is supported by a study at GSTT which found that homozygous MTHFR variants for 1286 and 677 pushed TPMT wild types into the TPMT phenotype carrier range.[243] As MTHFR variants have been associated with reducing TPMT activity, it is likely that the mechanism by which MTHFR variants are involved in hepatotoxicity, is independent of hypermethylation. Instead, an alternative pathway of hepatotoxicity, due to changes in the Methionine Cycle leading to reduced glutathione, is explored further in Chapter 6.[261]

The findings in this study suggest that MTHFR variants play a role in thiopurine hepatotoxicity and therefore the role of these variants in other disease processes warrants further discussion. In this study, the G allele for MTHFR 359+160 (rs17367504) predisposed to hepatotoxicity but in other studies the G allele has been associated with a protective effect against pre-eclampsia, a condition which causes liver injury.[292] The G allele is located within a binding site for 16 transcription factors according to the UCSC genome browser http://genome.ucsc.edu/ and therefore likely to be a gene with a functional significance.

The MTHFR 1654-80 (rs17375901) SNP shows the least degree of interpopulation phenotypic diversity of all the MTHFR SNPs.[293] It has been associated with a susceptibility for atrial fibrillation through scar tissue formation but not neural tube defects as other MTHR SNPs have been.[294, 295]

MTHFR 1298 A>C (rs1801131) polymorphisms leads to the substitution of glutamine to alanine in the C-terminal regulatory domain resulting in a decrease in MTHFR activity. Studies suggest that AA variants with rheumatoid arthritis are more likely to respond to MTX in South Asian populations.[296] This can be explained by the mechanism via which MTX acts as an immunosuppressant, which is partly through the inhibition the Methionine Cycle. MTX has also been shown to have increased efficacy in MTHFR 677 CC variants with TT variants having a fourfold increased risk of non-response to MTX.[297]

Both 677 TT and 1298 CC variants are associated with a 15% reduction in colorectal cancer risk.[298] Both MTHFR polymorphisms have been associated with the extent of UC, with the 677 TT and 1298 AC variants being more prevalent in distal colitis and the CC allele being more common in extensive colitis.[299] This was not accounted for in our analysis.

Both 677 TT and 1298 CC variants were found to be more prevalent in patients with NAFLD and non-alcoholic steatohepatitis (NASH) compared to healthy controls, correlating with hyperhomocysteinaemia.[300]

The study can be criticised for the fact that 21 patients with hepatotoxicity with unknown methylation status could not be included in some of the analyses. Prospective data collection would have ensured that metabolite levels would have been available for all patients. Although the overall cohort was appropriately powered, certain phenotypic groups could have been too small to determine the true effect size. In addition, although there were multiple subgroup comparisons, any significant differences found between genotypes would have lost significance when corrected for multiple testing (Bonferonni correction). However, the association found between MTHFR and CYP1A2 variants and hepatotoxicity from thiopurines is still a novel finding. The mechanism by which these associations occur are explored further in the subsequent chapters.

Summary of study findings:

- There are 2 types of thiopurine hepatotoxicity, one involving hypermethylation and the other without
- Both types have a > 85% chance of resolving when switched to low dose thiopurine with allopurinol
- Thiopurine hepatotoxicity was more common in patients with a higher MeMP in univariate analysis
- Smoking was shown to be protective against hepatotoxicity
- MP use, increasing age and weight were also significantly associated with thiopurine hepatotoxicity
- CYP1A2*F variants predicted hypermethylators with hepatotoxicity compared to hypermethylators
- CYP1A2*C variants were four times less common in hepatotoxics compared to the global minor allele frequency
- MTHFR 360+169 variants predicted hepatotoxics from non-hepatotoxics and were three times more common compared to the global minor allele frequency
- Both CYP1A2 and certain MTHFR variants are associated with thiopurine hepatotoxicity, suggesting the mechanisms by which thiopurine hepatotoxicity occurs

Chapter 5: The relationship between smoking and thiopurine metabolism

5.1 Introduction

Demethylation by CYP450 enzymes, is the process by which MeMP are converted back to MP (see Section 1.16), a biochemical process proven *in vitro* by both Elion and Blaker et al.[233,234] CYP1A2*F variants increased CYP450 enzyme activity in studies on acute liver injury induced by Polygonum multiflorum, whilst CYP1A2*C variants decreased enzyme activity. [301-303] Smoking has been shown to be an inducer of CYP1A2*F variants.[238]

The aim was to analyse MeMP concentrations in smokers compared to non-smokers. It was hypothesised that patients who smoked whilst on thiopurines would demethylate more than non-smokers and therefore have lower MeMP concentrations.[235-237] It was also hypothesised that patients with the CYP1A2*F variant would demethylate more if smokers.

5.2 Patient recruitment and summary of methods

A cohort of both smokers and non-smokers were recruited in series as they came to clinic. Some smokers were also identified from a database of patients on thiopurines. Smoking status of IBD patients who had ever been on thiopurines were collected as they attended the IBD clinic. A questionnaire was attached to patients' medical notes and filled in by the attending IBD clinician.

Data were collected regarding: the amount smoked, IBD-type, ethnicity, drug therapy, TPMT (pmol/h/mgHb) and weight (Kg) for analysis. TGNs and MeMP values were recorded for each patient from when they had first been measured through to December 2015. Values of either zero TGN or zero MeMP were not included as this would just indicate poor compliance and could bias the results unnecessarily. Metabolite differences between smokers and non-smokers were analysed according to the first TGN, first MeMP, average of first three TGNs, first three MeMPs and the corresponding MeMP:TGN ratios. If patients had either stopped smoking or been switched to LDTA, metabolites after that time point were not analysed. Patients who were on tioguanine or LDTA were excluded. Patients smoking cannabis only were excluded.

A random selection of smokers were consented to be part of the CYP450 genetics study according to Pharmacogenetics of AZA study protocol, (REC reference LREC 06/Q0707/84) and genotyped for CYP1A2*F (163C>A) and *C (3860G>A). A blood sample was obtained in an EDTA blood tube and then stored at -70°C. When ready to analyse, the samples were thawed and then DNA extracted. Realtime TaqMan PCR was performed and genotypes validated using Sanger sequencing as per the methods outlined in Section 2.2 – 2.5. Statistical analysis was performed using Fisher's exact tests for categorical variables and an Independent samples t-test for parametric variables as outlined in Section 2.11. Allelic variances were assessed using a 2 x 2 web based contingency table (http://graphpad.com/quickcalcs/contingency1.cfm.

5.3 The cohort

237 patients had smoking data collected of whom 112 were smokers and 125 were non-smokers. Comparisons between both groups are made in Table 5.3. There were significant differences seen between the 2 groups for the percentage of patients who had had previous surgery and the percentage of hypermethylators. The percentage of patients with a normal TPMT activity (≥26 pmol/h/mgHb) was 10% higher in the smokers, however, mean TPMT was similar across both groups. Otherwise, both groups were similar for the other variables.

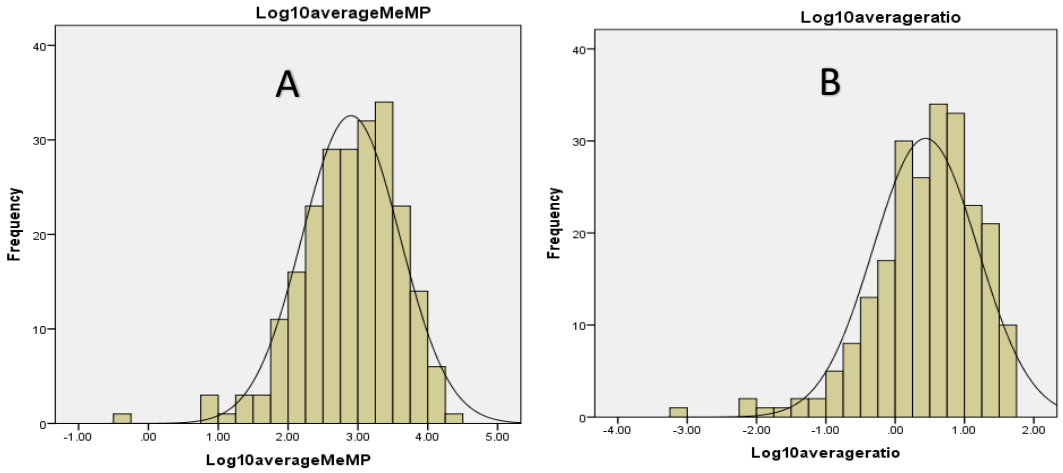
Table 5.3: Comparison between the smokers and non-smokers

Variable	Smokers (n=112) (SD)	Non-smokers (n=125) (SD)	P-value
% Males	49.1%	46.4%	0.8*
% CD	82.1%	72.1%	0.1*
% Previous surgery	47.3%	28.1%	**<0.01***
Mean age	33.4 (1.1)	30.2 (1.2)	0.5‡
Mean TPMT (pmol/h/mgHb)	34.4 (1.0)	33.9 (0.8)	0.8‡
% with normal TPMT activity (≥26)	72.3 %	62.4%	0.8*
% Caucasian	87.5%	79.2%	0.2*
% Hypermethylators	18.8% (n=21)	35.2% (n=44)	**<0.01***
% Hepatotoxicity	14.3% (n=16)	13.6% (n=17)	0.9*

* Two-tailed Fisher's exact test; ‡Independent samples t-test.

5.4 Metabolites in smokers versus non-smokers

Figures 5.4 shows the distribution of average MeMP and TGN:MeMP in the cohort which required adjustment by logarithm 10 (plus a constant of 1 for TGN:MeMP) in order to become normally distributed and ready for statistical analysis. Both groups are compared in Table 5.4 with significantly higher average 1st MeMP and MeMP:TGN ratios found in non-smokers (P = 0.03, Independent samples t-test).



Figures 5.4: The Log_{10} of the values for average MeMP (A) (pmol/8x10^8 RBC) and the TGN:MeMP (B), with a now normal distribution. Because some of the average TGN:MeMP ratios were 1, which would not be logged, a constant of 1 was also added.

Table 5.4: Compares significance differences between the logarithm 10 of first TGN and MeMP, average of first three TGNs and first three MeMPs, and the MeMP:TGN ratio (plus a constant 1) between smokers and non-smokers.

	Smokers (SD)	Non-smokers (SD)	P-value*
Log_{10} 1st TGN	2.4 (0.3)	2.41 (0.3)	0.9
Log_{10} average 1st MeMP	2.7 (0.7)	2.96 (0.8)	**0.03**
Log_{10} average 1st three TGNs	2.4 (0.3)	2.44 (0.2)	0.9
Log_{10} average 1st three MeMPs	2.8 (0.7)	2.97 (0.7)	0.1
Log_{10} average MeMP:TGN ratio + constant 1	0.6 (0.4)	0.72 (0.5)	**0.03**

‡Independent samples t-test.

5.5 Candidate gene analysis of CYP1A2 polymorphisms

The smokers (n=82) were then genotyped for CYP1A2 variants to determine if variants predisposed to demethylation.

Table 5.5.1: Comparison of CYP1A2*C and CYP1A2*F variants between hypermethylators and normomethylators in smokers.

	N	CYP1A2*C(rs2069514)			CYP1A2*F(rs762551)		
		GG (%)	GA (%)	AA (%)	AA (%)	AC (%)	CC (%)
Smokers	82	76 (93)	5 (6)	1 (1)	40 (49)	31 (38)	11 (13)
Hypermethylators	15	15 (100)	0	0	8 (53)	5 (33)	2 (13)
Normomethylators	67	61 (91)	5 (7)	1 (2)	32 (48)	26 (39)	9 (13)

There were no minor alleles for CYP1A2*C in hypermethylators whereas the MAF for CYP1A2*C was 5% in the normomethylators. The MAF in the hypermethylators for CYP1A2*F was 30% whereas it was 33% in the normomethylators. There were no significant differences between genotypes for CYP1A2*C or CYP1A2*F between hypermethylators and normomethylators in the smoking cohort in either a dominant or recessive model. Table 5.5.2 and Figure 5.5 compare metabolite differences between CYP1A2 variants of which there were none which reached significance.

Table 5.5.2: Compares the logarithm of average first MeMP, the first three TGNs and MeMPs and the average MeMP:TGN ratio (plus a constant 1) based on the first 3 metabolites for both CYP1A2*C and CYP1A2*F.

		CYP1A2*C				CYP1A2*F			
	N	GG (SD)	GA (SD)	AA	P<0.05	CC (SD)	CA (SD)	AA (SD)	P<0.05
Log_{10} 1st MeMP	76	2.8 (.7)	2.6 (.5)	2.2	No‡	2.6 (.8)	2.9 (.5)	2.9 (.9)	No‡
Log_{10} average 1st three TGN	82	2.5 (.3)	2.4 (.1)	2.4	No‡	2.4 (.3)	2.5 (.3)	2.5 (.2)	No‡
Log_{10} average 1st three MeMP	79	2.8 (.7)	2.9 (.4)	2.2	No‡	2.7 (.8)	2.9 (.5)	3.0 (.7)	No‡
Log_{10} average MeMP:TGN ratio + constant 1	82	0.6 (.5)	0.5 (.3)	0.2	No‡	0.6 (.4)	0.6 (.5)	0.8 (.5)	No‡

‡Independent samples t-test.

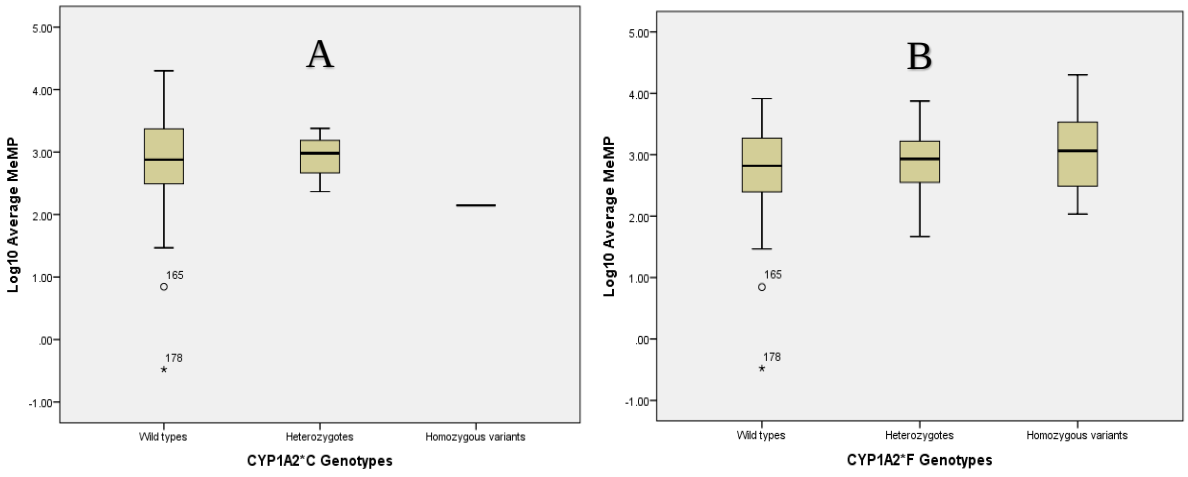


Figure 5.5: Box plots comparing the logarithm of the average MeMP (First 3 MeMP following starting thiopurines) in smokers according to the 3 genotypes for CYP1A2*C (A) and *F (B).

The cohort was then restricted to standardise against TPMT heterozygotes and therefore patients with a TPMT of < 26 pmol/h/mgHb were excluded. This analysis was performed to determine if significant differences may arise between the two groups. However, significant differences were less for Log_{10} first MeMP but more for Log_{10} average MeMP:TGN ratio + constant 1. 1^{st} MeMP and average MeMP:TGN ratio remained significantly higher in non-smokers (Table 5.5.3).

Table 5.5.3: Compares the logarithm of first TGN, first MeMP average first three TGNs and MeMPs and the average MeMP:TGN ratio between smokers and non-smokers after excluding TPMT heterozygotes.

	Smokers (SD) (n=81)	Non-smokers (SD) (n=80)	P-value
Log_{10} 1^{st} TGN	2.4 (0.3)	2.4 (0.3)	0.9
Log_{10} 1^{st} MeMP	2.8 (0.7)	3.0 (0.7)	**0.04**
Log_{10} average 1^{st} three TGN	2.4 (0.3)	2.4 (0.2)	0.9
Log_{10} average 1^{st} three MeMP	2.9 (0.7)	3.1 (0.6)	0.08
Log_{10} average MeMP:TGN ratio + constant 1	0.6 (0.4)	0.8 (0.4)	**0.02**

5.6 Discussion

In this study, smokers had reduced MeMP and MeMP:TGN ratios compared to non-smokers. Overall, there were significantly more hypermethylators in the non-smokers compared to the smokers (35% vs 18%, P <0.01, Fisher's exact test). These findings support the hypothesis that smoking upregulates the demethylation of MeMP mediated by CYP450 enzymes thereby reducing the number of hypermethylators.

Smoking was hypothesised to upregulate CYP450 mediated demethylation in CYP1A2*F variants. However, there was no difference in the number of CYP1A2*C and *F variants in smokers who hypermethylated compared to those who did not, neither were metabolite concentrations different between groups.

MeMP concentrations were found to be lower in smokers than non-smokers, despite the smoking group having a higher percentage (72% versus 62%) of patients with normal TPMT activity. This separates out any potential hypermethylation occurring through increased TPMT activity in the non-smoking group; therefore, the lower MeMP in smokers can be put down to demethylation only. Although there were no significant differences in TPMT activity in my study (Table 5.3), others have found increased TPMT activity in smokers, thought to be due to elevated S-adenosylmethionine (SAM), based on findings of elevated SAM in human lung epithelial-like cells exposed to cigarette smoke.[290] An increased TPMT activity in smokers would balance out the reduction in MeMP concentrations through increased demethylation and might explain why differences in metabolite concentrations were not as significant.

There were no differences in the number of patients with thiopurine hepatotoxicity between smokers and non-smokers. This runs contrary firstly, to the findings in Chapter 4, where thiopurine hypermethylation and hepatotoxicity was less common amongst smokers and secondly, to the hypothesis that hepatotoxicity occurs as a result of formaldehyde release during demethylation. Instead, it suggests that although smoking increases demethylation, hepatotoxicity does not arise as a result of this process.

Normally, 5% of patients develop thiopurine hepatotoxicity whereas in this cohort, 14% of patients had hepatotoxicty.[143] This difference could be explained firstly, by the increased proportion of hypermethylators (27% versus 20% in the normal population) and secondly, if patients with thiopurine

hepatotoxicity, as well as hypermethylators, were more likely to attend the IBD clinic and hence be recruited to the study.

TGN concentrations were no different between smokers and non-smokers despite significant differences in MeMP. There are several reasons to explain why TGNs were not increased. Firstly, any increase in MP concentrations as a result of demethylation could be shunted towards the formation of thiouric acid (TUA) rather than TGN. Secondly, MP may be excreted as urinary MP and thirdly, smoking may inhibit other enzymes required for TGN formation. Either way, smoking does not increase TGNs and hence the clinical reality of this is that smoking would not alter the efficacy of thiopurines other than by reducing the potential side effects associated with hypermethylation.

There are several limitations to this study. Firstly, all patients should have been TPMT genotyped, to allow for standardisation against TPMT variants. Secondly, the recruitment of smokers and non-smokers should have been more randomised, as a proportion of both smokers and non-smokers were highlighted for recruitment from a database for a study on hypermethylation. Thirdly, the retrospective nature of the study meant that metabolite concentrations were not measured using a standardised approach and compared for other variables, such as dose changes and drug compliance. Lastly, other studies have suggested that the oral contraceptive pill (OCP) and caffeine both increase the activity of CYP1A2 enzymes.[304] These are potential confounding factors which were not analysed for in this study.

Overall, the study is underpowered. The MAF for CYP1A2*C in the smoking cohort was 4% which is below the 20% that the initial power calculations were based on (see Table 2.10). Therefore, to achieve a three-fold effect size using a P-value of 0.05 to reject the null hypothesis, the standard differences (SD) would be 0.21 with a sample size required of 600 (Figure 5.6).

$$P = \frac{P1+P2}{2}$$

$$P = \frac{0.04+0.008}{2} = 0.024$$

$$SD = \frac{P1 - P2}{\sqrt{P\,(1-P)}}$$

$$SD = \frac{0.04 - 0.008}{\sqrt{0.024\,(1-0.024)}}$$

$$SD = 0.21$$

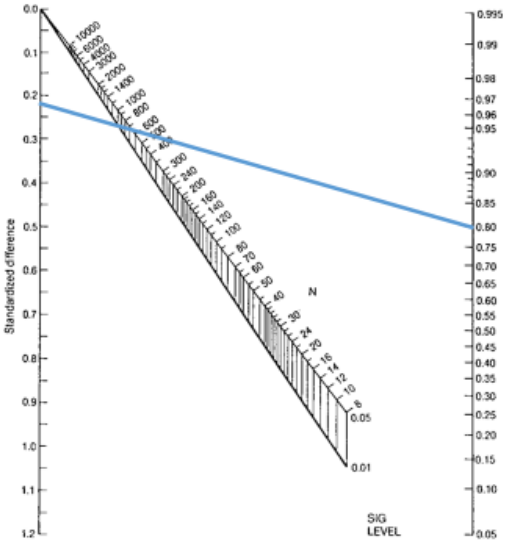


Figure 5.6: The calculation for standard difference (SD) if the MAF was 0.04 and a 5-fold difference would need to be observed. The Gore and Altman normogram is used to calculate sample size if a power of 80% was required.[305]

Other than smoking, the potential role for drugs, such as the COCP, and caffeine to affect CYP1A2-mediated demethylation should also be studied, as these too may have an impact on the efficacy of thiopurines.[304]

In this study, smokers were found less likely to hypermethylate and have lower MeMP. The demethylation of MeMP has been shown to occur by CYP450 enzymes. Smoking has been proven to induce CYP450 activity in CYP1A2*F variants, which in this context would increase demethylation. The correlation between this study and other studies makes the association between CYP1A2 enzyme-mediated demethylation and smoking a convincing one; the size of the cohort was too small to determine if variants for CYP1A2 affected hypermethylation in the smoking group (Table 5.5.1).

The clinical relevance of this study is uncertain, but the findings support the impact of CYP1A2 enzyme-mediated demethylation on thiopurine metabolism and how phenotypic differences can be induced by smoking. However, no relationship was observed with regards to thiopurine hepatotoxicity. A larger prospective study, with a standardised methodology, is required to confirm this association.

Summary of study findings:

- Smokers had lower MeMP than non-smokers
- Hypermethylators were more common in the non-smokers
- Amongst smokers, there was no correlation between in CYP1A2 variants and hypermethylation
- Demethylation appears to be upregulated in smokers

Chapter 6: Measurement of SAM by mass spectrometry in patients on thiopurines

6.1 Introduction

S-adenosylmethionine (SAM) is a cofactor for the TPMT methylation of MP to MeMP, and is an essential requirement of the TPMT assay.[271] Many studies suggest a role for SAM in causing liver disease. For example, a reduced SAM:SAH (s-adenosylhomocysteine) ratio is thought to contribute towards alcohol-induced liver injury.[306, 307] In alcoholic hepatitis, therefore, it is proposed that the administration of SAM might replenish depleted glutathione concentrations in hepatic tissue, hence reducing liver toxicity.[308] The administration of SAM has been demonstrated in placebo controlled trials to improve transplant-free survival of patients with cirrhosis.[309]

In this study, the role of SAM in thiopurine hepatotoxicity is investigated. The aim was to determine metabolite differences for SAM, SAH, homocysteine (HCys), and methionine (Met) between patients on thiopurines and correlate these compounds with Pyroglutamtate (PG), a marker of reduced glutathione and hepatotoxicity.

The hypothesis was that hepatotoxicity in patients on thiopurines occurs as a result of reduced glutathione following alterations in SAM and SAH and that the concentrations of these compounds would differ between normomethylators, hypermethylators and hypermethylators with hepatotoxicity.

6.2 Patient recruitment and summary of methods

The inclusion criteria for this study required patients to have abnormal metabolite profiles (MeMP:TGN) or LFTs as a result of thiopurine treatment at the time that blood samples for the study were taken. To achieve this, samples taken for the future measurement of compounds using LC-MS, were also sent for LFT, MeMP and TGN measurements in order to confirm that patients still fitted the group that they were recruited into. Samples for LC-MS were taken in a heparinised tube on ice, and immediately spun down and separated into plasma, buffy coat and RBCs into Eppendorf tubes; RBCs were washed twice with 0.9% saline. WB samples were also reserved for measurement of compounds. The Eppendorf tubes were stored at -70°C.

Patients who had normal LFTs and metabolite profiles were also included in the study for comparison. Healthy staff from the Purine Laboratory at GSTT, were included as a control group (n=5, 3 males). Patients were consented using the Pharmacogenetics of AZA studies (REC Ref LREC 06/Q0707/84).

Patients were excluded if they were on tioguanine or LDTA or if they had an underlying chronic liver disease. Subjects recruited were assigned a group as defined in Table 6.2. As the data is small and non-parametric, results are expressed as medians and not tested for significance.

Table 6.2: Definition of patients and controls recruited.

Group	Definition
Hypermethylators with Hepatotoxicity	Patients with ALT >56 IU/L and MeMP:TGN > 20 and a temporal relationship of hepatotoxicity to the start of thiopurine
Hepatotoxicity only	Patients with ALT >56 IU/L and MeMP:TGN < 11 and a temporal relationship of hepatotoxicity to the start of thiopurine
Hypermethylators only	Patients with ALT < 56 IU/L but MeMP:TGN > 20
Normomethylators	Patients on thiopurines with ALT < 56 IU/L and MeMP:TGN < 11
Controls	Volunteers without IBD and not on a thiopurine

6.3 The cohort

Twenty-five patients with IBD on thiopurines had their concentrations of SAM, SAH, HCys and Met measured along with five healthy members of the laboratory staff as controls. Of the 25 patients (AZA=20, MP=5), 12 patients had normal metabolite profiles (males=10, MP=1), 6 were hypermethylators with normal LFTs (males=3, MP=2), and 6 were hypermethylators with hepatotoxicity (males=3, MP= 2). One patient, recruited as being on AZA, was found to have zero MeMP and was therefore assigned an active IBD, non-thiopurine phenotype; their compounds were still measured. Three of the patients with both hypermethylation and hepatotoxicity had compounds re-measured after a switch to LDTA (mean time of re-measurement after starting LDTA was 218 days (SD 88)). Variables of interest between the groups are compared in Tables 6.3.1 and 6.3.2.

Table 6.3.1: The medians and ranges for MeMPs, TGNs, MeMP:TGN ratios and ALTs for the patients on thiopurines (n=24).

Group	MeMP (pmol/8x10^8 RBC)	TGN (pmol/8x10^8 RBC)	MeMP:TGN	ALT (IU/L)
Normomethylators	728 (126-5832)	347 (42-629)	2.0 (1-10)	37.5 (13-55)
Hypermethylators	5574 (1510-11431)	280 (119-471)	21.5(13-32)	20.5 (18-28)
Hypermethylation with hepatotoxicity	10908 (2913- 29383)	352 (153-533)	33.0 (11-72)	92.5 (76-272)

Table 6.3.2: Medians (IQRs) and ranges for TPMT, weight and age between the groups.

Groups	TPMT (pmol/h/mgHb)	Weight (kg)	Age (years)
Normomethylators	35.0 (IQR 7, 29-42)	72.5 (IQR 16.0, 50-85)	35.5 (IQR 16, 22-57)
Hypermethylators	38.8 (IQR 8, 30-48)	67.9 (IQR 18.3, 56-81)	31.5 (IQR 25, 21-54)
Hypermethylation with hepatotoxicity	37.5 (IQR 4, 35-40)	77.6 (IQR 34.9, 68-112)	38 (IQR 23, 28-54)

6.4 Measurement of SAM/SAH/HCys and Met

It was clear from the calibration assays that the recovery of these compounds in plasma was much higher than in RBC and WB (Table 2.7.4). Plasma was separated from blood samples as discussed in Section 2.7. Compounds of the Methionine Cycle were measured in these samples using LC-MS calibrated against deuterated internal standards. Plasma SAM, SAH and the SAM:SAH ratios for all patient groups are shown in Table 6.4.1. Figure 6.4.1 shows the differences in median plasma SAM concentrations between patients on thiopurines compared to controls.

Table 6.4.1: Medians (IQRs) of plasma SAM, SAH and the SAM:SAH ratios.

Group	Plasma SAM nmol/L	Plasma SAH nmol/L	Plasma SAM:SAH
Controls	85.7 (15.2)	16 (3.1)	4.95 (1.24)
Normomethylators	104.5 (29.3)	17.9 (3.1)	5.98 (0.96)
Hypermethylators	99.3 (26.7)	18.8 (14.5)	5.42 (2.65)
Hypermethylators with hepatotoxicity	101 (36.6)	18.4 (6.4)	5.73 (2.2)

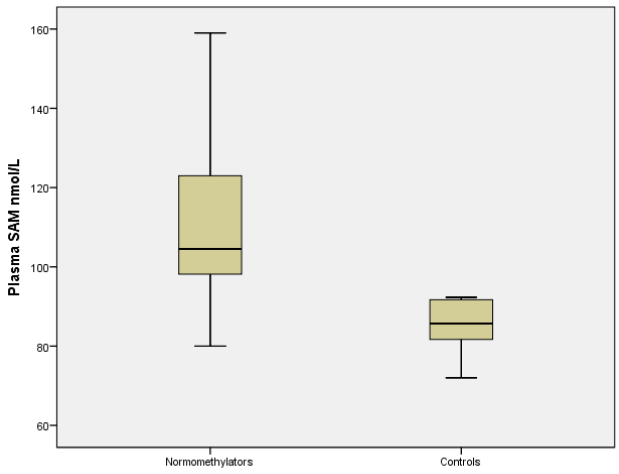


Figure 6.4.1: Box plot comparing the median differences between concentrations of plasma SAM in controls (n = 5, median 85.7 mmol/L, IQR 15.2) and normomethylators (n = 12, median 104.5 nmol/L, IQR 29.3).

There was a week negative correlation between plasma SAM and TGN concentrations (r^2 =0.33) (Figure 6.4.2). There was more plasma SAM in patients on thiopurines compared to controls (Figure 6.4.3).

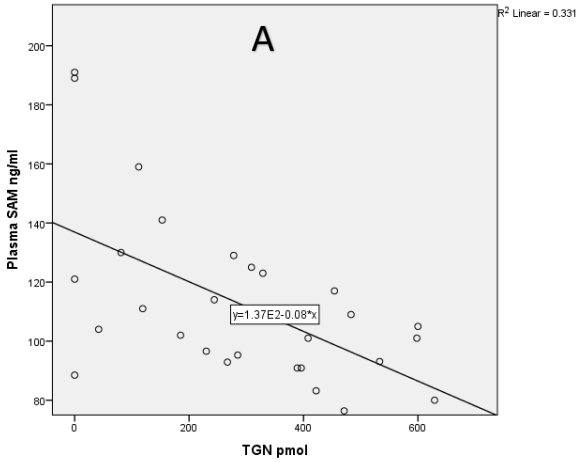


Figure 6.4.2: Correlates Plasma SAM relative to TGNs.

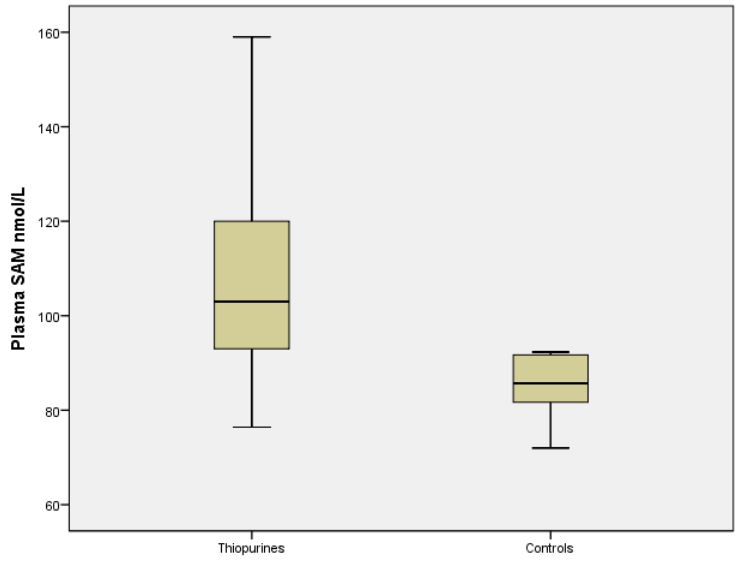


Figure 6.4.3: The median differences in plasma SAM concentrations (n = 24, median 103 nmol/L, IQR 29) between patients on thiopurines compared to controls (n = 5, median 85.7, IQR 15).

HCys and Met were also measured as described in Section 2.7, the concentrations of which are shown in Table 6.4.2; correlations between compounds were almost linear (i.e no correlation) between HCys and both plasma SAM and the SAM:SAH ratio (Figure 6.4.4).

Table 6.4.2: Median (IQR) Plasma Met and HCys levels.

Group	Met µmol/L	HCys µmol/L
Controls	30.4 (10.8)	9.41 (4.7)
Normomethylators	32.8 (21.4)	12.6 (3.9)
Hypermethylators	40.7 (18.2)	11.7 (3.4)
Hypermethylation with hepatotoxicity	34.0 (21.0)	9.17 (5.0)

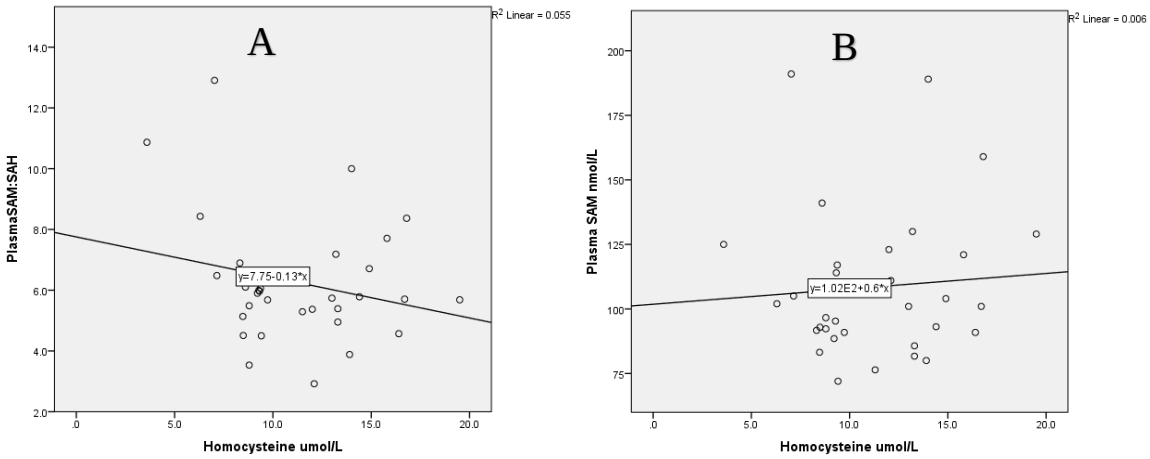


Figure 6.4.4: The correlation between increasing plasma SAM (A) and reducing SAM:SAH ratio (B) with increasing HCys concentrations (r^2 = 0.006 and 0.055 respectively).

Three of the patients who were hypermethylators with hepatotoxicity had plasma SAM and SAH measured after switching to LDTA and once LFTs had normalised (mean ALT 38 IU/L) (Table 6.4.3). The switch to LDTA increased concentrations of both metabolites (Figure 6.4.5).

Males had higher SAH concentrations than females (In the study, the gender balance was similar between controls and patient groups). However, there was no difference in plasma SAM concentrations (Table 6.4.4). There was a poor correlation (r^2 = 0.18) between plasma SAM concentrations and weight.

Table 6.4.3: Changes in median concentrations of SAM, SAH and SAM:SAH before and after LDTA in 3 patients.

Group (n=3)	Plasma SAM nmol/L	Plasma SAH nmol/L	SAM:SAH
Before LDTA	109	20.6	5.78
After LDTA	189	15.7	10.0

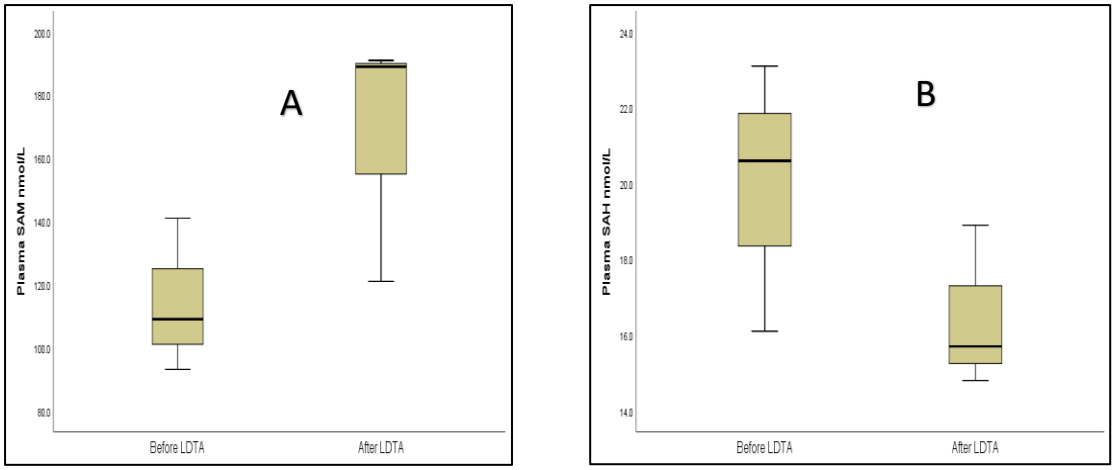


Figure 6.4.5: Boxplots comparing the changes to median plasma SAM (A) (109 to 189 nmol/L) and SAH (B) (20.6 to 15.7 nmol/L) before and after LDTA in 3 hypermethylating patients with hepatotoxicity.

Table 6.4.4: Compares median (IQR) concentrations of compounds in plasma between males and females in the cohort

	Plasma SAM nmol/L	Plasma SAH nmol/L	Plasma SAM:SAH
Males	102.5 (29.0)	18.3 (5.3)	5.69 (2.03)
Females	93.1 (37.2)	16.0 (3.0)	6.26 (1.99)

6.5 Measurement of 5-MTHF

Plasma 5-methyltetrahydrofolate (5-MTHF) concentrations were also measured in the samples using HPLC as described in the method by Sobczynska-Malefora et al.[283] This was to assess whether 5-MTHF correlated with compounds from the Methionine Cycle (SAM, SAH, HCys and Met) (Table 6.5.1). Correlations between compounds and 5-MTHF were almost linear (Figure 6.5). There were also no differences in plasma MTHF before and after LDTA (Table 6.5.2) and between any of the thiopurine groups and controls.

Table 6.5.1: Median (IQR) 5-MTHF concentrations.

Group	5-MTHF nmol/L
Controls	17.2 (11.5)
Normomethylators	20.0 (21.7)
Hypermethylators	25.4 (39.0)
Hypermethylation with hepatotoxicity	23.3 (14.5)

Table 6.5.2: Compares median 5-MTHF concentrations before and after LDTA.

Group (n=3)	5-MTHF nmol/L
Before LDTA	24.8
After LDTA	19.7

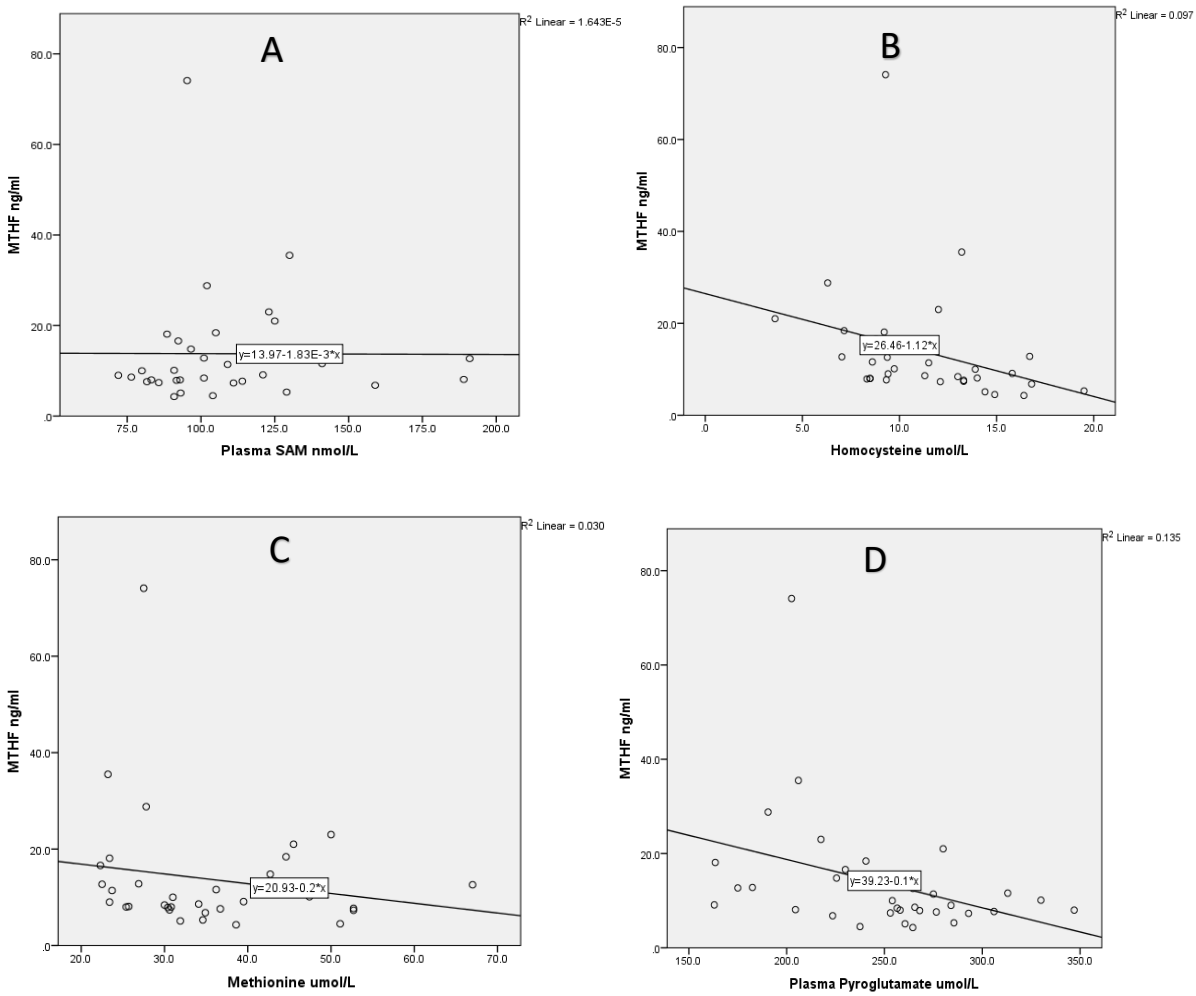
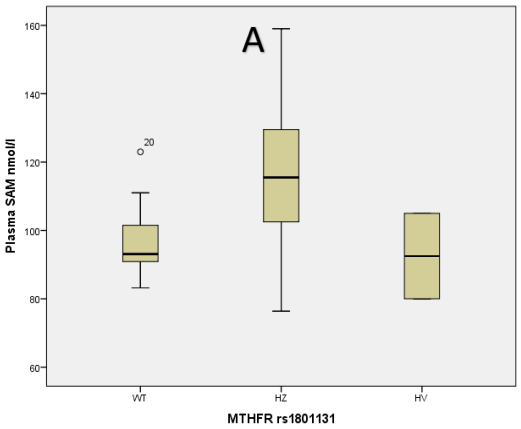
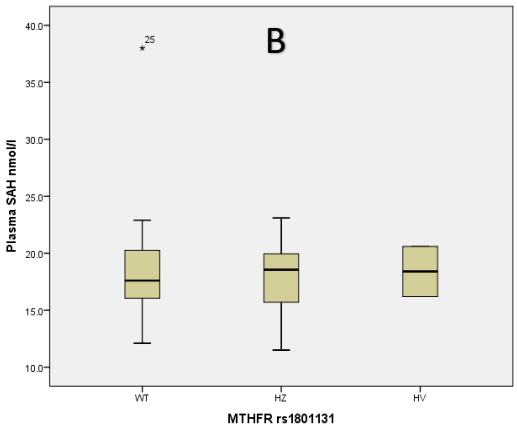


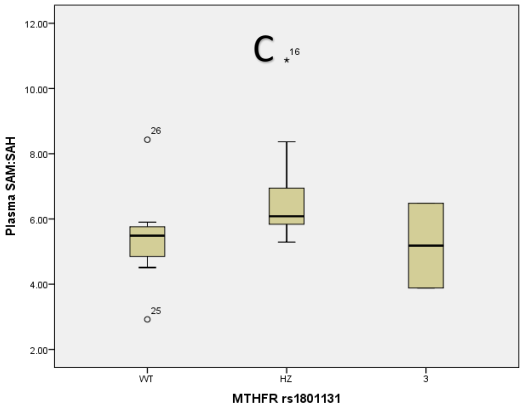
Figure 6.5: Four correlation curves correlating 5-MTHF with concentrations of plasma SAM (A) (r^2 = 1.64), HCys (B) (r^2 =0.097), Met (C) (r^2 = 0.03), and plasma PG (D) (r^2 = 0.135).

6.6 Comparisons between compounds and MTHFR genotype

25 patients (including the IBD patient with zero MeMP) were analysed for plasma SAM and SAH according to four MTHFR variants (rs1801131, rs1801133, rs17375901, and rs17367504). Carriers (n = 12) for the rs1801131 variant (1286 A>C) and rs1801133 (677 C>T) had higher plasma SAM concentrations and SAM:SAH ratios than wild types (n = 11). Differences were not seen for the other 2 variants, rs17375901 (1654-80 G>A) and rs17367504 (359+160 A>G).

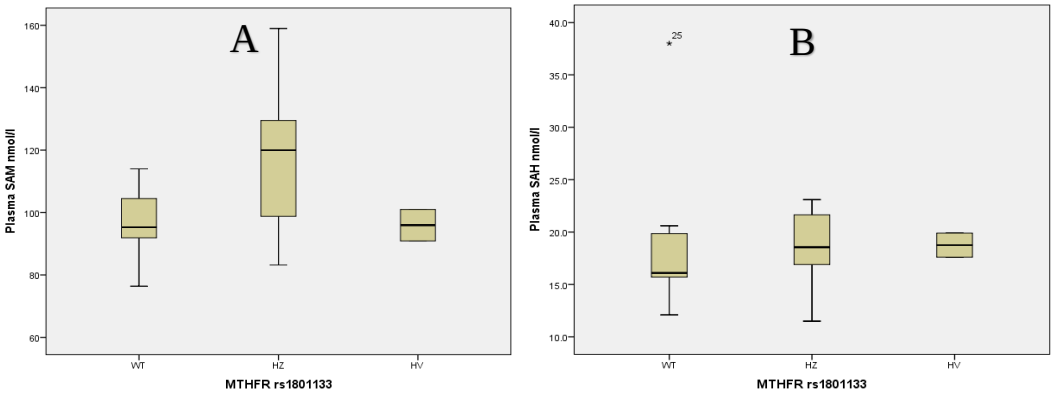


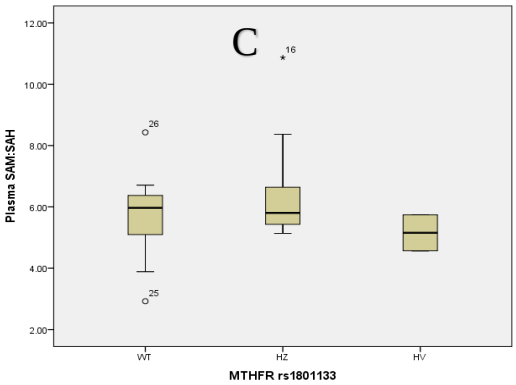




Compound	Genotype	N	Median SAM (nmol/L)	IQR (nmol/L)
SAM	AA	11	93.1	11.1
SAM	AC	12	116	28.0
SAM	CC	2	92.5	Range 80-105
SAH	AA	11	17.6	4.6
SAH	AC	12	18.6	4.7
SAH	CC	2	18.4	Range 16.2-20.6
SAM:SAH	AA	11	5.49	1.21
SAM:SAH	AC	12	6.08	1.29
SAM:SAH	CC	2	5.18	Range 3.88-6.48

Figure 6.6.1: The median differences in plasma SAM concentrations (A) (nmol/L), plasma SAH concentrations (B) (nmol/L) and the SAM:SAH ratio between MTHFR variants for rs1801131 (1286 A>C).





Compound	Genotype	N	Median SAM (nmol/L)	IQR (nmol/L)
SAM	CC	11	95.3	14
SAM	CT	12	120	32
SAM	TT	2	96.0	Range 91-101
SAH	CC	11	16.1	5.1
SAH	CT	12	18.6	11.6
SAH	TT	2	18.8	Range 17.6-19.9
SAM:SAH	CC	11	5.97	1.97
SAM:SAH	CT	12	5.80	1.51
SAM:SAH	TT	2	5.15	Range 4.57-5.74

Figure 6.6.2: Median differences in plasma SAM concentrations (A) (nmol/L), plasma SAH concentrations (B) (nmol/L) and SAM:SAH ratio (C) between MTHFR variants for rs1801133 (677 C>T).

6.7 Measurement of PG, glutamate and glutamine

PG, glutamate and glutamine were measured in the plasma, RBC and WB samples (Tables 6.7.1 - 6.7.2); Only PG was measured against its deuterated standard. The compounds were not assessed for recovery in plasma, RBC and WB samples and therefore results in all samples are displayed. RBC PG concentrations were higher in hypermethylators with hepatotoxicity compared to controls and normomethylators but similar between hypermethylators and hypermethylators with hepatotoxicity. There were no differences in glutamate concentrations in plasma, WB or RBC between the different groups (Table 6.7.2). WB glutamine was higher in hypermethylators with hepatotoxicity compared to controls (Table 6.7.3).

RBC PG concentrations were higher in IBD compared to non-IBD patients (Figure 6.7.2). However, concentrations of glutamine and glutamate were similar (Table 6.7.4).

RBC PG concentrations were higher in patients on thiopurines compared to those not on thiopurines (Figure 6.7.3). Glutamate or glutamine concentrations, however, were similar (Table 6.7.5).

Table 6.7.1: The median differences (IQR) for PG between the different groups for thiopurine metabolism when measured using the deuterated standard.

Group	Plasma PG µmol/L	WB PG µmol/L	RBC PG µmol/L
Controls	23.2 (2.9)	76.3 (17.5)	99.1 (19.5)
Normomethylators	20.8 (4.7)	78.0 (26.7)	137 (41.0)
Hypermethylators	19.1 (13.6)	65.9 (75.4)	159 (69.4)
Hypermethylation with hepatotoxicity	23.1 (3.7)	109 (268)	176 (75.9)

Table 6.7.2: Compares median (IQR) glutamate in plasma, WB and RBC between the 3 groups and controls.

Group	Plasma glutamate µmol/L	WB glutamate µmol/L	RBC glutamate µmol/L
Controls	77.7 (38.6)	451 (162)	434 (302)
Normomethylators	85.1 (47.6)	489 (133)	372 (351)
Hypermethylators	61.7 (76.6)	509 (266)	350 (545)
Hypermethylation with hepatotoxicity	93.9 (157)	544 (397)	535 (559)

Table 6.7.3: Compares median (IQR) glutamine in plasma, WB and RBC.

Group	Plasma Glutamine µmol/L	WB Glutamine µmol/L	RBC Glutamine µmol/L
Controls	532 (163)	321 (181)	161 (156)
Normomethylators	549 (161)	365 (115)	260 (189)
Hypermethylators	513 (133)	325 (141)	263 (161)
Hypermethylation with hepatotoxicity	562 (202)	489 (291)	391 (149)

Tables 6.7.4: Compares median (IQR) PG, glutamate and glutamine in plasma, WB and RBC between non-IBD (n=5) and IBD patients (n=25).

Group	Plasma PG µmol/L	WB PG µmol/L	RBC PG µmol/l
Non-IBD	23.2 (2.9)	76.3 (17.5)	99.1 (19.5)
IBD	21.0 (6.4)	80.7 (47.5)	152.5 (48.1)

Group	Plasma Glutamate µmol/L	WB Glutamate µmol/L	RBC Glutamate µmol/L
Non-IBD	77.7 (38.6)	451.0 (161.5)	434 (302)
IBD	84.4 (50.5)	480.0 (140.5)	386.5 (347)

Group	Plasma Glutamine µmol/L	WB Glutamine µmol/L	RBC Glutamine µmol/L
Non-IBD	532.0 (162.5)	321.0 (181.0)	161.0 (156.0)
IBD	532.5 (130.5)	366.5 (155.5)	329.5 (189.3)

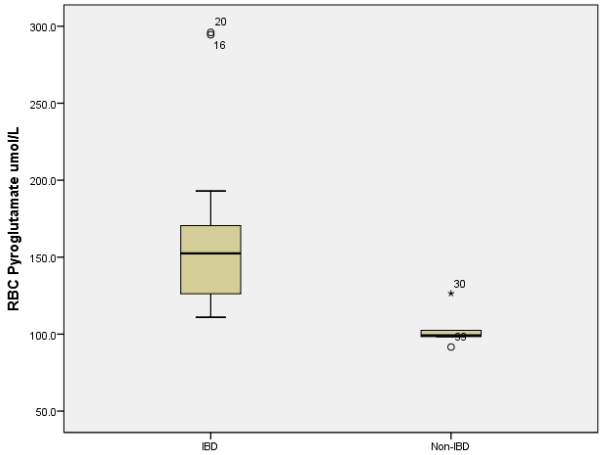


Figure 6.7.2: Compares median RBC PG between patients with IBD (median 154 µmol/L, IQR 44.3 µmol/L) and non-IBD (median 99.1 µmol/L, IQR 19.5 µmol/L).

Tables 6.7.5: Compares median (IQR) PG, glutamate and glutamine in plasma, WB and RBC between non-thiopurine (n=6) and thiopurine samples (n=24).

Group	Plasma PG µmol/L	WB PG µmol/L	RBC PG µmol/L
Non-thiopurines	22.8 (3.2)	78.0 (15.4)	101 (35.9)
Thiopurines	21.5 (6.8)	81.5 (49.4)	154 (52.1)

Group	Plasma Glutamate µmol/L	WB Glutamate µmol/L	RBC Glutamate µmol/L
Non-thiopurines	83.6 (35.5)	461 (128)	374 (356)
Thiopurines	83.8 (51.6)	489 (145)	429 (222)

Group	Plasma Glutamine µmol/L	WB Glutamine µmol/L	RBC Glutamine µmol/L
Non-thiopurines	531.5 (122)	372 (170)	218 (201)
Thiopurines	534 (139)	365 (138)	304 (166)

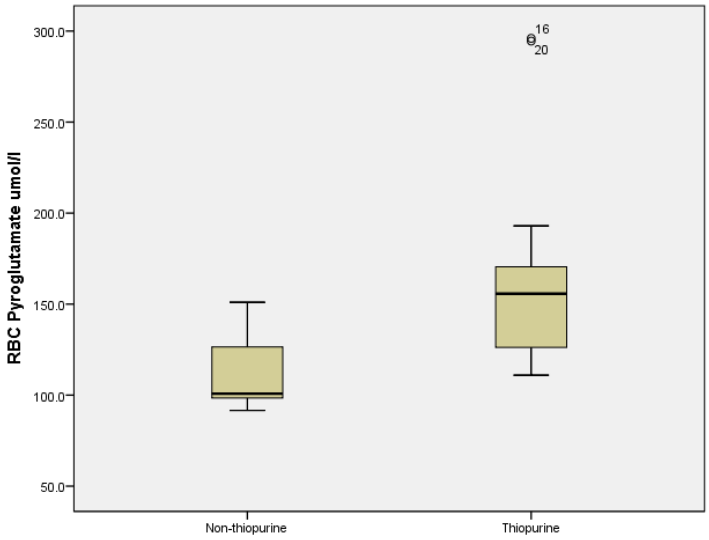


Figure 6.7.3: Median differences in RBC PG concentrations between patients on thiopurines (median 156 µmol/L, IQR 48.1 µmol/L) and not on thiopurines (median 100 µmol/L, IQR 35.9 µmol/L).

Compounds were compared between 3 patients with hypermethylation and hepatotoxicity compared to the same 3 patients after switching to LDTA. RBC PG and glutamine concentrations were all lower after switching to LDTA (Table 6.7.6 and Figure 6.7.4). The differences in the median concentrations of RBC PG and RBC glutamine between all the main groups are shown in Figures 6.7.5 – 6.7.6). Lastly, there were no differences between RBC PG concentrations between patients on AZA and patients on MP in hypermethylators or hypermethylators with hepatotoxicity.

Table 6.7.6: Shows medians of PG, glutamine and glutamate concentrations before and after LDTA

	RBC PG µmol/L	RBC glutamine µmol/L	RBC glutamate µmol/L
Before LDTA	187	467	459
After LDTA	115	137	401

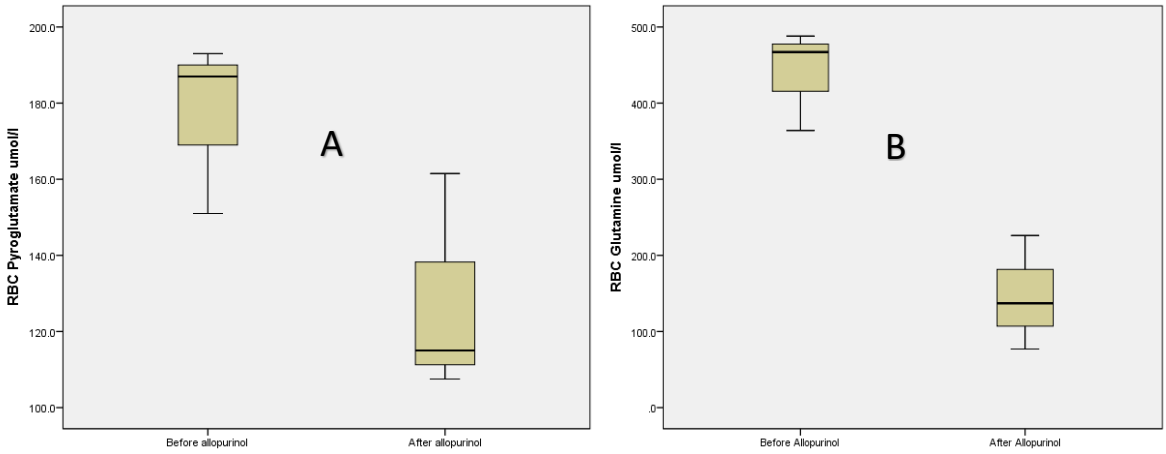


Figure 6.7.4: RBC PG concentrations (A) in three patients with hypermethylation and hepatotoxicity before (median 187 µmol/L, range 151-193 µmol/L) and after LDTA (median 115 µmol/L, range 108-162 µmol/L) and RBC glutamine concentrations (B) in three patients with hypermethylation and hepatotoxicity before (median 467 µmol/L, range 364-488 µmol/L) and after LDTA (median 137 µmol/L, range 76.9-266 µmol/L).

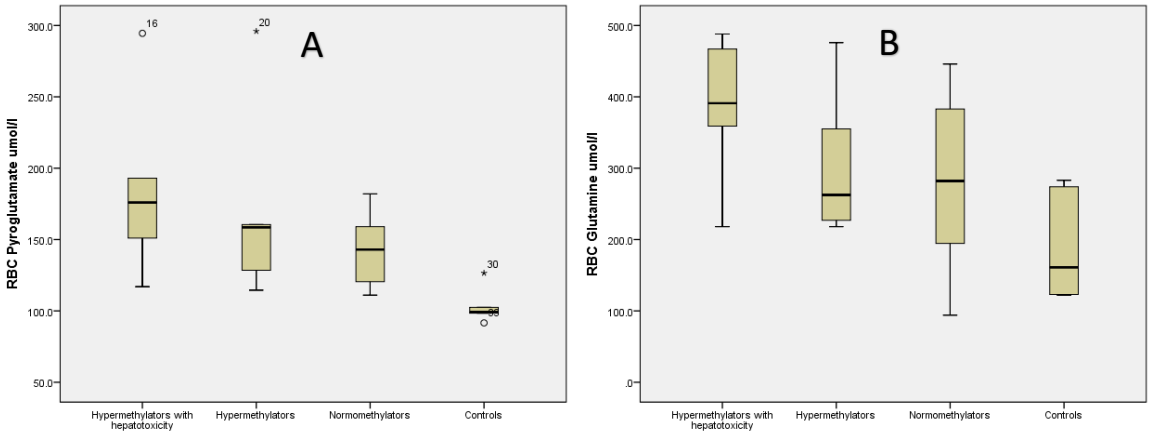


Figure 6.7.5: Median differences between the three different groups and controls for RBC PG (A) and glutamine (B) concentrations. The concentrations of RBC PG (A) in the groups (expressed as µmol/L) were as follows: hypermethylators with hepatotoxicity = median 176, IQR 45; hypermethylators = median 159, IQR 27; normomethylators = median 143, IQR 28; controls = median 99. 1, IQR 7. The concentrations of RBC glutamine (B) in the groups (expressed as µmol/L) were as follows: hypermethylators with hepatotoxicity = median 391, IQR 110; hypermethylators = median 263, IQR 125; normomethylators = median 282, IQR 190; controls = median 161, IQR 158.

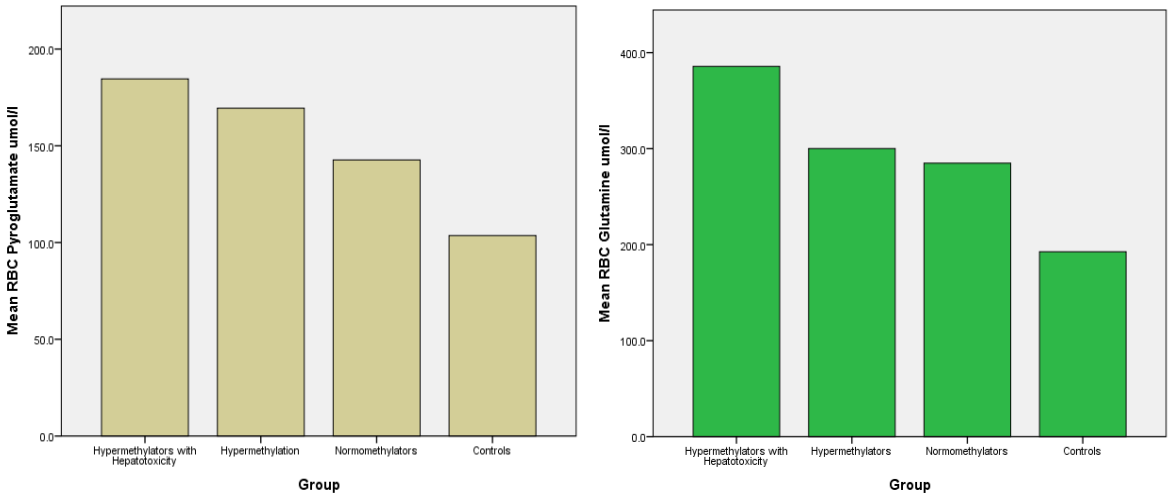


Figure 6.7.6: Decreasing concentrations of both RBC PG and RBC glutamine within the cohort.

6.8 Discussion

This is a novel study in that it is the first time SAM has been measured in patients on thiopurines. As such, comparisons with the literature are not possible. The assay developed to measure SAM and SAH was also new to our institution. The accuracies of the assay were acceptable (improving to 99.4-125% with the deuterated standard) with the CV of SAM best when measured in plasma.

The recovery of SAM and SAH was best seen in plasma and their measurement in WB and RBC afforded nothing different. The median concentrations of plasma SAM in controls was 85.7 nmol/L, with a median plasma SAH of 16 nmol/L and a SAM:SAH ratio of 5:1. These concentrations are consistent with those measured in the study by Klepacki et al.[270]

Plasma SAM was higher in patients on thiopurines, compared to controls, with even higher concentrations when measured in hypermethylators with hepatotoxicity. However, plasma SAM concentrations were similar between the thiopurine groups. Likewise, plasma SAH and the methylation potential (SAM:SAH) were no different between any of the thiopurine groups either.

Plasma HCys concentrations measured in controls were the same as reported in previous studies.[259] HCys did not differ between any of the groups and there was no differences between genders as has been reported in previous studies.[259] Met concentrations, however, were higher in hypermethylators than in controls .

Although the concentrations of 5-MTHF between groups were the same, 5-MTHF variants for two genotypes (677 C>T and 1286 A>C) were found to have higher plasma SAM concentrations. This finding, along with the findings of other studies, suggests a role for 5-MTHF variants in thiopurine metabolism.[243]

Plasma SAM and TGNs were shown to be inversely proportional to each other on the correlation curves, although the correlation was only weakly positive (Figure 6.4.2). This finding can be explained by a hypothesis whereby, as SAM concentrations increase, TPMT activity and MeMP concentrations increase, thereby reducing the concentrations of MP available for TGN formation.

The switch to LDTA in three hypermethylators with hepatotoxicity patients increased plasma SAM and the SAM:SAH ratio, restoring their concentrations back to baseline.

There were gender differences seen only between concentrations of plasma SAH and no correlation found between plasma SAM and increasing weight.

There was an increase in RBC PG between groups: hypermethylation and hepatotoxicity > hypermethylation > normomethylation > controls (Figure 6.7.6).

Patients on thiopurines had higher RBC PG concentrations than patients not on thiopurines and there was a trend for PG in WB and RBCs to be higher in patients on thiopurines. The switch to LDTA in three patients with hypermethylation and hepatotoxicity reduced RBC PG and glutamine concentrations. Again, this suggests a normalisation of the concentrations of these compounds after LDTA. The similarity seen between SAM, PG and glutamine concentrations in response to LDTA, confirms an inter-play between these compounds as hypothesised.

RBC PG concentrations were no different between patients on AZA or MP. Since raised PG is synonymous with reduced glutathione, this finding goes against the hypothesis that glutathione is reduced in the conversion of AZA to MP by GST or via the activity of XO during the conversion of MP to TUA (Figure 1.11);[310] the latter releases superoxidase anions which are reduced by glutathione peroxidase at the expense of a decrease in hepatocyte GSH.[149] Experiments on cultured rat hepatocytes showed that high doses of AZA reduced GSH, and led to the depletion of ATP in mitochondria causing hepatocyte injury and necrosis. However, against the XO hypothesis is that hepatotoxicity is not seen in patients with Lesch-Nyhan Syndrome, where there is excessive formation of uric acid. In the TOPIC study, MP was more likely to cause hepatotoxicity than AZA (22.7 versus 11.9%) and my study also showed that MP use was greater than AZA within the hypermethylators with hepatotoxicity group (see Section 4.3).[288]

The study could be improved with regards to the methodology. Firstly, it is small, although significant differences between compounds were still obtained. Secondly, the timing at which the samples were taken from patients was not well defined. For example, some patients who had hypermethylation with hepatotoxicity initially, had normal LFTs when SAM and SAH were measured. This meant that some patients who were hypermethylators with hepatotoxicity were assigned to the hypermethylator only category. This might explain why concentration differences of compounds between the groups were not as pronounced, as patients had been bled whilst transitioning between the groups. In the future, patients crossing between groups would be excluded. Thirdly, PG was measured with its deuterated standard after three freeze-thaw cycles compared to SAM and SAH, which was measured

after only one. Whilst it is not clear as to the difference this might make, the results are likely to be less accurate, the more freeze thaw cycles occur. The assay was also not assessed for recovery of compounds in plasma, RBC and WB.

Measurement of SAM and PG needs performing in patients with thiopurine hepatotoxicity in the absence of hypermethyation to determine if similar changes in compounds occur. If the changes to the concentrations of these compounds are similar, it would suggest that the mechanism by which hepatotoxicity occurs is the same and vice versa. Knowing this would be crucial to understanding more about the mechanisms of thiopurine hepatotoxicity.

Both plasma SAM and RBC PG concentrations were raised in patients with IBD compared to patients without IBD. Although the patients with IBD were almost all on thiopurines too, further studies are needed to confirm that IBD itself is not a cause of elevated plasma SAM and RBC PG concentrations, as this would reduce their usefulness as a biomarker for abnormal thiopurine metabolism in IBD patients.

The implications of higher concentrations of SAM on the methylation of 6-TG bases, thereby potentially enhancing the cytotoxic effects of thiopurines – for better or worse, also needs further research.[111]

In summary, this study has found that concentrations of plasma SAM, RBC PG and RBC glutamine, increase in patients who abnormally metabolise thiopurine, especially in patients with hypermethylation and hepatotoxicity. Concentrations returned to baseline in those patients commenced on LDTA. PG is thought to be a surrogate marker for reduced glutathione which in turn is found in association with thiopurine hepatotoxicity.[273, 275] Therefore, the compounds measured are potential biomarkers for thiopurine hepatotoxicity and the findings of this study fit with a mechanism proposed by which thiopurine hepatotoxicity might occur (Figure 6.8).

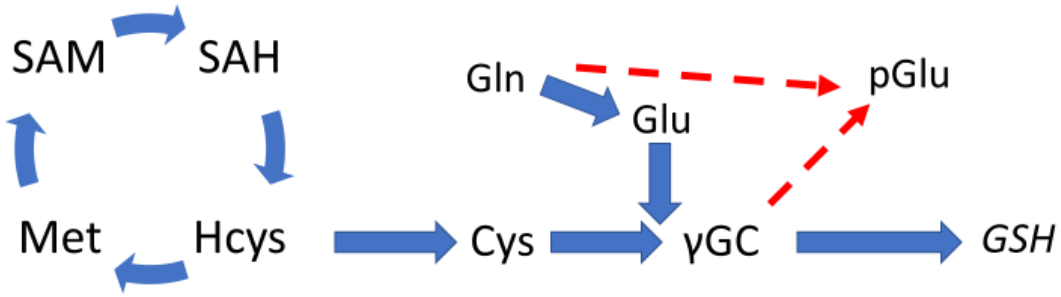


Figure 6.8: The Methionine Cycle and the formation of Cys (Cysteine) and subsequently GSH (glutathione) as a potential mechanism by which changes in concentrations of SAM can cause reduced glutathione. The formation of GSH requires Glu (Glutamate) and Cys to react forming γGC (γ-Glutamyl cysteine). Where Gln (Glutamine) is not being used up to form Glu, it cyclizes to pGlu (pyroglutamate). Likewise, where glutathione is not being produced, γGC will also be converted into pGlu.

One issue with applying this study clinically, is that although the assay is accurate and rapidly reproducible, the current separation and storage techniques of the blood sample, make the use of these compounds, as biomarkers, impractical for day to day clinical use.

In conclusion, this is the first study to investigate the potential role played by SAM in thiopurine metabolism, by measuring the compounds directly. Since, SAM is a well-known cofactor for TPMT, the study is an important step in improving our knowledge of thiopurine metabolism.

Summary of study findings:

- Plasma SAM, RBC glutamine and RBC PG increased in patients on thiopurines
- Plasma SAM, RBC glutamine and RBC PG were highest in hypermethylators with hepatotoxicity
- Plasma SAM was higher in MTHFR variants
- High plasma SAM, RBC glutamine and RBC PG concentrations corrected in patients switched to low dose thiopurine with allopurinol
- Abnormal thiopurine metabolism involves an increase in SAM and glutamine followed by reduced glutathione, implicating the involvement of the Methionine Cycle and Transulfuration Pathway in abnormal thiopurine metabolism and hepatotoxicity

Chapter 7: The use of MRE to diagnose NRH

7.1 Introduction

Thiopurines are a recognised cause of NRH, although some studies have associated thiopurines with a higher prevalence of NRH than others.[165, 167] In practice, screening for NRH in IBD patients, who are on thiopurines, is difficult. Concerns over the diagnosis, results in withdrawal of the drug and a risk of relapse in IBD. The European Crohn's and Colitis Organisation (ECCO) recommend performing yearly MRIs on all patient on TG. Although gadolinium-enhanced MRI has been demonstrated to have reasonable sensitivity and specificity, the gold standard for diagnosis remains a liver biopsy. However, liver biopsies are invasive and carry a 1% risk of bleeding.

The aim was to assess the feasibility of MR elastography alongside primovist-enhanced MRI in patients with biopsy-proven NRH and to compare MRI measurements with controls. The accuracy of fibroscan at diagnosing NRH was also examined. The hypothesis was that MRE and specific sequences such as T1 mapping, R2* and ADC could quantitively diagnose NRH better than fibroscan.

7.2 Patient recruitment and summary of methods

This was a multicentre study involving four sites, to recruit patients with NRH diagnosed on liver biopsies within the last year. Such patients were found using searches of histology databases and approached by the Site Investigator to be part of the study. If the patient agreed to take part in the study, they were met at least 24 hours before any investigations commenced, to take a history, perform an examination and to gain their informed consent. Previous portal pressure and spleen measurements taken at the time of diagnosis were also obtained.

All patients had an FBC and LFTs within a week of MRI scanning. Patients had three consultations: the first to gain their consent to enter the study and examine them, the second to take some blood tests (LFTs and FBC) and perform the MRI scan and the third, to perform the fibroscan. Patients underwent MRI and MRE sequences as described in Section 2.8. The 4 sequences (T1 mapping, R2*, ADC and MRE) were quantitatively analysed and compared against those of healthy controls. Statistical analyses were performed using an independent samples t-test for parametric data and a Mann-Whitney U test for non-parametric data. The SD for the MRE parameters is expressed as root mean square (RMS).

Research ethics permission was obtained and assigned the reference 15/L0/138. Each centre (GSTT, King's College Hospital, Royal Sussex County Hospital and The Royal Free Hospital) required separate agreement from their individual Research and Development committees.

Although the study opened in September 2015 and was due to close in August 2016, it was extended by a year due to poor recruitment and to allow time for recruitment from the Royal Free Hospital which was not an initial recruitment site.

7.3 The cohort

In total, six patients (four males, median age 55, range 47 -74 years) were recruited to this study over a two-year period along with five controls (five males, median age 35, range 35 -37 years) as the comparator. All patients had a liver biopsy confirming NRH with minimal or no fibrosis within the last year.

Patient 1:

A 47-year old male with ileocolonic CD and perianal disease since 1994. He had had a right hemicolectomy 20 years ago with a temporary ileostomy in 2015. He continued to have active inflammation at his anastomosis. His treatment at the time of enrolment was infliximab 10 mg/kg, 8 weekly, which he had been on since 2012 (although initially started at 5 mg/kg and then dose-escalated in 2015). He had been on AZA in 2012 which was stopped after one year due to thrombocytopenia. He had no other medical problems. He is an artist and denied any alcohol intake or smoking. Investigation for his persistent thrombocytopenia, included an MRCP in 2015 which showed a normal liver but periportal oedema and 13.3 cm splenomegaly. He started to develop ascites in early 2016 and therefore had a transjugular liver biopsy with portal pressure measurements; the liver biopsy confirmed NRH. On examination, he had a midline laparotomy scars and mild pedal oedema. There was 1-2 cm splenomegaly. His weight was 73.3 kg.

Patient 2:

A 47-year old female with HIV diagnosed in 2005 and complicated by tuberculosis in 2006 and cryptococcal meningitis in 2008, which presented as seizures. She had been commenced on didanosine (DDI) in 2005, a purine analogue and a reverse transcriptase inhibitor used for its reduced likelihood of provoking seizures. She had been on this for four years through to 2009, when she was found at OGD to have grade 1 varices. In 2012, she developed ascites and in 2016 found to have grade

2 varices which were banded. An ultrasound showed a nodular liver with 14.5 cm splenomegaly, patent portal vein and no ascites. She then proceeded to liver biopsy which confirmed NRH secondary to previous didanosine. At the time of enrolment, she was also taking warfarin, kaletra, kivexa, vitamin D supplements, Epilim, spironolactone and propranolol. She had also been on emtricitabine from 2005 to 2009. She denied smoking and rarely drank alcohol. Examination was entirely normal. Her weight was 80 kg.

Patient 3:

A 57-year old male with CD diagnosed 22 years ago with a previous right hemicolectomy and primary anastomosis at diagnosis. Post-operatively he was treated with AZA for a year which was then switched to mesalazines for a few years and then stopped; he has been in remission since. In 2015, he presented with dysphagia and found at OGD to not only have oesophagitis, but also oesophageal varices which were banded. He had further investigations for portal hypertension until a liver biopsy in July 2016 confirmed NRH. His only medications at enrolment were propranolol and omeprazole. He denied smoking and consumed 12 units of alcohol weekly. On examination, he had a midline laparotomy scar with no stigmata of chronic liver disease. His weight was 92 kg.

Patient 4:

A 74-year old female with common variable immunodeficiency (CVID) and granulomatous lung disease with elevated angiotensin converting enzyme levels since 1996 but no formal diagnosis of sarcoidosis. Two years ago, she developed abnormal LFTs and thrombocytopenia. An OGD in 2015 showed oesophageal varices. She underwent a liver biopsy in December 2015 which confirmed NRH. Otherwise, her past medical history includes osteoporosis and type 1 diastolic dysfunction. At the time of enrolment, she was on regular infusions of Privigen (immunoglobulins), azithromycin, omeprazole, ramipril and vitamin D supplements. She rarely drank alcohol and never smokes. On examination, she had 2 cm of splenomegaly but no ascites. Her weight was 53 kg.

Patient 5:

A 55-year old man with CVID diagnosed in 2011 and a background of autoimmune haemolytic anaemia. He had a family history of Evans syndrome. He was diagnosed initially in 2013 with NRH on a liver biopsy but he had remained stable until an episode of spontaneous bacterial peritonitis caused liver decompensation and deterioration of his AIHA in January 2016 requiring treatment with rituximab. A transjugular liver biopsy in March 2016 confirmed the diagnosis of NRH. Otherwise, he has mild heart failure and was norovirus colonized. His medications include Intratect infusions

(immunoglobulins), prednisolone, alendronic acid, vitamin D and folic acid supplements. On examination, there were no stigmata of chronic liver disease. His weight was 79.4 kg.

Patient 6:

A 53-year old male diagnosed with ileal CD in 2013 and treated with infliximab 5 mg/kg 8 weekly through to 2016 and concomitant AZA between January 2015 and January 2017. He developed persistently abnormal LFTs with thrombocytopenia in 2017. A liver screen was normal. An US showed splenomegaly and he underwent transjugular liver biopsy in 2017 which confirmed NRH in the absence of fibrosis. He had no other medical problems. His medications at enrolment were Pentasa only. He smoked 20 cigarettes per day and drinks alcohol rarely, admitting to around 30-40 units weekly up to his diagnosis of CD. On examination, there were no stigmata of chronic liver disease. His weight was 89.9 kg.

Characteristics of the patients recruited are shown in Table 7.3.

All 5 controls were healthy with no relevant medical problems. One has chronic immune thrombocytopenia with no splenomegaly and is not on treatment. Another has IgA nephropathy in remission.

Table 7.3: Characteristics of the patients recruited. Platelet count, ALT and ALP are those values taken on the day of or very close to the date of the MRE scan.

	Age	Disease	Spleen size(cm)	Thiopurines (Last dose)	HVPG (mmHg)	Platelets ($x10^9$/L)	ALT (IU/L)	ALP (IU/L)	Fibroscan MLS (IQR)
1	47	CD	13.3	AZA (2013)	8	138	32	158	9.2 (1.2)
2	47	HIV	14.5	DDI (2009)	8	79	19	112	4.1 (0.2)
3	57	CD	12.8	AZA (1995)	Not done	120	19	64	4.8 (0.6)
4	74	CVID	21	None	10	84	31	211	12 (1.1)
5	55	CVID	18	None	13	90	92	249	Not done
6	53	CD	16	Aza (2017)	6	104	273	172	5.1 (0.9)

HVPG: Hepatic venous pressure gradient; mmHg: millimetres of mercury; MLS: Mean liver stiffness (measured is kilopascals): MLS; IQR: Interquartile range.

7.4 Results

Data was first compared between three small ROIs in the three MRI sequences between patients (18 ROIs in total) and controls (15 ROIs in total, except for in the ADC sequences where ROIs were only measured in 9). The mean intensities of the pixels were normally distributed. An independent samples t-test showed no significant differences in mean pixel intensity (Table 7.4.1).

Table 7.4.1: Comparing the mean pixel intensities across three sequences between patients and controls for all small ROIs.

Sequence	Group	ROIs	Mean pixel intensity	SD	P-value‡
T1	Patients	18	827	187	0.5
	Controls	15	866	122	
R2*	Patients	18	31.0	11.4	0.1
	Controls	15	37.0	7.9	
ADC	Patients	18	1144	162	0.09
	Controls	9	1269	194	

‡Independent samples t-test.

Mean pixel intensity was then compared between the whole ROI in the three different sequences between patients and controls. As these were non-parametric, a Mann-Whitney U-test was used to assess for significant differences (Table 7.4.2).

Table 7.4.2: Comparing the mean pixel intensities across 3 sequences between patients and controls for whole ROIs.

Sequence	Group	ROIs	Mean pixel intensity	SD	P-value≠
T1	Patients	6	907	188	0.5
	Controls	5	923	132	
R2*	Patients	6	29.4	11.4	0.2
	Controls	5	37.4	6.1	
ADC	Patients	6	1280	159	0.7
	Controls	3	1290	131	

≠ Mann-Whitney U-test.

Mechanical vibrations showed good wave penetration for all the patients except for Patient 1. This was most likely due to his ascites. The in-house software provided data on non-linearity, attenuation and velocity based on the viscoelastic parametric maps. All small ROIs (top, middle and bottom) were compared for mean differences in pixel non-linearity, velocity and attenuation and there were no significant differences. When combining ROIs, the data was larger and therefore normally distributed. Comparisons were made between patients, controls and nodules. There were still no significant differences when comparing patients and controls (Table 7.4.3). However, the non-linearity and attenuation was significantly higher in the nodules than in the controls (mean non-linearity was 42.2 (SD 0.90) in nodules versus 18.3 in controls (SD 9.5), P = 0.015, Mann-Whitney U-test) (Mean attenuation was 0.06 (SD 0.03) in nodules versus 0.04 (SD 0.01) in controls, P = 0.02, Mann-Whitney U-test). Mean shear wave velocity differences were approaching significance (P = 0.09, Mann-Whitney U-test). All were significantly different when comparing nodules and the patients themselves (Table 7.4.4). One of the two patients with nodules had a significantly higher pixel intensity in T1 mapping when compared to all other patients and controls. The nodules of both patients had significantly higher non-linearity, lower velocity and higher attenuation than controls.

Table 7.4.3: Comparing non-linearity, attenuation (α, mm^{-1}) and velocity (C, m/s) between patients and controls of small ROIs.

MRE	Patient/Control	ROIs	Mean	SD	P-value‡
Non-linearity	Patient	18	17.9	5.94	0.9
	Control	15	18.2	9.49	
Attenuation	Patient	18	0.04	0.009	0.6
	Control	15	0.04	0.009	
Velocity	Patient	18	1.14	0.12	0.1
	Control	15	1.08	0.09	

‡Independent samples t-test.

Table 7.4.4: Comparing non-linearity, attenuation (α, mm^{-1}) and velocity (C, m/s) between patients and nodules.

	Patients/Nodule	ROIs	Mean	SD	P-value≠
Non-Linearity	Patients	18	17.9	5.9	P =0.01
	Nodules	2	42.4	0.9	
Attenuation	Patients	18	0.04	0.009	P =0.01
	Nodules	2	0.06	0.003	
Velocity	Patients	18	1.14	0.12	P =0.02
	Nodules	2	0.92	0.08	

≠Mann-Whitney U-test.

Figures 7.4.1 and 7.4.2 show no pattern to the attenuation and velocity of whole and small ROI measured in patients and controls.

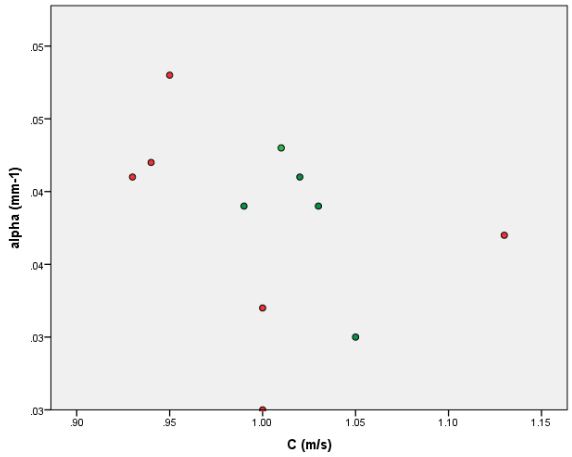


Figure 7.4.1: A scatter graph plotting MRE measured shear wave attenuation α against the MRE shear wave velocity C in the whole ROIs of both patients and controls (n=11).

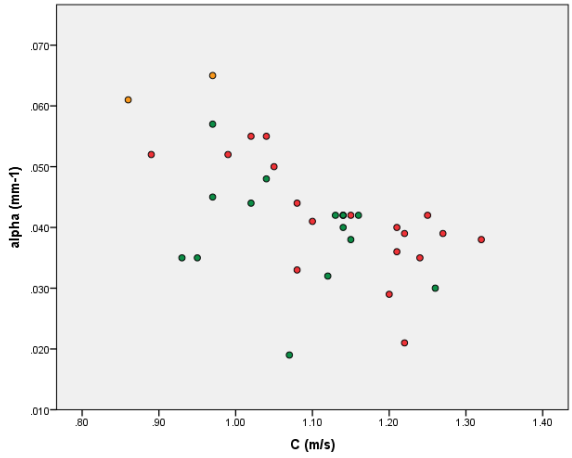


Figure 7.4.2: A scatter graph plotting MRE measured shear wave attenuation α against the MRE shear wave velocity C in all small ROIs of both patients (red dots), nodules (orange dots) and controls (green dots) (n=35).

The data was then assessed for differences in heterogeneity between patients and controls in case NRH presents itself more by the heterogeneity of the liver architecture rather than discrete areas of increased liver stiffness. To assess for this, the SD of the data were analysed (the SD for MRE parameters is expressed as root mean square (RMS)). When comparing separate sequence and ROI SDs (non-parametric), none were significantly different between patients and controls (Table 7.4.5). When combining all ROI SDs for different sequences, there were no significant differences between patient and controls for T1, R2* or ADC mapping sequences (Table 7.4.6). Similarly, there were no significant differences when comparing the range of pixel intensities between the three sequences (Table 7.4.7).

Table 7.4.5: Comparing the means of the RMS for non-linearity, attenuation (α, mm^{-1}) and velocity (C, m/s) between patients and controls in both whole and small ROIs.

MRE	Patients/Controls	ROIs	Mean of RMS	SD	P-value‡
Non-linearity	Patients	24	10	4.7	0.6
	Controls	20	10.7	4.8	
Attenuation	Patients	24	0.02	0.02	0.8
	Controls	20	0.03	0.03	
Velocity	Patients	24	0.18	0.09	0.1
	Controls	12	0.22	0.07	

‡Independent samples t-test.

Table 7.4.6: Comparing the mean of the SD between patients and controls in the 3 sequences for both whole and small ROIs

Sequence	Patients/Controls	ROIs	Mean of SD	SD	P-value‡
T1	Patients	24	173	106	0.2
	Controls	20	132	79.4	
R2*	Patients	24	15.4	5.7	0.3
	Controls	20	13.9	3.9	
ADC	Patients	24	206	96.2	0.2
	Controls	12	241	83.7	

‡Independent samples t-test.

Table 7.4.7: Comparing the mean of the range differences between patients and controls in the three sequences for both whole and small ROIs.

Sequence	Patients/Controls	ROIs	Mean of range difference	SD	P-value‡
T1	Patients	24	1490	1150	0.4
	Controls	20	1230	1030	
R2*	Patients	24	117	96.3	0.2
	Controls	20	87.2	48.2	
ADC	Patients	24	1130	592	0.2
	Controls	12	1360	556	

‡Independent samples t-test.

7.5 Discussion

The data obtained from the MRE scan analysis was consistent between patients and controls. The results were also similar to where these parameters have been measured in other organs (e.g kidneys).[311] This suggests that as an imaging modality, MRE is both accurate and reproducible. The MRI sequences (T1, R2* & ADC) and MRE were, however, unable to discriminate between patients and controls and therefore, will not diagnose NRH. There was also no significant difference in heterogeneity between patients and controls. The scatter graphs (Figure 7.4.1 – 7.4.2) show that even when combining both the MRE shear wave attenuation and the velocity together, they were unable to discriminate between patients and controls. However, where there were nodules, there were significant differences found between both the MRI sequences and MRE parameters. The nodules had significantly higher MRE shear wave attenuation and significantly lower MRE shear wave velocity than in both patients and controls.

Patient 3 was an outlier on the scatter graph (Figure 7.4.1). It is possible that increasing the number of patients and controls, that the scatter graphs might have been more useful at discriminating between them. However, when the number of cases was increased, as in Figure 7.4.2, there was still no discrimination possible.

Fibroscan readings were raised in one patient, equivocal in another and normal in three patients. Therefore, fibroscans were not able to diagnose NRH either.

Previous studies have shown that MRE is able to diagnose hepatic fibrosis.[285] Therefore, MRE may still be helpful in differentiating patients with portal hypertension in cirrhosis compared to non-cirrhotic portal hypertension.

Overall, the study was affected by difficulties in recruiting patients. This emphasises the need for multicentre collaboration when it comes to researching rare diseases. There was only one patient with IBD diagnosed with NRH from two large IBD centres (King's College Hospital and GSTT) over the period relevant to the study. This either suggests that NRH is rarely a problem for IBD patients on thiopurines or that we are not diagnosing it appropriately.

Even though NRH is rare, it is still important to be vigilant when it comes to monitoring IBD patients on thiopurines, especially TG. All the patients recruited were thrombocytopenic and four out of six had abnormal LFTs. Splenomegaly occurred in four out of six. Any of these findings should prompt further investigations, a strategy for which will be discussed in Chapter 8.

Summary of study findings:

- Fibroscans were not able to diagnose NRH
- MRI using primovist and MRE were not able to differentiate between NRH and normal liver in healthy controls
- NRH should be considered as the cause of abnormal LFTs and/or thrombocytopenia in patients on thiopurines
- Liver biopsies with portal pressure measurement remain the best way to diagnose NRH

Chapter 8: Conclusions and future work

8.1 Summary of findings

Liver inflammation accounts for a significant burden of disease for patients with IBD (30%). The causes are mostly thiopurine-related or due to flares of disease. However, IBD patients also have an inherent risk of developing PSC and patients with CD have been found to be more likely to suffer from fatty liver and biliary stones.[39] In this chapter, I will summarise the main findings of the thesis and then address each of the main aims with what was achieved. Lastly, ideas for future work will be discussed.

The first aim of this thesis was to assess the burden of liver inflammation in our own cohort of IBD patients and, based on this, propose a systematic approach by which abnormal LFTs can be investigated to determine the cause most efficiently (see Section 8.2: A model for managing liver inflammation in IBD). The second aim was to determine both the genetic and biochemical reasons for why some patients get hepatotoxicity from thiopurines, whilst others do not (see Section 8.3: The potential mechanisms for thiopurine hepatotoxicity). This would allow clinicians to predict the patients most at risk (see Section 8.4: A model for predicting thiopurine hepatotoxicity). The final aim of the thesis was to develop a screening tool for NRH, thereby avoiding the need to biopsy the liver (see Section 8.5: Investigating for NRH). Figure 8.1 summarises the main research findings across all studies.

Summary of main research findings:

- Two thirds of IBD patients with abnormal LFTs had CD and a third had UC - therefore, abnormal LFTs are more common in patients with CD.
- Inflammation is mostly hepatocellular, except in biliary diseases such as PSC or choledocholithiaisis, where inflammation is cholestatic.
- Abnormal LFTs account for low mortality in IBD. There is, therefore, the time to investigate patients using a strategic approach.
- One third of abnormal LFTs are due to thiopurines, one third due to disease flares and one third due to other causes. (PSC accounts for 7% of other causes).
- Flares of disease are a common cause of abnormal LFTs, affecting patients with CD more than patients with UC (71% versus 28%).
- 60% of thiopurine hepatotoxicity occurs with hypermethylation whilst 40% occurs with normomethylation. Although still hepatocellular, hepatotoxicity in normomethylators has a significantly higher ALP than hepatotoxicity with hypermethylation, suggesting a cholestatic component to this type of hepatotoxicity. There were also significant differences in the number of MTHFR variants between each type. These findings suggest that there are likely to be two different mechanisms for thiopurine hepatotoxicity.
- Thiopurine hepatotoxicity occurs after a median period of around 92 days in hypermethylators compared to 42 days with normomethylators- this suggests a more immunoallergic mechanism for hepatotoxicity in the normomethylators.
- Thiopurine hepatotoxicity is more common in:
  - -Hypermethylators, especially with higher MeMP and MeMP:TGN ratios
  - -Heavier patients and older patients
  - -Non-smokers
  - -Patients with UC
  - -Patients with a fatty liver on ultrasound
  - -CYP1A2*F variants in hypermethylators
  - -MTHFR 359+160 variants
- Thiopurine hepatotoxicity does not correlate with TPMT.
- Switching to LDTA is effective at normalising LFTs in over 85% of hepatotoxicity with normomethylation and in over 95% of hepatotoxicity with hypermethylation. A minority of patients continue to get hepatotoxicity and therefore, all patients switched to LDTA should continue to be monitored.
- Reducing the dose of the thiopurine was 100% effective for both types of hepatotoxicity although this was based on a smaller sample size.
- Smokers were less likely to be hypermethylators than non-smokers.
- Plasma SAM and PG concentrations are raised in patients on thiopurines and increase further in hypermethylators and in patients with hypermethylation and hepatotoxicity. PG and SAM concentrations return to baseline after switching to LDTA.
- MRE was not able to diagnose NRH – however, based on the results of previous studies, it could differentiate between NRH and portal hypertension caused by cirrhosis.

Figure 8.1: Summary of main research findings.

8.2 A model for managing liver inflammation in IBD

A third of the population have been shown to have at least one abnormal LFT with less than 4% having significant liver disease.[312] The Birmingham and Lambeth Liver Evaluation Test Strategy (BALLETS) study, a prospective analysis of patients with abnormal LFTs in Primary Care, found that only 5% had abnormal liver screens, including autoimmune, viral and metal storage blood tests. Yet there are also concerns that 50% of patients with end stage liver disease miss being detected early and so the British Society of Gastroenterology Guidelines recommend full investigation of the underlying aetiology at first detection.[313] Therefore, there exists a dichotomy between the unnecessary cost of extensive liver screens versus the cost of missing potentially unwell liver patients.

In Chapter 3, a large cohort of IBD patients with abnormal LFTs (assumed to represent a third of the IBD population) were analysed and the cause of liver inflammation assigned to specific disease processes: Flares, thiopurines, PSC etc. Two thirds of the cohort had abnormal LFTs unrelated to an underlying chronic liver disease, 90% of which settled within 4 months (2.7 months where the cause was thiopurines and 3.7 months where the cause was flares of disease). This is as opposed to those patients in the BALLETS study where 75% of patients continued to have abnormal LFTs 2 year later. Radiology and liver screens diagnosed the cause of liver disease in 20% of my cohort (66/329) and bloods test alone in only 3.6% (12/329). Based on the uniqueness of abnormal LFTs in IBD, I support an investigative strategy with a more judicial use of liver screens based on the clinical context, which prioritises the more intensive investigations for those patients at risk of liver failure (Figure 8.2).

The following case illustrates how the findings from this thesis can be used to manage patients with IBD and abnormal LFTs more effectively:

A 45-year old with biopsy-proven ulcerative pancolitis since 2011 attended clinic for consultation following a colonoscopy, which had shown active disease throughout the colon. He reported five times daily bowel frequency with rectal bleeding. He had been on 200 mg AZA since his diagnosis as well as asacol 2.4 g. LFTs were found to be elevated (ALT 150 IU/L, ALP 200 IU/L but bilirubin was 15 µmol/L). His LFTs six months ago were all normal. On examination, his weight was 100 kg, observations were all normal and his abdominal examination was unremarkable.

The potential causes for his abnormal LFTs are as follows:

- The AZA – this could be either be causing hepatotoxicity directly or through the development of NRH.
- The flare or activity of his disease – this was shown to be a major contributory factor in causing abnormal LFTs in our cohort of patients.
- PSC – affecting 7% of all patients with abnormal LFTs, this is even more common in patients with ulcerative pancolitis.
- Asacol – hepatotoxicity from mesalazines affects 2% of patients.
- NAFLD is a growing cause of liver disease in developed countries and patients with IBD are more at risk than the general population.
- Other causes of liver disease such as: choledocholithiasis, haemochromatosis, IgG4 disease, viral hepatitis or alcoholic liver disease.

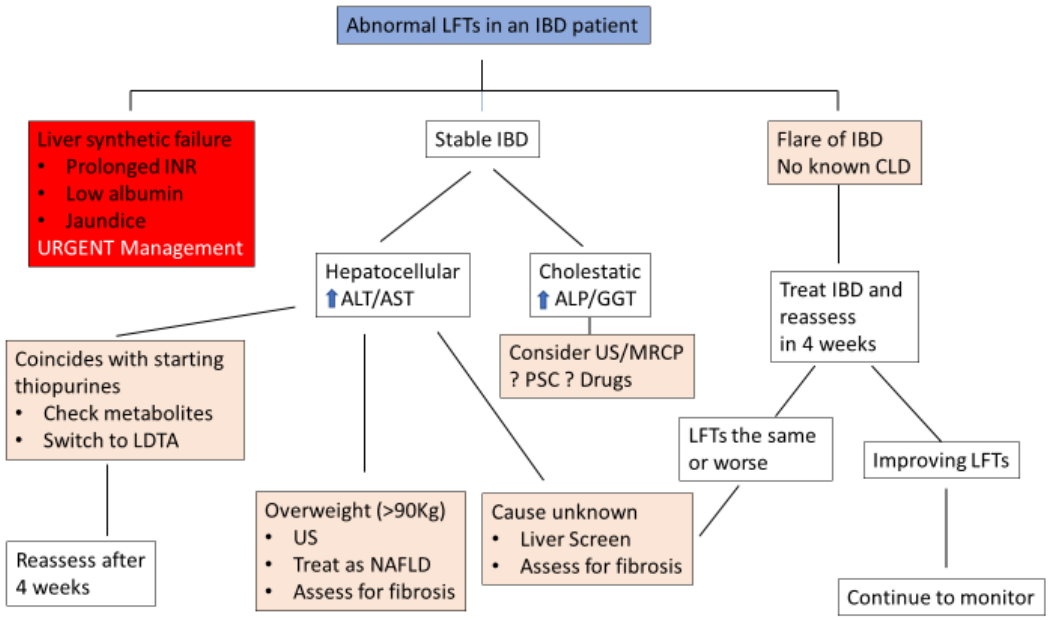


Figure 8.2: A decision tree to guide clinicians on how to investigate abnormal LFTs in IBD patients. It assumes that a history and examination has taken place and that common causes of abnormal LFTs, such as alcohol, have been excluded. Where there are signs of liver failure, these patients should undergo intensive investigation and management. However, the investigation of abnormal LFTs, in the absence of liver failure, should undergo a systematic approach. When the diagnosis is confirmed, patients should be assessed for fibrosis by either a FIB-4 score, a fibroscan or liver biopsy. INR: International normalised ratio; CLD: Chronic liver disease; US: Ultrasound; PSC, Primary sclerosing cholangitis; MRCP: Magnetic resonance cholangiopancreatography; LDTA: Low dose thiopurine with allopurinol; NAFLD: Non-alcoholic fatty liver disease.

The clinician first decides to treat the flare of disease with a course of oral steroids and plans to repeat the blood tests along with thiopurine metabolites (TGN and MeMP) in 4 weeks. In the meantime, asacol is withheld. 4 weeks later, his symptoms have resolved completely, but LFTs remain as elevated. Thiopurine metabolites return within the therapeutic range (TGN 350 pmol/L, MeMP 1250 pmol/L, MeMP:TGN 3.5). The LFTs are predominantly hepatocellular, so biliary disease is less likely and so an ultrasound of the liver and an MRCP are not requested. The normal metabolites do not exclude the fact that this could be thiopurine-related. Indeed, 40% of patients with hepatotoxicity in this thesis had normal metabolites. The fact that hepatotoxicity has occurred after many years of AZA treatment does not exclude the fact that the hepatotoxicity is thiopurine-related. The patient is switched to LDTA (50 mg AZA, 100 mg allopurinol). The LFTs and metabolites are rechecked and are still abnormal. Given the findings that 85% of patients who were hepatotoxicity only, normalised their LFTs with LDTA, the clinician decides to stop the AZA completely; a month later, LFTs remain abnormal. Our cohort study suggested that the mean time to normalisation of LFTs following stopping thiopurines is 2.7 months. Therefore, the clinician continues to monitor for improvement, but also requests an ultrasound scan of the liver and a liver screen to exclude a chronic liver disease or biliary stones. A month later the results return; the ultrasound scan and liver screen are normal for the liver, other than the spleen being enlarged at 16 mm.

At this point the patient has worsening bowel frequency and rectal bleeding due to a flare of his colitis, having stopped all treatment. The clinician considers either restarting the AZA or escalating to a biologic. The cause of abnormal LFTs remains undiagnosed and therefore the patient is at risk of liver fibrosis and liver failure if the aetiology is not corrected. NRH is considered as a potential diagnosis, as this is associated with long-term thiopurine use.[165] Indeed, on closer inspection of his blood tests, it is noted that his platelet count has been slowly dropping and is now 96 x 10^9/L. He is referred to the Liver and Haematology teams for their opinion. The Haematologists find larger-sized platelets on the blood film and the Liver team recommend performing an oesophageal-gastro-duodenoscopy (OGD). This revealed the presence of portal hypertensive gastropathy and grade 1 oesophageal varices. A transjugular liver biopsy with portal pressure measurements is performed. The histology returns with findings in keeping with NRH and the pressure measurements are mildly elevated at 10 mmHg suggesting a sinusoidal lesion. The case highlights the potential differentials and complexities of abnormal LFTs in IBD and how my research findings can be used to reach a diagnosis systematically. Although NRH is rare, the presence of thrombocytopenia, or the absence of other causes of CLD, should prompt the clinician to investigate for NRH whether it be through a liver biopsy or MRI.

8.3 The potential mechanisms for thiopurine hepatotoxicity

The existence of biochemical and genetic markers of thiopurine hepatotoxicity would have been useful in managing the case above. In this thesis, I have found that compounds of the Methionine Cycle, namely RBC PG and plasma SAM, are potential biomarkers and that CYP1A2*F and MTHFR variants are potential genetic markers of thiopurine hepatotoxicity.

I hypothesised that the Methionine Cycle was the mechanism through which thiopurine hepatotoxicity occurs because of the close association between SAM and TPMT in thiopurine metabolism. Indeed, plasma SAM concentrations were raised in patients with thiopurine hypermethylation and hepatotoxicity, as were RBC PG concentrations, which correlate with reduced glutathione, a marker of hepatotoxicity in many studies. The switch to LDTA dramatically altered the concentration of these compounds with RBC PG and glutamine concentrations returning to their baseline. My hypothesis is also supported by my candidate gene analysis where MTHFR variants had significantly higher plasma SAM concentrations and were more common in patients with thiopurine hepatotoxicity.

I also hypothesised that hepatotoxicity arises as a result of the release of formaldehyde in the demethylation of MeMP to MP by CYP1A2 enzymes. Formaldehyde caused toxicity in rat livers through the depletion of glutathione resulting in hepatocyte mitochondrial damage.[150, 151] The demethylation of drug metabolites by CYP450 has also been proven to release formaldehyde in hepatocyte mitochondria.[152] This hypothesis would explain the correlation between hypermethylation and hepatotoxicity at higher concentrations of MeMP, as more substrate is available to demethylate. The hypothesis is supported by the finding that CYP1A2*F variants, a phenotype associated with ultra-rapid demethylation, were more likely to develop hepatotoxicity in hypermethylators (Table 8.3 and Chapter 4). Hepatotoxicity would be more likely, therefore, to occur in smokers in whom the CYP1A2*F variant has been shown to be induced. However, whilst MeMP was found to be lower in smokers, there were no differences for hepatotoxicity and, if anything, hepatotoxicity (and hypermethylation) was found to be higher in non-smokers in Chapter 4, although this may be due to methodology of the study.

Table 8.3: The hypothesis for the effects of CYP1A2 variants on formaldehyde release through demethylation.

CYP450 variants	Demethylation of MeMP	Formaldehyde release	Effects on the liver
CYP1A2*C	Decreased	Decreased	Less hepatotoxicity
CYP1A2*F	Increased	Increased	More hepatotoxicity

Perhaps coincidentally, the release of formaldehyde during demethylation is enhanced by high concentrations of S-methyl donor compounds, such as SAM.[152] The two mechanisms I have hypothesised as causing thiopurine hepatotoxicity may not be independent of each other and explain why the switch to LDTA resolves over 80% of thiopurine hepatotoxicity (Figure 8.3).

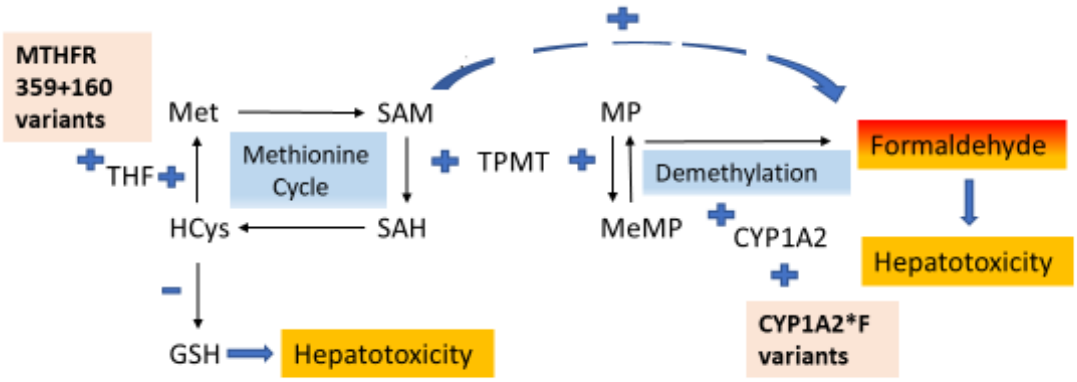


Figure 8.3: The two mechanisms for thiopurine hepatotoxicity: one via the Methionine Cycle and reduced glutathione and the other CYP1A2 mediated demethylation and the release of formaldehyde. MTHFR and CYP1A2*F variants have been shown to make hepatotoxicity more likely. SAM may enhance the release of formaldehyde. MP: mercaptopurine; MeMP: methylmercaptopurine; SAM: S-adenosylmethionine; TPMT: thiopurine methyltransferase; SAH: S-adenosyl homocysteine; HCys: Homocysteine; Met: Methionine; THF: Tetrahydrofolate; MTHFR: Methytetrahydrofolate reductase; GSH: Glutathione synthetase.

8.4 A model for predicting thiopurine hepatotoxicity

From my research, patients who were heavier or who were older, were more likely to develop thiopurine hepatotoxicity if they were hypermethylators. Similarly, a fatty liver on ultrasound due to NAFLD or alcoholic liver disease, gives patients a higher risk of hepatotoxicity from thiopurines. Indeed, over half of those patients with thiopurine hepatotoxicity had a fatty liver on ultrasound. This suggests that a "double-hit" from two or more factors (e.g fatty liver plus thiopurine or alcohol plus thiopurine) may increase the risk of hepatotoxicity. The multifactorial nature of this "double-hit" hypothesis explains why it is so difficult to predict thiopurine hepatotoxicity through genetics alone.

If the "double-hit" theory is true, then patients with active IBD or flares would be more likely to develop hepatotoxicity than patients in remission. However, thiopurines are more likely to be started in patients with active disease. Bastida et al concluded that concomitant steroids were risk factors for hepatotoxicity, possibly due to an increase in fatty liver-related insulin resistance.[287] Below are the factors that predict thiopurine hepatotoxicity in a univariate analysis. Table 8.4.1 shows odds radios for the risk factors which predict both types of thiopurine hepatotoxicity. Table 8.4.2 shows the odds ratios for risk factors which predict thiopurine hepatotoxicity in hypermethylators only.

Table 8.4.1: Odds ratios (OR) and statistical significance of variables predicting hepatotoxicity as calculated according to Altman, 1991 (www.medcalc.org/calc/odds_ratio.php) in univariate analysis.

Variable	Hepatotoxicity (N=92)/Total N	OR	95% CI	P-value
Age at starting thiopurine > 35	>35 = 54/113 < 35 = 38/153	2.8	1.6-4.7	P<0.001
UC	UC = 37/84 CD = 51/174	1.9	1.1-3.3	P=0.02
Non-smoking	Non-smoking =78/182 Smoking= 14/84	3.8	1.9-7.1	P<0.001
Hypermethylating	Hypermethylating=36/97 Non-Hypermethylating=31/144	2.2	1.2-3.8	P<0.009
Weight > 75 kg	>75 = 48/105 <75 = 40/152	2.4	1.4-4.0	P=0.001
Thiopurine type	AZA = 66/219 MP = 26/47	1.9	1.1-3.2	P=0.03

Table 8.4.2: Odds ratios (OR) and statistical significance of variables predicting hepatotoxicity in patients who are hypermethylators as calculated according to Altman, 1991 (www.medcalc.org/calc/odds_ratio.php) in univariate analysis.

Variable	Hepatotoxicity (N=36)	OR	95% CI	P-value
Age at starting thiopurine > 35	>35 = 20/36 <35 = 16/61	3.5	1.5-8.4	0.005
Weight > 75 kg	>75 = 21/43 <75 = 15/52	2.4	1.0-5.5	0.048
MeMP > 8000	>8000 = 19/38 <8000 = 17/58	2.4	1.0-5.7	0.043

In a univariate analysis, age, UC, non-smoking, hypermethylation, weight and thiopurine type were all predictors of hepatotoxicity. All of these except UC were predictors in a multivariate analysis (Table 4.6.1).

For hypermethylators, the only predictors for hepatotoxicity were age, weight and MeMP in a univariate analysis. These findings suggest that extreme hypermethylates who are overweight and older are more at increased risk of thiopurine hepatotoxicity.

Added to this are the risks associated with certain genotypic variants. For example, the OR for thiopurine hepatotoxicity from MTHFR variant 359+160 T>C is 1.94 (95% CI 1.1-3.5). This means that patients with this variant might have up to a 3.5 x risk of hepatotoxicity than wild types. Similarly, hypermethylators could be up to 6 x more likely to get hepatotoxicity if they have a variant for CYP1A2*F 163C>4 (OR 2.7, 95% CI 1.1-6.4); another “double hit” hypothesis. The effects of this variant may further be inducible by smoking or high caffeine consumption.

We can also hypothesise the OR associated with certain thresholds of plasma SAM and RBC PG concentrations (Table 8.4.3). Figure 8.4 shows how receiver operative curves (ROC) can be used to assess the use of SAM and PG concentrations to predict abnormal thiopurine metabolism. Plasma SAM concentrations are a poor predictor of abnormal thiopurine metabolism whilst RBC PG concentrations are a fair predictor of abnormal metabolism.

Table 8.4.3: The OR, CI and P-values of thiopurine hepatotoxicity according to a randomly chosen threshold of 100 µmol/L for plasma SAM and 165 µmol/L for RBC PG in the 12 patients (six with hepatotoxicity, six without).

Variable	Hepatotoxicity or non-hepatotoxicity (n=12)	OR	95% CI	P-value
Plasma SAM > 100 µmol/L	<100 =3/6 >100 =3/6	1.0	0.1-9.6	1.00
RBC PG >165 µmol/L	<165 = 2/7 >165 = 4/5	10.0	0.7-154	0.10

Table 8.4.4: The sensitivities and specificities of randomly chosen PG concentrations to predict hepatotoxicity in patients on thiopurines.

RBC PG levels µmol/L	True Positives for hepatotoxicity	False Positives for hepatotoxicity	Sensitivity	Specificity
129	5/6= 0.8	4/6 =0.7	0.8	0.3
165	4/6 = 0.7	1/6 = 0.2	0.7	0.8
193	1/6 = 0.2	1/6 = 0.2	0.2	0.8

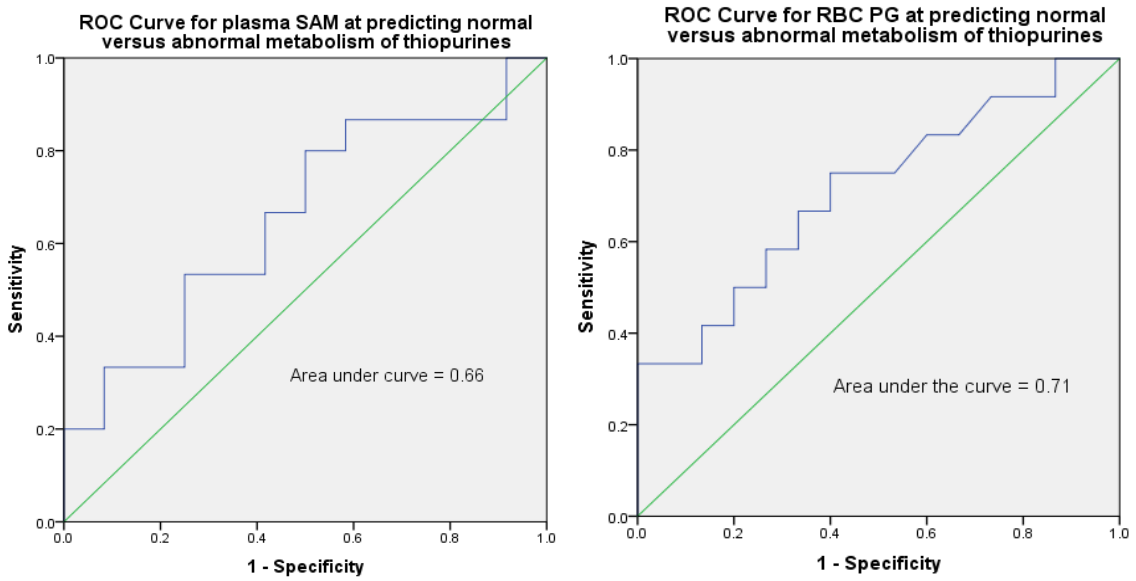


Figure 8.4: Receiver operator characteristic (ROC) curves for plasma SAM and RBC PG at predicting abnormal metabolism of thiopurines (hypermethylation or hypermethylation with hepatotoxicity). Area under the curves (AUC) suggest that plasma SAM concentrations are a poor predictor of abnormal thiopurine metabolism whilst RBC PG concentrations are a fair predictor of abnormal metabolism (http://gim.unmc.edu/dxtests/roc2.htm).

In summary, although genes are important, they should be interpreted alongside other risk factors for thiopurine hepatotoxicity such as age, smoking, IBD-type, weight and coexistent liver problems. SAM and PG concentrations may increase in some patients more than others and could be used as an early indicator of future thiopurine hepatotoxicity once a patient has been started on a thiopurine. A combination of MTHFR variants leading to higher plasma SAM and reduced glutathione, hypermethylation as a result of higher SAM, and then CYP1A2*F variants with the release of formaldehyde through demethylation, could be the ideal combination needed to cause thiopurine hepatotoxicity.

8.5 Investigating for NRH

MRE failed to detect NRH in my patient cohort. A guide for how patients with suspected NRH is suggested in Figure 8.5. Generally, NRH should be considered in two main situations:

1) If a patient develops thrombocytopenia of unknown cause or splenomegaly on US with a history of thiopurine use.
2) If a patient develops abnormal LFTs where the cause remains unknown with a history of thiopurine use.

Of the cohort of patients with abnormal LFTs and IBD, 18 patients had unexplained thrombocytopenia (range 84-148 x 10^9) at some point following treatment with thiopurines over the last 10 years. Of these, one patient had a platelet count <100 x 10^9/L. Whilst most of the patients have now normalised their platelet counts, three remain low. Of these three, two had normal MRIs and continued on thiopurines, but one had unexplained splenomegaly and has stopped. There are two other patients with a history of thiopurine use with low platelet counts but normal LFTs, one with biopsy-proven NRH (and enrolled into the NRH and MRE study) and one with a platelet count of 118 who has a normal MRI and who is having ongoing monitoring. This means that 20 patients have had unexplained thrombocytopenia who needed management as per the guide below (Figure 8.5).

Of the 40 (12%) patients with unexplained abnormal LFTs, nine were on thiopurines. The question is whether these nine patients should also be investigated for NRH. The Dubinsky study would suggest that 76% could have NRH, although this study was based on thioguanine.[165] Figure 8.5 is also a guide for how patients with unexplained abnormal LFTs on thiopurines, could be investigated for NRH.

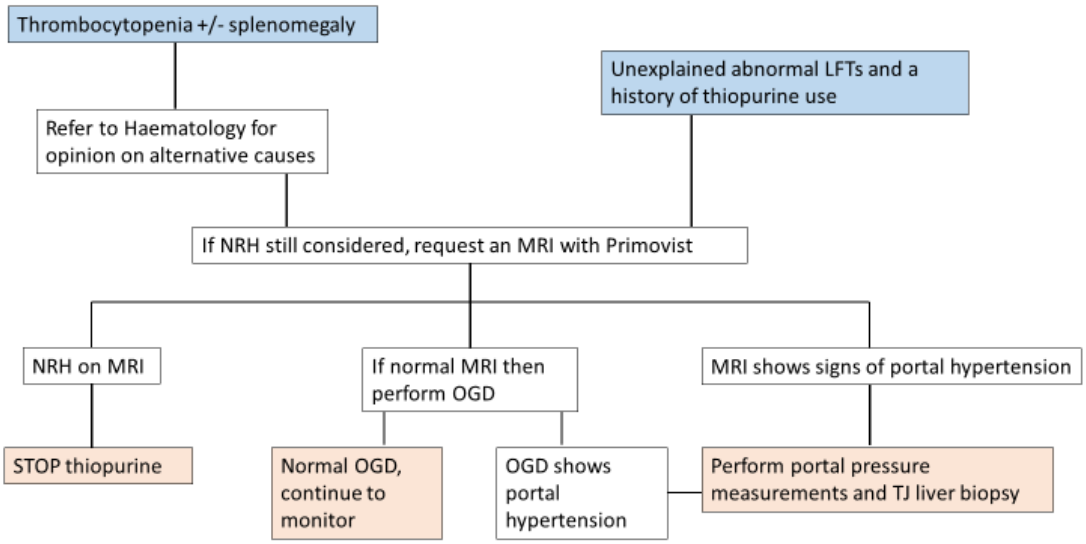


Figure 8.5: The investigation of NRH according to two main types of presentation: those patients with thrombocytopenia and splenomegaly and those patients with unexplained abnormal LFTs. Portal pressure measurements with trans-jugular (TJ) liver biopsy remains the definitive means of diagnosis. The pink squares represent final endpoints. OGD: oesophageal-gastro-duodenoscopy.

50-70% of patients with NRH show signs of portal hypertension.[155] However, it is not known what proportion of patients with NRH progress to liver failure once the drug is stopped. From the four patients in my study who had thiopurine-induced NRH, the symptoms occurred up to 10 years after the drug was stopped, which suggests that the disease can progress even in the absence of the cause; this suggests that we may see more NRH develop in the future. There is therefore a need to diagnose patients earlier, before complications arise.

8.6 Future work

The main findings in this thesis are that there appears to be two types of hepatotoxicity from thiopurines: one occurring with hypermethylation and the other without hypermethylation. The hepatotoxicity that occurs with hypermethylation correlates with high MeMP and is associated with CYP1A2*F variants. The hepatotoxicity that occurs in the absence of hypermethylation, and affects all TPMT genotypes, is associated with MTHFR variants. Whether the mechanisms leading to both types of hepatotoxicity are different, needs further research. MTHFR variants, with their downregulating effects on TPMT activity, are not enough to explain why one type of thiopurine hepatotoxicity occurs with hypermethylation and the other does not. Hypermethylation with hepatotoxicity is more hepatocellular (higher ALT) and less acute, suggesting that the pathology may be different.

Two mechanisms for hepatotoxicity have been described in this thesis, one as a result of reduced glutathione and the other through the release of formaldehyde (Figure 8.3); these mechanisms may or may not be independent of each other. How the two types of hepatotoxicity correlate with these two mechanisms for hepatotoxicity should be researched further, perhaps by observing cellular differences using hepatocyte models *in vitro*.

In this thesis, we showed that RBC PG and plasma SAM concentrations were raised in hypermethylators with hepatotoxicity. We do not know whether they are also raised in hepatotoxicity without hypermethylation or even flares of disease. Although, our results showed that patients with IBD had higher RBC PG and plasma SAM concentrations than non-IBD patients, the IBD patients were almost all on thiopurines. Comparisons of SAM and PG concentrations are therefore needed between both types of thiopurine hepatotoxicity and between patients with both active and inactive IBD.

The mechanism by which LDTA resolves hepatotoxicity is another area that needs further research. Traditionally, it was thought to occur through the inhibition of XO by allopurinol. However, the Purine Laboratory at St Thomas' has suggested that it occurs as a result of TPMT inhibition mediated via TX. What is certain, is that LDTA is effective at circumventing all causes of thiopurine hepatotoxicity. Indeed, a trial of LDTA is one way by which clinical dilemmas such as the ones above could be solved.

It would also be interesting to assess what proportion of patients with thiopurine hepatotoxicity can be safely dose-reduced or dose-split, thereby avoiding the need to switch to LDTA or stop thiopurines

entirely. These patients would of course need to be carefully monitored. The success of split-dosing in patients who hypermethylate is discussed further in the Appendix.

Larger prospective studies are required to examine MTHFR and CYP1A2 variants in more detail, standardising against factors such as weight and age. Alternatively, a candidate gene analysis for these variants could be repeated in a different cohort of patients. The PRED4 study is a much larger study of patients with the aim of understanding the genetics associated with all side effects from thiopurines in patients with IBD and it recruited patients with higher LFTs (elevation of ALT to > 5 X ULN (i.e males > 295 IU/L, females > 225 IU/L).

The "double hit" hypothesis is an interesting idea that also deserves further research, especially with regards to whether active disease predisposes to thiopurine hepatotoxicity. There are a few ways that this could be achieved. Firstly, one could compare rates of hepatotoxicity in patients where thiopurines were started when in remission, such as post-operatively, with patients who have active disease endoscopically. The second way is to make comparisons with a group of patients who are in remission on a biologic, where thiopurines are often added as concomitant therapy. Additionally, thiopurines also tend to be added to biologics to prevent antibody formation and hence there may be an association between adding a thiopurine and underlying active disease.[314, 315] The third way is to make comparisons with a non-IBD cohort. For example, hepatotoxicity from AZA in ANCA positive vasculitis patients has been studied.[316] Of a cohort of 67 patients, 21 (31%) developed worsening LFTs on AZA. Although this is much higher than the 5% hepatotoxicity rate in IBD, 48% of these patients had abnormal LFTs to begin with and the median age was 69 with 73% being female. Although this does not help determine the potential role of active IBD in thiopurine hepatotoxicity, it does support the "double-hit" hypothesis, where age, gender and pre-existing liver disease are all factors which predispose to thiopurine hepatotoxicity. A retrospective study performed at GSTT on 41 patients with chronic immune-thrombocytopenia treated with AZA, with no underlying IBD, found three (7%) patients with thiopurine hepatotoxicity, less than the prevalence seen in IBD. [317]

The introduction to this thesis discussed how *in vitro* studies had demonstrated reduced mitochondrial glutathione is associated with cell necrosis in hepatocytes exposed to AZA which then reversed when cells were pre-incubated with NAC, allopurinol and vitamin E analogues (see Section 1.11).[149] NAC has already been shown to prevent hepatotoxicity in rats exposed to AZA.[318] A study could be performed where patients with thiopurine hepatotoxicity are given NAC and assessed for resolution of hepatotoxicity. NAC is an anti-oxidant which increases GSH in hepatocytes and has been shown to be

beneficial in a number of diseases such as cystic fibrosis and paracetamol toxicity. NAC has a lower susceptibility to oxidation, and dissolves in water. [319] A review of 46 placebo-controlled trials show that NAC administration in humans is not associated with significant adverse effects. It is available in aerosol, oral and intravenous form and is almost completely absorbed when given orally.[320]

A third of the patients with abnormal LFTs in the context of IBD were associated with disease flares. The exact mechanism for why this should occur remains unknown, but it occurs more commonly in patients with CD. One explanation is that inflammation within the bowel tracks transmurally across to the liver causing a hepatitis, as part of the Gut-Liver axis. The mechanism by which PSC is thought to occur is either through lymphocyte-trafficking up the enterohepatic circulation by increased gut permeability or through dysbiosis, although this occurs more commonly in UC.[43] A retrospective analysis of liver biopsies taken from patients with unexplained abnormal LFTs during a flare of their IBD may help explain exactly what mechanisms are occurring.

The role of MRE as a non-invasive screening modality for NRH needs investigating further. Whilst results from this very small cohort of patients are unremarkable, a larger sample size may yield more significant results. Ideally, a single blinded multicentre study should be performed where patients with biopsy-proven NRH undergo MRE and are compared to a non-NRH control population. The true impact of NRH for patients is unknown and could be researched further. Lastly, the prevalence of NRH in all liver biopsies has been reported as 4.4% and 15% of biopsies performed for unexplained abnormal LFTs.[321] Around one third of these patients had portal hypertension at diagnosis and 65% had an identifiable cause. In a large Dutch study, 0.6% of explants were found to have NRH, suggesting that patients who go into liver failure requiring transplantation are rare.[158] Hepatocellular cancer has been reported in a patient with NRH suggesting it could be a premalignant condition.[179] There is generally a poor inter-observer agreement between Histopathologists in diagnosing NRH on biopsy and therefore knowing what happens to true NRH patients would be valuable.[322]

Prior to this research, the clinical unmet need was to determine the prevalence, mechanisms and risk factors for liver inflammation in IBD and how we might better screen for them in our patients. This thesis summarises the causes of liver disease which IBD patients suffer from based on the largest cohort study of IBD patients with abnormal LFTs ever performed. It provides an evidence-based strategy to investigate abnormal LFTs in IBD. It distinguishes the two types of thiopurine hepatotoxicity and uses biochemical and genetic analyses to support two main mechanisms by which hepatotoxicity might be occurring. These are the first studies attempting to understand the role of

the Methionine Cycle in patients with abnormal thiopurine metabolism. The thesis also highlights phenotypic risk factors for thiopurine hepatotoxicity, such as age and weight. Lastly, it adds to our knowledge of MRE as a potential screening tool for diagnosing NRH.

It is only through ongoing research that we can optimise the efficacy of thiopurines further. Although our use of thiopurines in IBD is being superseded by some biologics, they still offer a therapeutic benefit for IBD patients. The research performed on thiopurines is a good example of how science can get the best out of an old drug. And so, 70 years after thiopurines were first discovered, we find ourselves using them more than ever.

Appendix:

Split dosing of thiopurines as a means of circumventing hypermethylation

Introduction

Hypermethylation of thiopurine drugs, azathioprine (AZA) and mercaptopurine (MP), occurs in 20% of patients and is associated with an increased likelihood of hepatotoxicity, myelotoxicity and other side effects.[143] Previous studies have demonstrated significant reductions in MeMP (11785 versus 5324 pmol/8x10^8 RBC) through dose splitting whilst maintaining TGNs within a therapeutic range (between 230 and 450 pmol/8x10^8 RBC).[144] Dose splitting involves patients halving the dose such that they take half of the dose in the morning and half at night (e.g 100 mg is split to 50 mg morning and night). Split dosing is a useful alternative to the more conventional approach of switching to allopurinol whilst reducing to 25% the dose of the AZA or MP, otherwise known as low dose thiopurine and allopurinol (LDTA). Allopurinol is a xanthine oxidase inhibitor designed exactly for this purpose before it was used in the treatment of gout.[233] It blocks the breakdown of MP to thiouric acid (TUA) thereby increasing the amount of the substrate for breakdown to thioguanine nucleotide (TGN). By the reducing the dose of either AZA or MP, the hypothesis is that the enzymes which breakdown MP are able to be more efficient, thereby reducing the MP available for methylation by thiopurine methyl transferase (TPMT). This has been shown to successfully prevent hypermethylation.[182, 185] In my thesis, I have shown how LDTA not only reduces hypermethylation but can resolve thiopurine hepatotoxicity.

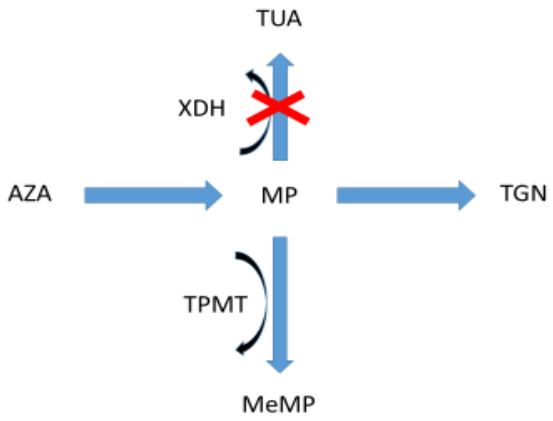


Figure 1: Allopurinol mediated xanthine dehydrogenase (XDH) inhibition and of thiouric acid (TUA) formation thereby allowing the dose of AZA and MP to be reduced by 25% forming the same concentration of TGN but little or no MeMP.

There is a very small risk of toxic epidermal necrolysis and DRESS syndrome (Drug reaction with eosinophilia and systemic symptoms) with allopurinol.[187] There is also very little safety data on allopurinol use in pregnancy and switching to LDTA involves patients taking another medication which has repercussions in terms of drug compliance.[323] For these reasons, dose splitting is an alternative for patients and clinician who may have concerns about switching to LDTA.

The aim of this study was to assess the efficacy of dose splitting in hypermethylators and patients with thiopurine induced nausea in our cohort of patients on thiopurines with inflammatory bowel disease at GSTT.

Methods

Pharmacy databases were searched for all patients in the last 10 years who had allopurinol prescribed from the Gastroenterology department at GSTT. These were screened for more than once a day dosing. The hospital records of the patients that these prescriptions were applicable to were then retrospectively analysed for IBD patients, dosing changes, reasons for dose splitting, and changes in MeMP and TGN concentrations. The overall success of dose splitting was assessed by the author, based on an improvement in side effects, resolution of hypermethylation and lack of the need to switch to LDTA. Patients were also followed up for loss of response from the time of dose splitting to the present day.

Statistics were performed on SPSS using a paired t-test for significance defined as a P-value of < 0.05.

Results

37 patients were found from hospital records who split dosed but complete data in terms of MeMP and TGN concentrations before and after dose splitting were only available in 35 patients. 22 were female (62,9%), mean age 36 years (SD 11.8, range 23-65). TPMT was available for 27 patients only (mean 37.2, SD 5.9). 21 patients had Crohn's disease, 13 had ulcerative colitis and one had indeterminate IBD. Mean dose of AZA (n=32) was 141 mg (SD 46.0) and 66.7 mg (SD 28.9) for MP (n=3). Dose splitting was concluded to be successful in 13 patients (37.1%). A reduction in the MeMP:TGN but not to below a ratio of 11 was achieved in a further 8 patients (22.9%). A further two patients (5.7%) had an improvement in nausea with dose splitting but the MeMP:TGN ratio did not improve. Over a median follow-up period of 12 months, one patient lost response on a dose split regime. 12 patients (34.3%) were switched to LDTA.

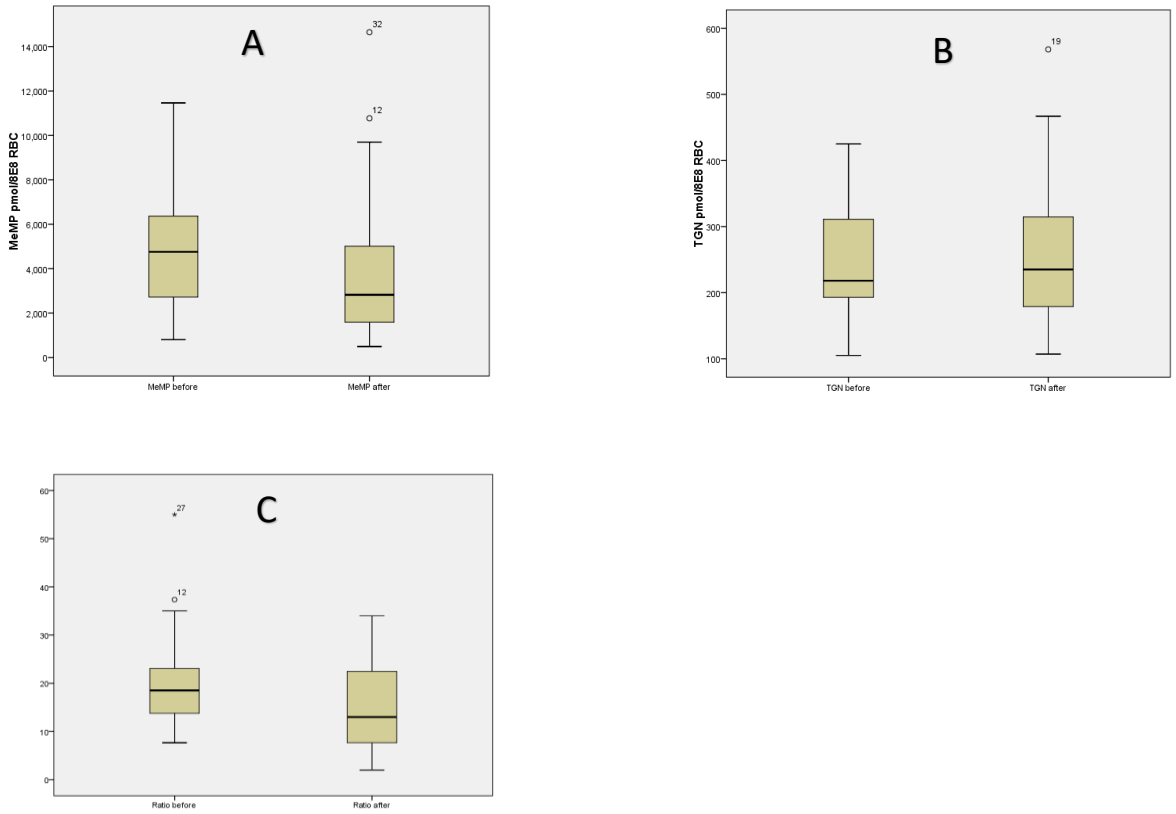


Figure 2: Comparisons between median MeMP (A), TGN (B) and MeMP:TGN (C) before and after dose splitting.

Table 1: The change in MeMP, TGN and MeMP:TGN after dose splitting.

	Before dose splitting (SD)	After dose splitting (SD)	P-value¥
MeMP	4888 (2430)	3829 (3321)	0.04
TGN	243 (73.0)	257 (101)	0.4
MeMP:TGN	20.2 (9.8)	14.4 (9.3)	0.003

¥Paired samples t-test.

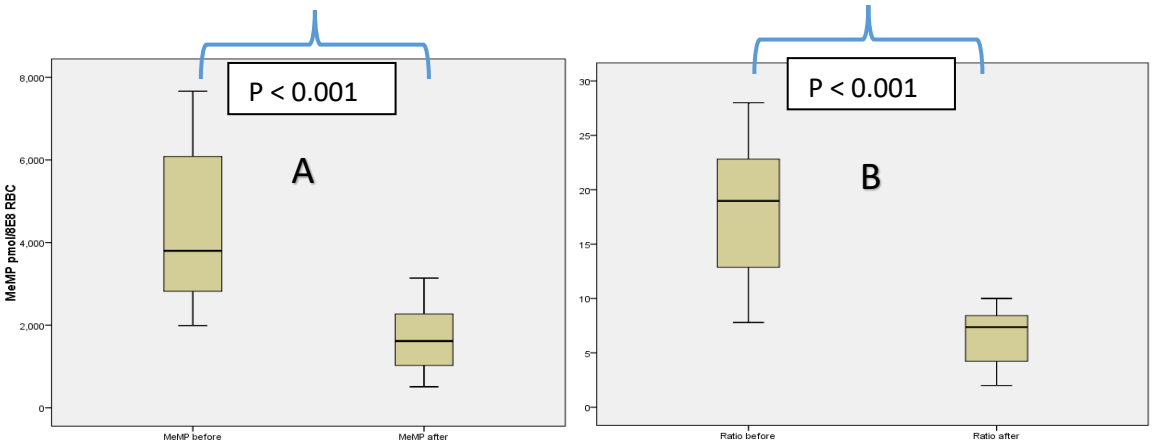


Figure 3: The significant reduction of median MeMP (A) (mean MeMP was 4510 before versus 1611 pmol/8 x10^8 RBC after, P < 0.001, paired samples t-test) and MeMP:TGN ratio (B) (mean 18.0 versus 6.8, P < 0.001, paired samples t-test) in the subgroup of 13 patients who responded to dose splitting. There was no significant change in mean TGN concentrations (251 versus 234 pmol/8 x10^8 RBC, P = 0.1, paired samples t-test.).

In the subgroup of patients with complete success of dose splitting, the results are more significant.

Of the cohort, two patients were hypermethylators with hepatotoxicity. Neither of these responded to dose splitting and both were switched to LDTA.

Conclusion

This was a retrospective study which collected data based on one metabolite result either side of the dose split. Although patients' notes were followed up for a median of one year for dose splitting success and loss of response, success was based on too few metabolites measurements. Determining success or loss of response based on the author's opinion was prone to bias. There is also no data on use of concomitant therapies such as biologics or mesalazines.

Overall, there was a significant reduction in MeMP concentrations and the MeMP:TGN ratio after dose splitting although true success was only achieved in a third of patients. There is clearly a subgroup of patients who respond extremely well to dose spitting. This supports the belief that the state of hypermethylation may occur through several mechanisms, not just through TPMT but also a lack of demethylation or alterations in the balance between exporter and importer pumps of particular compounds. Dose splitting may improve hypermethylation attributed to one of these mechanisms and not the other. Switching to LDTA was shown in my thesis to resolve 95% of patients with hypermethylation and hepatotoxicity and so it is comparatively more successful. However, allopurinol is associated with side effects and is not validated as safe in pregnancy. Dose splitting is therefore an alternative so long as metabolites are monitored afterwards. Dose splitting is not advised in patients with hepatotoxicity as there is no guarantee of normalising LFTs. Some patients hypermethylate in the absence of symptoms of hepatotoxicity. There is no data to suggest how these patients should be managed since high MeMP concentrations are thought to have no direct bioactivity. For this group of patients, dose splitting is a harmless option.

In conclusion, these findings represent a reasonable sized cohort of patients who have undergone dose splitting with a significant overall reduction in hypermethylation whilst maintaining TGN concentrations within a therapeutic range. There is evidence to suggest that a subgroup respond better than others, however, larger prospective studies are needed to truly assess the efficacy of dose splitting.

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