12-2018

Is TPMT Testing a Predictor of Duration of Use or Long-Term Benefit of Thiopurine Agents?

Shatoya R. Wilson
Grand Valley State University

Is TPMT Testing a Predictor of Duration of Use or Long-Term Benefit of Thiopurine Agents?

Shatoya Renee Wilson

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

Partial Fulfillment of the Requirements

For the Degree of

Master of Health Science

Biomedical Science

December 2018

Dedication

This is dedicated to my sister and father, for their support and love. I thank you for listening to me when I needed to practice with or vent to someone.

I would also like to dedicate this to my thesis committee, who helped me grow as a student and researcher. For your guidance, I am grateful.

Acknowledgements

I would like to thank my thesis committee, Dr. Debra Burg, David Chesla, and Dr. John Capodilupo for their mentorship and guidance. I would also like to thank the Spectrum Health Department of Research for providing the research database from which my study was obtained. Special thanks to Spectrum Health biostatisticians Brandon George and Jessica Parker for their statistical consulting.

Abstract

Background

Inflammatory Bowel Disease (IBD) affects as many as 1.3 million Americans and includes both ulcerative colitis (UC) and Crohn's disease (CD). Recent practices have moved towards using immunomodulators early in the course of disease to prevent disease progression. Patients have shown improved outcomes on immunomodulators compared to aminosalicylates, and reduced risk for tachyphylaxis compared to corticosteroid treatment. Thiopurine agents such as azathioprine and 6-mercaptopurine are immunomodulators often used to treat IBD. Unfortunately, many patients experience cytotoxicity from thiopurine use. Recent studies have shown that patients deficient in thiopurine-S-methyltransferase (TMPT), produce an overabundance of the therapeutic end product, 6-thioguanine nucleotide (6-TGN), causing toxicity leading to apoptosis in all types of immune cells. In this study we investigated whether pre-emptive genotyping for TPMT would allow customized doses of thiopurines based on TPMT status and whether knowing this genotyping would reduce the risk of adverse events without compromising disease control.

Methods

A retrospective study using an available data set identified 30 adults over 18 with CD or UC. Patients were included in the study if their TPMT genotype and phenotype, 6-TGN and 6-MeMPN levels, and disease exacerbation were recorded. Using Fisher's Exact test for statistical analysis, genotype was compared to phenotype (enzyme activity), metabolite levels, and toxicity/exacerbation. Phenotype was compared to metabolite levels.

Results

Genotype and enzymatic activity were not independent of each other. Subjects with a mutant TPMT genotype were more likely to respond to thiopurine therapies. Genotype was not

indicative of high risk for hepatotoxicity, and there was no significant relationship between enzyme activity and metabolite levels. Lastly, there was no relationship between genotype and toxicity.

Conclusions

Multiple studies have studied dose escalation based on genotype alone. However, genotype does not take into consideration confounding factors that might impact patient outcomes when using thiopurine therapy for IBD. This would include rare deficiency alleles, true phenotypic expression, other genetic polymorphisms, and most importantly polypharmacy. Thiopurine interaction with ASAs, benzoic acid derivatives, non-steroidal anti-inflammatory drugs, and methotrexate have been shown to influence TPMT enzyme activity. Whether this is due to allosteric inhibition or competitive inhibition has yet to be discovered.

Table of Contents

List of Tables

List of Figures

Chapter 1 Introduction

Introduction

The digestive tract is primarily responsible for the mechanical and enzymatic break down of food, extraction of nutrients, and removal of waste products. Inflammation within the digestive tract may disrupt any one of these processes. Inflammatory bowel disease (IBD) is a group of inflammatory conditions affecting the gastrointestinal (GI) tract, and while the exact etiology of IBD is unknown, three characteristics define IBD: (1) an altered response to gut microorganisms, (2) a dysregulated immune response, and (3) genetic predisposition for the disease (1-4). The two primary forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC) (44). Common symptoms of IBD include abdominal pain, weight loss, diarrhea, and colonic perforation (1-2). The anatomic location of the inflammation is important for distinguishing CD from UC. UC is limited to the colon and starts in the distal sigmoid colon. Disease development is uniform from the rectum through the colon and symptoms include bloody diarrhea, abdominal pain, weight loss, ulceration, and bleeding (1-3). UC cannot only progress to colorectal cancer (CRC), but also predisposes patients to CRC. The longer inflammation persists, the greater the chance it will progress to CRC. According to a 2012 review by Dilip et al. (5), the lifetime incidence of colon cancer in patients with UC is 2.5% at 10 years, 7.6% at 30 years, and 10.8% at 50 years (5-6). While gender and age of onset are considered risk factors for the development of colon cancer, the location of the lesions predicts the aggressiveness of the disease (7). In contrast with UC, CD is characterized by segmental and occasionally randomized spread, termed "skip" lesions, throughout the GI tract with the most common site of involvement being at the ileocecal valve. Additional manifestations of CD are fistulas, fissures, strictures, abscesses, anemia, fever, arthritis, and skin changes.

There are four categories of medicinal therapy used to treat IBD: aminosalicylates (ASA), corticosteroids, immunomodulators, and biologics. Aminosalicylates reduce

inflammation by inhibiting cyclooxygenase and prostaglandins, which are involved in mediating inflammation, anaphylactic reactions, vasoconstriction, and vasodilation (8-10). Corticosteroids prevent inflammation and cause general immune suppression, especially of T cells. Immunomodulators modulate the immune response toward adaptive rather than maladaptive activities and induce, amplify, attenuate, or prevent the immune response, depending on therapeutic goals (11). Biological agents are typically monoclonal antibodies that act as TNF-α antagonists and integrin receptor antagonists (12). As a group, biologics are the most recently developed medicinal therapies approved for the treatment of IBD and they are typically a last resort because they may act on more than one target, cause an immune response, are expensive, and run the risk of overtreatment of patients with milder IBD (12-13).

In recent years, a change in the treatment goals for IBD has generated intense discussion. In the past, treatment was initiated only in response to acute flares of the disease and were aimed at reducing clinical symptoms. This was considered the "step-up" approach, which refers to a sequential treatment strategy that usually begins with a less toxic but potentially less effective therapy choice such as aminosalicylates, with progression to the more effective but potentially more toxic treatment strategies such as prednisone, immunomodulators and biologics as patients begin to fail the previous line of therapy. The paradigm for IBD treatment has now shifted to preventing damage to the intestinal wall to prevent disease progression and disability. This has become known as the "top-down" approach. It encourages the initial use of more aggressive, highly effective, but potentially more toxic treatment strategies early in the course of chronic illness because studies have shown that this approach decreases the long-term rates of surgery and hospitalization.

Thiopurines, which fall under immunomodulators, are situated in the middle of the therapy pyramid. If treatment is started with immunomodulators, which are more effective than

ASA and less toxic than biologic agents, it is possible in the course of chronic illness to prevent disease progression and disability. However, genetic predisposition can not only determine susceptibility to disease state, but also impacts the ability to metabolize thiopurines (14-16). The clinical efficacy of thiopurine as an immunomodulator is highly dependent the degree to which the body metabolizes the drug, either converting it to therapeutic levels of the active product or toxic levels that cause adverse effects such as leukocytopenia (14-17). Thiopurines are metabolized in the liver and gut by one of three competing enzymes: thiopurine-S-methyltransferase (TPMT), xanthine oxidase (XO), or hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) (18-19). HGPRT is responsible for converting the pro-drug to the 6-mercaptopurine (6-MP) therapeutic form, while the other two enzymes lead to inactivation of thiopurines.

TPMT is the major enzyme for inactivation of thiopurines, since the amount of XO is negligible in hematopoietic tissues. TPMT enzymatic activity is a major factor in determining whether the amount of 6-TGN produced is in the therapeutic range or the toxic range because its catabolism of 6-MP controls how much remains for HGPRT to convert to 6-TGN. The degree to which TPMT is able to inactivate 6-MP is determined by the genotype of the patient. Ethnicity may also influence enzyme activity, as some variant alleles are more common among certain ethnic groups. While people of all ethnicities can get IBD, the disease burden is lower in ethnic minorities compared to non-Hispanic whites (20). In the Caucasian population, approximately 89% of the population is homozygous for wild type TPMT, associated with normal levels of enzymatic activity. Approximately 0.3% are homozygous for mutations in TPMT and thus have negligible enzyme activity, meaning they have a true deficiency of TPMT. The remaining 11% are heterozygous and exhibit reduced enzymatic activity (21-22). In patients who exhibit reduced

TPMT activity, higher levels of active 6-MP are available to be converted to 6-TGN, producing toxic levels that cause myelosuppression as well as bone marrow and liver toxicity (23-26).

TPMT genotyping prior to using these drugs would help to identify individuals with low or absent TPMT activity so that toxicity could be prevented by adjusting the dose of 6-MP or Aza prescribed (14,17, 21-22,27). In fact, guidelines for TPMT genotyping and associated thiopurine dosing were created in 2011 by the Clinical Pharmacogenetics Implementation Consortium (CPIC), which is part of the National Institutes of Health's Pharmacogenomics Research Network (28). Because the CPIC guidelines are relatively new, it is likely that not all gastroenterologists have implemented TPMT genotyping prior to prescribing thiopurine treatments for their IBD patients, leaving room for improvement regarding safety, efficacy and cost to the patient.

Purpose

To address the usefulness of TPMT genotyping, we proposed a retrospective study to determine the extent to which TPMT genotyping predicts phenotype, drug toxicity, and/or disease exacerbation. The first question addressed whether or not genotype is a good predictor of phenotype. TPMT genetic testing detects variations in the TPMT gene that predict its activity. Phenotypes are categorized as high, normal, or low enzyme activity and can be measured in RBCs. However, there may be other factors influencing TPMT enzyme activity (phenotype) and its potential for bone marrow toxicity. TPMT enzyme activity can be inhibited by interactions with several drugs, including naproxen, ibuprofen, ketoprofen, furosemide, sulfasalazine, mesalamine, olsalazine, mefenamic acid, thiazide diuretics, and benzoic acid inhibitors, some of which are frequently used in combination therapy with thiopurines. Because of the potential confounding effects of polypharmacy, further data is needed to determine the reliability of

predicting phenotype from genotype. The second question asked whether genotype is a prognostic indicator of drug toxicity and/or exacerbations. One of the benefits of preemptive TPMT enzyme genotyping is that doses can be customized based on genotype status to reduce the likelihood of adverse events without compromising disease control. We investigated whether the genotype could predict elevated exacerbations such as abdominal pain, diarrhea, and weight loss.

Significance

Exacerbations of CD and UC have a substantial economic impact on annual healthcare expenditures. Direct and indirect annual costs in the U.S. were estimated to be $1.6 to $31.6 billion in 2014, as reported by the Crohn's and Colitis Foundation of America (CCFA). Although treatment expenses make up a significant portion of the cost of IBD, studies have shown that inappropriate treatment, suboptimal treatment, and lack of adherence to therapeutic regimens also contribute to the cost burden (29). Costs for IBD include hospitalizations, the eventual need for surgery due to disease complications, and frequent physician visits. The staggering economic burden of IBD makes it imperative to couple early diagnosis with effective treatment at the onset of symptoms (29-31). To this end, preemptive genotyping to generate customized doses of thiopurines based on TPMT status would reduce the likelihood of adverse events without compromising disease control.

Chapter 2 Review of Literature

Characteristics of IBD

The exact etiology and pathology of IBD remain unknown, but available evidence suggests that an abnormal immune response against the microorganisms in the intestine is responsible for the disease in genetically susceptible individuals (32). Pathologic alterations in the intestinal microbiome trigger an aberrant mucosal immune response in genetically predisposed individuals, leading to the development of chronic intestinal inflammation. These pathologic alterations in gut microbial composition seen in IBD are referred to as intestinal "dysbiosis." There is a shift from predominantly "symbiont" microbes to potentially harmful "pathobionts." Research suggests that perturbations in the gut microbiome are an essential factor triggering inflammation in IBD rather than being merely a consequence of the chronic inflammation (33). Abundant evidence supports the integral role that the intestinal microbiome plays in the pathogenesis of IBD, including dysbiosis observed in individuals with IBD. In addition, the majority of genetic polymorphisms for IBD susceptibility are associated with host mucosal barrier function and are involved in host–microbiome interactions (33).

The gut microbiota is the largest and most diverse community of microbes in the human body. The intestinal microbiota, or microorganism population of the intestine, constitutes only a fraction of the complexity of the intestinal microbiome. This diverse array of microbiota expresses a variety of microbial genes resulting in gene products that can interact with the host environment. The composition of the gut microbiome changes over time, and no single microbe or microbial milieu has proven to be the specific cause of IBD. The immediate question that follows is how the host responds to dysbiosis. Host genetic factors, specifically those pertaining to the innate arm of immunity, are predicted to play a role in the pathogenesis of IBD. The intestinal microbiota is a major source of immune stimulation, and the colonic epithelium lies in

close proximity to a high density of diverse microbes (34). The continual communication between host cells and host microbes is essential for normal homeostasis, but imbalance can cause deleterious effects and contribute to intestinal inflammation (35-36).

Host immune factors play a role in maintaining or reacting to mucosal inflammation. It has long been suspected that an individual's genotype contributes to overall susceptibility to developing IBD. Studies evaluating specific genetic variants have highlighted the interplay between the gut immune system and intestinal microbiota. For example, genetic variations in NOD2 (nucleotide-binding oligomerization domain 2) correlate with decreased IL-10, an important anti-inflammatory cytokine (33). Genetic polymorphisms of NOD2 have demonstrated an inability to respond to bacterial muramyl dipeptide (MDP), reducing downstream activities of NF-κB activation and subsequent inflammation (34). This contributes to down-regulation of inflammatory responses, with chronic stimulation of NOD2 acting to tolerize cells against bacterial stimulation and ultimately down regulating other pathogen recognition receptors (PRRs). Additional polymorphisms in toll-like receptors (TLRs) have been identified and associated with pancolitis in UC patients (34). The innate immune response produces inflammation that in turn allows for the recruitment of the adaptive arm of the immune response into the tissue, causing dysregulation.

Dysregulation of the immune response in the intestine plays a critical role in the pathogenesis of IBD. Dysregulation involves a wide range of cells including cytokines, T regulatory cells, and T helper cells that are likely to be associated with disease progression (32). Dysregulation can lead to an imbalance in the T helper cells subsets, an increase in the cytotoxic activity of cytotoxic T cell and natural killer cells, and increased activation of antigen presenting cells that present to T cells (32). The dysfunction of the mucosal immune system plays a central role in both the induction and persistence of chronic inflammation by producing pro-

inflammatory cytokines. These pro-inflammatory cytokines are potent stimulators of the intestinal mucosal effector functions. Under inflammatory conditions, a large number of activated immune cells infiltrate the intestinal tract.

Genome-wide association studies (GWAS) have been successful in, identifying 99 over-lapping genetic risk loci (37) involved in IBD. Twenty-eight of these are shared between CD and UC. Analysis of the genes and genetic loci implicated in IBD indicate several functions that are crucial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defense, innate immune regulation, reactive oxygen species (ROS) generation, autophagy, regulation of adaptive immunity, endoplasmic reticulum (ER) stress and metabolic pathways associated with cellular homeostasis (Figure 1) (37). These actions are coordinated at four different levels in the epithelium: microbial sensors, recruitment of mediators, signal amplification, and transducers and effectors (37). The gut has many tiers of defense against invasion by microbes, including the epithelial barrier, and the innate and adaptive immune responses. These components are all closely interrelated and may all be susceptible to genetic variations, including variations in drug metabolism.

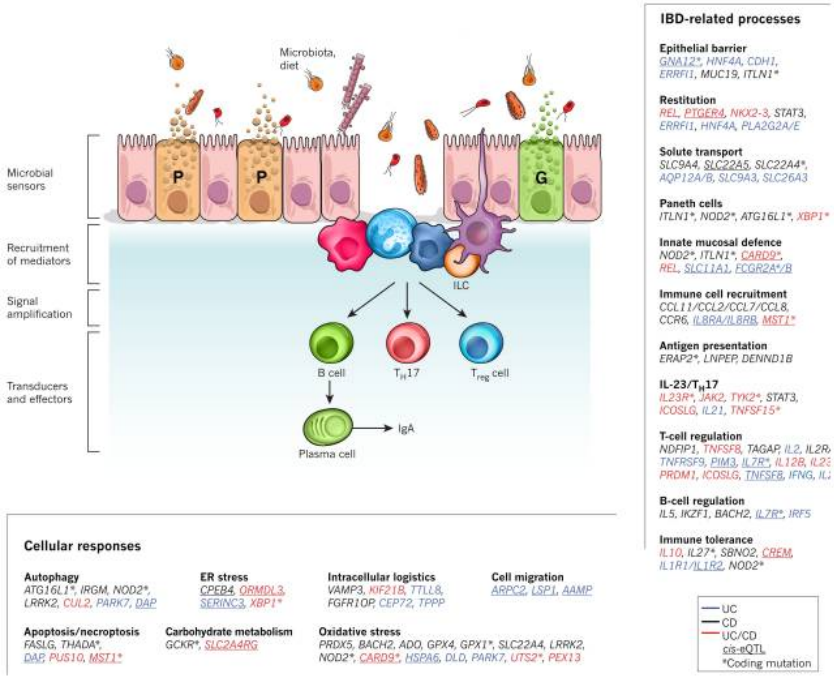


Figure 1. *A model for IBD pathways based on GWAS*. Intestinal homeostasis involves the coordinated actions of epithelial, innate and adaptive immune cells. Barrier permeability permits microbial incursion, which is detected by the innate immune system, which then orchestrates appropriate tolerogenic, inflammatory and restitutive responses in part by releasing extracellular mediators that recruit other cellular components, including adaptive immune cells. Genetic variants, the microbiota and immune factors affect the balance of these signals. Genes in linkage disequilibrium (r2 > 0.8) with IBD-associated single nucleotide polymorphisms (SNPs) were manually curated and classified according to their function(s) in the context of intestinal homeostasis and immunity. Text color indicates whether the genes are linked to risk loci associated with Crohn's disease (CD; black), ulcerative colitis (UC; blue) or both (red). Asterisk denotes corresponding coding mutations; cis-eQTL effects are underlined. G, goblet cell; P, Paneth cell. (37)

Metabolomics of Thiopurines

Two of the most common drugs in the thiopurine class of immunomodulators are azathioprine (AZA) and 6-mercaptopurine (6-MP). AZA is the pro-drug form of 6-MP, where an imidazole ring has been added to the 6-MP molecule to facilitate uptake by the liver (Figure 2) (16). Glutathione, a physiologically important antioxidant and reducing agent, rapidly converts AZA to 6-MP by a non-enzymatic, nucleophilic attack of the thioether bond (16,38).

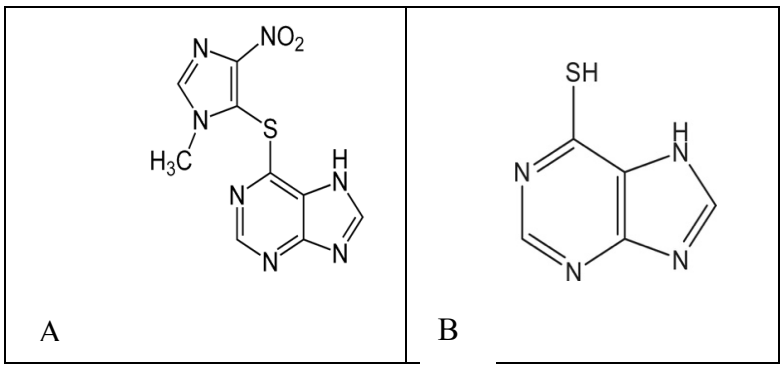


Figure 2. *A) AZA molecular structure. B) 6-MP molecular structure (39).*

Once AZA has been converted to 6-MP, the 6-MP is metabolized in the liver and gut by one of three competing enzymes: thiopurine-S-methyltransferase (TPMT), xanthine oxidase (XO), or hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) (18-19). TPMT inactivates 6-MP by the methylation of 6-MP to generate the inactive metabolite known as 6-methyl-mercaptopurine (6-MeMP). XO converts 6-MP to inactive 6-thioruate (6-TU), which is excreted in the urine (22, 40). In contrast, HGPRT converts 6-MP to the active metabolite 6-thioguanine nucleotide (6-TGN) (18-19, 22, 40). Both the therapeutic activity of 6-MP and AZA, as well as its toxicity to bone marrow and liver, are a result of the 6-TGN metabolite (Figure 3) (41-43). When 6-TGN levels are maintained in the range of 230-400 pmol/8 x 10^8 RBC, a positive therapeutic response is achieved and IBD goes into remission. On the other hand, when 6-TGN levels exceed 400 pmol/8 x 10^8 RBC (44), the metabolite causes liver toxicity and suppression of hematopoiesis in the bone marrow.

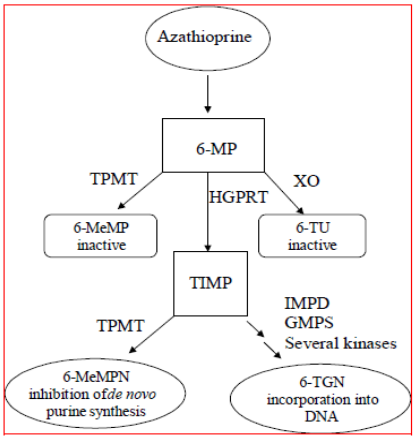


Figure 3. *AZA metabolism pathway.* Competing pathways result in either inactivation of 6-MP by the enzymes TPMT or XO, or conversion of 6-MP to metabolically active 6-TGN for incorporation of cytotoxic nucleotides into DNA (45).

Achieving a therapeutic response

There are three mechanisms by which thiopurines achieve a therapeutic response. Taken together, these three mechanisms prevent activation and proliferation of the T-lymphocytes implicated in the pathogenesis of IBD and induce them to undergo apoptosis (Figure 4). First, 6-TGN, a purine analog arrests the cell cycle and triggers apoptosis by being incorporated into DNA in place of adenosine and guanine, leading to chromosome damage (46). Second, when 6-TGN is incorporated into base pairs, this causes reduced stability, through small changes in local DNA structure, and increased levels of methylation, activating the DNA mismatch repair system (46). Third and most importantly, 6-thioguanine triphosphate nucleotide (6-TGTP), one of the thioguanine nucleotides, acts as a direct antagonist of Rac1, a factor that dampens the inflammatory cascade through its effects on NF-κB and signal transducer and activator of transcription 3 (STAT-3) (46).

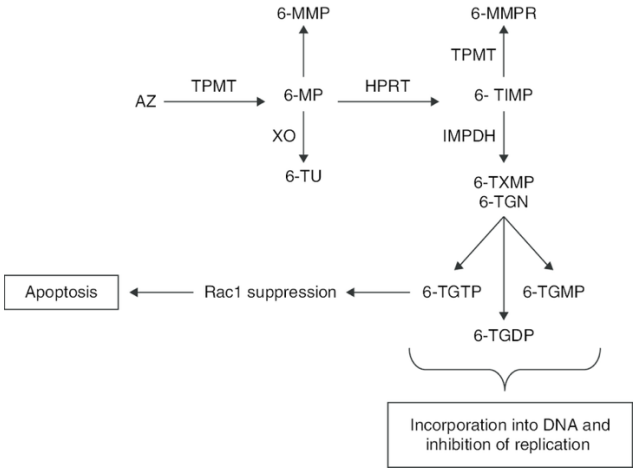


Figure 4. Action of 6-TGN (43).

Balance between efficacy and toxicity

The immunosuppressive properties of thiopurines are most probably mediated through their interference with protein synthesis and nucleic acid metabolism, as well as by their cytotoxic effects on lymphoid cells (47). 6-MP must be transformed into a ribonucleotide, which functions as a purine antagonist. However, 6-MP undergoes rapid and extensive catabolic oxidation by xanthine oxidase (XO), limiting the absolute bioavailability of 6-MP (47). As such, the bioavailability can range from 5-37%. The anabolic transformation of 6-MP into active metabolites is mediated by the competing pathways catalyzed by TPMT and HPGRT. Therefore, the same pathways that lead to efficacy in treatment can also lead to toxicity. Low TPMT activity has been associated with increased cytotoxicity because it allows 6-MP metabolism to be shunted towards the excessive production of 6-TGN via the enzyme HPGRT.

Genetic polymorphism

TPMT enzymatic activity is a major factor in determining whether the amount of 6-TGN produced is in the therapeutic or toxic range because its catabolism of 6-MP determines how much is available to convert to 6-TGN. TPMT enzyme activity can be measured in the clinical

setting and characterized as deficient (<10mU/L), low (20–67mU/L), normal (68–150mU/L), and high (>150mU/L) (44). The level of enzyme activity is determined by the genotype of the patient. Ethnicity may also influence enzyme activity, as some variant alleles are more prominent among certain ethnic groups when compared to others. In the Caucasian population, approximately 89% of the population is homozygous for wild type TPMT, associated with normal levels of enzymatic activity. Approximately 0.3% are homozygous for mutations in TPMT and thus have negligible enzyme activity, meaning they have true deficiency of TPMT. The remaining 11% are heterozygous and exhibit reduced enzymatic activity (21-22). In patients who exhibit reduced TPMT activity, higher levels of active 6-MP are available to be converted to 6-TGN, shifting 6-TGN from a therapeutic to a toxic range that causes liver toxicity and myelosuppression (23-26).

There are at least 20 genetic polymorphisms of TPMT identified within the human population that lead to varying degrees of reduced enzyme activity (38). Among these, TPMT 2, 3A, 3B, and 3C are the most common mutations (48). The reduced enzyme activity of these variants causes the affected individuals to be particularly susceptible to thiopurine toxicity due to overproduction of 6-TGN. Thus, TPMT activity correlates inversely with 6-TGN levels in erythrocytes and presumably other hematopoietic tissues. Since these cells have negligible xanthine oxidase activities, TPMT methylation is the only inactivation pathway available (38, 44). This leads to increased levels of 6-TGN in the red blood cells and bone marrow toxicity that leads to clinical symptoms of myelosuppression, anemia, bleeding tendency, leukopenia and infection (22,75). Therefore, it is not recommended that patients with a known TPMT deficiency be treated with AZA or 6-MP (28).

Polypharmacy drug interactions and TPMT activity

Adverse effects can occur that are unrelated to the main pharmacological action of a drug. Such adverse effects are termed idiosyncratic and are often initiated by metabolites of the parent drug or by other indirect mechanisms (49). In recent years, with the advent of novel therapeutic agents to treat a host of disorders, including inflammatory bowel disease and others, the potential for serious clinically relevant drug reactions has increased. Novel drugs such as thiopurines were originally designed to treat a specific disease but were later repurposed to treat additional diseases without further clinical trial research. A significant number of adverse events can be explained by drug interactions (49). For example, some drug interactions are caused by one drug competitively inhibiting the rate of metabolism of another drug. Other interactions can be mechanistic, in which one or more drugs become synergistic without any change in actual drug levels (49).

Thiopurines and ASA are two of the most widely used drugs for IBD, and they are often used in combination therapy. *In vitro* studies have shown that ASAs and their metabolites can inhibit the activity of TPMT (50). 5-ASA is inactivated by the n-acetyl transferase 1 (NAT1) isoform in colonic mucosa. NAT1 catalyzes the transfer of an acetyl group from acetyl-CoA to various aryl amine and hydrazine substrates. This enzyme helps metabolize drugs and other xenobiotics, substances that are foreign to the body. Polymorphisms in this enzyme can be classified as low, intermediate, or normal just like TPMT. The inheritance of a slow acetylator genotype could lead to a reduced inactivation of 5-ASA (50), which in turn allows 5-ASA to inhibit the activity of recombinant TPMT *in vitro* (51-52). In one study by Stocco et al. (50), patients with NAT1 slow metabolizer phenotype had significantly higher 6-TGN levels in comparison with rapid NAT1 metabolizers. A study completed by Hande et al. (53), concluded that 5-ASA therapy was associated with higher 6-TGN levels in adults and children with IBD in remission on Aza or 6-MP. It is possible that NAT1 influences TGN concentrations

independently of mechanisms involved in 5-ASA metabolism; i.e. allosteric binding at certain concentrations of ASAs.

In an *ex vivo* study by Xin et al. (54), the inhibitory potential of sulfasalazine (SZ), 5-aminosalicylate, and 5-ASA's main metabolite Ac-5-aminosalicylate on TPMT activities were investigated in patients with IBD. This inhibitory potential could be the consequence of its common ASA-prodrug structure, which may determine the binding affinity to TPMT. This proposed binding and inhibition is speculated to be reversible. In addition, they assessed whether there was a difference in the ASA drug interactions between thiopurine free patients or patients on long-term treatment with thiopurines. What they discovered was that while all three ASAs had inhibitory effects on TPMT activity (Figure 5), SZ was the strongest inhibitor (Figure 6, top) (54). There was no difference in thiopurine-free patients (Figure 6, bottom). Because ASAs are relatively slow in being eliminated from the body (54, 74), it is possible that the adverse effects occurring from thiopurine therapy could be the result of drug interactions between thiopurines and ASAs (55).

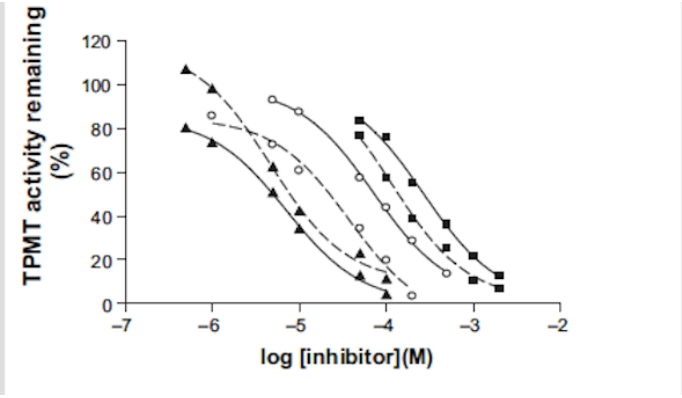


Figure 5. *Inhibition of TPMT activity in erythrocytes of two representative patients with IBD using the prodrug SZ, 5-ASA, and N-Ac-5-ASA*. Inhibition of thiopurine S-methyltransferase (TPMT) activity in erythrocytes of two representative patients with inflammatory bowel disease (IBD) by the prodrug sulfasalazine (SZ, black triangle), 5-aminosalicylate (5-ASA, black square) and its major acetylated metabolite (N-Ac-5-ASA, open circle). According to basal TPMT activity the two patients were phenotyped either as individuals with very high (62 units; solid lines) or with intermediate (20 units; broken lines) activity. (54)

Table 1. Inhibition of TPMT activity in erythrocytes from AZA-free patients with IBD by aminosalicylates

	Mean IC_{50} values (±s.d.) in μM of		
Group of IBD patients	SZ	5-ASA	Ac-5-ASA
Six patients with very high (>50 U) TPMT activity (60.4 ± 4.2 U)	9.4 (±3.1)	236* (±55)	73 (±20)
Six patients with normal (25–50 U) TPMT activity (37.9 ± 7.9 U)	17.4† (±10.9)	210 (±93)	74 (±16)
Six patients with intermediate (<25) TPMT activity (19.4 ± 4.6 U)	14.1 (±7.0)	129* (±54)	58 (±10)

* $P < 0.05$.
† See Table 2.
TPMT, thiopurine S-methyltransferase; IBD, inflammatory bowel disease; AZA, azathioprine.

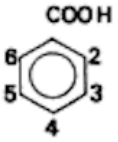
Table 2. Inhibition of TPMT activity in erythrocytes from IBD patients on AZA by aminosalicylates

	Mean IC_{50} values (±s.d.) in μM of		
Group of IBD patients	SZ	5-ASA	Ac-5-ASA
Six patients with very high (>50 U) TPMT activity (59.7 ± 6.8 U)	8.0 (±3.9)	227 (±89)	65 (±15)
Six patients with normal (25–50 U) TPMT activity (42.6 ± 5.5 U)	7.6† (±1.6)	147 (±51)	50 (±29)

†$P < 0.05$ compared with group of AZA-free patients (see Table 1).
IBD, inflammatory bowel disease; AZA, azathioprine; TPMT, thiopurine S-methyltransferase.

Figure 6. *Inhibition of TPMT activity in erythrocytes of AZA-free patients compared to patients on AZA*. **Table 1**) in all three groups, SZ was the strongest inhibitor (mean IC50 values ranged from 9 to 17 μM) if compared with 5-ASA (IC50 range: 129–236 μM) and Ac-5-ASA (IC_{50} range: 58–74 μM) in AZA-free patients. **Table 2**) in patients treated with AZA the assessment of the inhibitory action for the three aminosalicylates revealed that the corresponding IC_{50} values were very similar to those determined in AZA-free patients. (54)

In addition, benzoic acid derivatives have been shown to be potential inhibitors of TPMT activity and ASAs contain benzoic acid in their chemical structure (Figure 10). In a study by Woodson et al. (56), the benzoic acid derivative tropolone, also a catechol-O-methyltransferase (COMT) inhibitor, was shown to be a noncompetitive inhibitor of TPMT. A catechol structure contains a benzoic ring with an additional hydroxy group attached. This led to the suggestion that other COMT inhibitors might affect TPMT activity as well. COMT is one of several enzymes that degrades catecholamine, catechol estrogens, and various drugs and substances having a catechol structure. Woodson et al., furthered their studies of benzoic acid derivatives by looking at a series of benzoic acid derivatives known to inhibit TPMT. Each of the eight compounds tested inhibited TPMT activity (56). Concentrations needed to inhibit TPMT by 50% ranged from 20 μM to 2.1 mM (Figure 7).



Compound	IC_{50}	Substituents other than hydrogen			
		2	3	4	5
	mM				
DMHBA	0.02	—	OCH_3	OCH_3	OH
Veratric acid	0.10	—	OCH_3	OCH_3	—
m-Anisic acid	0.20	—	OCH_3	—	—
Salicylic acid	0.28	OH	—	—	—
Vanillic acid	0.48	—	OCH_3	OH	—
Benzoic acid	1.10	—	—	—	—
Gallic acid	1.70	—	OH	OH	OH
Acetylsalicylic acid	2.10	$OCOCH_3$	—	—	—

Figure 7: *Structures and IC_{50} for benzoic acid derivatives that inhibit TPMT (56).*

The authors then sought to determine whether there was a structure-activity relationship for benzoic acid inhibition of TPMT. Preliminary structure-activity analysis indicated that the carboxyl group was important for TPMT inhibition and that displacement of that group from the aromatic ring on a carbon side-chain decreased inhibition (Figure 8) (56). The addition of methoxy and/or phenolic hydroxyl substituents to the aromatic ring enhanced inhibition of TPMT activity (56-57). Thus, the authors concluded that TPMT may be catalyzing the methylation of nonheterocyclic aromatic sulfhydryl compounds other than the thiopurines and thiopyrimidines previously thought to be the only substrates for the enzyme, and this is possibly happening as a noncompetitive inhibition. Another study by Ames et al. (57), also looked at the structure-activity relationship for benzoic acid inhibitors and thiophenol substrates. They too, concluded that TPMT catalyzes the S-methylation of nonheterocyclic thiophenol derivatives. They also concluded that the most important factor in the binding of benzoic acid compounds to TPMT is the hydrophobicity of the first substituent, suggesting that the binding site on the enzyme for inhibitors includes a hydrophobic cleft (57). Interestingly enough, acetylsalicylic

acid, is also a benzoic acid derivative. This was one of the derivatives used in the Woodson et al. experiment that demonstrated the ability to inhibit TPMT activity.

Group	Compound	IC_{50}	Substituents other than hydrogen				
			1	2	3	4	5
		mM					
A	Benzoic acid	1.1	COOH	—	—	—	—
	Phthalic acid	2.3	COOH	COOH	—	—	—
	Isophthalic acid	2.3	COOH	—	COOH	—	—
B	*p*-Anisic acid	0.3	COOH	—	—	OCH_3	—
	p-Anisaldehyde	7.9	CHO	—	—	OCH_3	—
	Vanillic acid	0.5	COOH	—	OCH_3	OH	—
	Vanillin	1.9	CHO	—	OCH_3	OH	—
	Veratric acid	0.1	COOH	—	OCH_3	OCH_3	—
	Dimethoxybenzaldehyde	8.8	CHO	—	OCH_3	OCH_3	—
C	Vanillic acid	0.5	COOH	—	OCH_3	OH	—
	Homovanillic acid	5.5	CH_2COOH	—	OCH_3	OH	—
	Gentisic acid	0.5	COOH	OH	—	—	OH
	Homogentisic acid	5.0	CH_2COOH	OH	—	—	OH
D	Anisic acid						
	o-	>10	COOH	OCH_3	—	—	—
	m-	0.2	COOH	—	OCH_3	—	—
	p-	0.3	COOH	—	—	OCH_3	—

Figure 8. *Structure-activity relationship analysis for TPMT inhibitors*. A series of preliminary experiments was then conducted to study structure-activity relationships for benzoic acid inhibitors of TPMT. The first set of experiments (Table 2, Group A) demonstrated that compounds with more than one carboxyl group were less active than benzoic acid itself. The IC_{50} values for phthalic acid and isophthalic acid were twice that of benzoic acid (Table 2, Group A). The importance of the carboxyl group for TPMT inhibition was confirmed by comparison of the activities of benzoic acid derivatives with those of an analogous series of benzaldehyde compounds. Although all of the benzaldehydes studied were TPMT inhibitors, in each case the IC_{50} value of the corresponding benzoic acid derivative was considerably lower than that of the comparable benzaldehyde (Table 2, Group B). Exactly the opposite relationship between benzoic acid derivatives and benzaldehydes was found when similar compounds were tested as COMT inhibitors (18). Separation of the carboxyl group from the benzene ring by even a single carbon atom greatly reduced the inhibition of TPMT. The IC_{50} values of vanillic acid and gentisic acid were approximately 1 order of magnitude lower than those of homovanillic acid and homogentisic acid (Table 2, Group C). Finally, as expected, the relative positions of substituents on the ring played an important role in their effects on TPMT inhibition. When three different isomers of anisic acid were studied, the rank order of their potencies related to the position of the methoxy group was m ≥ p >> o (Table 2, Group D). (56)

Nonsteroidal anti-inflammatory drugs (NSAIDs) exhibit pharmacological effects similar to those of 5-ASA and have features in common with ASA and benzoic acid (Figure 9). It was demonstrated by Oselin et al. (58), that NSAIDs inhibit human TPMT *in vitro*, and clinically significant drug interactions may occur when thiopurines are used simultaneously with various

NSAIDs such as naproxen and mefenamic acid. This may be due to their structural determinants that are responsible for their pharmacological action in a manner similar to ASAs and benzoic acid derivatives (56, 58). ASA, benzoic acid derivatives, and NSAIDs have all been shown to be noncompetitive inhibitors of TPMT, with similar structural configurations. It is possible that they are inhibiting TPMT activity via binding the substrate based on structure and hydrophobicity (56, 58).

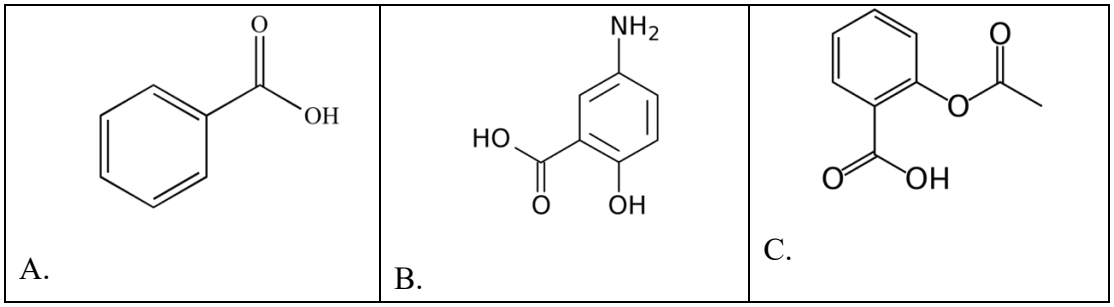


Figure 9. *A. Benzoic Acid, B. 5-ASA, C. NSAIDs (59-61).*

While not structurally similar to ASA, benzoic acid, or NSAIDs, methotrexate has been shown to bind TPMT and inhibit its enzyme activity (62). Methotrexate, a folic acid analog, was originally developed as a chemotherapy agent, but has been used in the treatment of autoimmune diseases such as rheumatoid arthritis and CD (63). Its mechanism of action is to competitively inhibit dihydrofolate reductase (DHFR), an enzyme that participates in tetrahydrofolate synthesis that is needed for essential purine and pyrimidine base biosynthesis (64-65). In the treatment of autoimmune disorders such as rheumatoid arthritis and CD, inhibition of DHFR is not thought to be the main mechanism, but rather multiple mechanisms are involved, including the inhibition of enzymes such as TPMT involved in purine metabolism. This leads to inhibition of T cell activation and suppression of intercellular adhesion molecule (ICAM) expression by T cells; selective down-regulation of B cells; increasing CD95 sensitivity of activated T cells; and inhibition of methyltransferase activity, leading to deactivation of enzyme activity relevant to immune system function (66-67).

Conclusion

Thiopurines and other immunomodulators are often used in co-therapy with other drug classes to treat IBD, including ASAs and biologics and the effect of polypharmacy on thiopurine is still being debated. Decreased thiopurine metabolism could be due to other enzymes coded in the genes that metabolizes a different class of drugs used to treat IBD, such as NAT1 and 5-ASA. Research has also debated the effect of structural similarities between thiopurines and other drug classes such as ASA and benzoic acid derivatives. It is possible that these structures either mimic thiopurines to bind at the same sight on TPMT or bind at an allosteric site. Further research is needed to deduce the location in which these molecules are binding and affecting the activity of TMPT and decreasing enzymatic activity.

Chapter 3 Methodology

Experimental Design

This was a retrospective study of CD and UC patients who have been treated with Aza or 6-MP, at Spectrum Health (SH). SH is a managed health care organization based in West Michigan and founded in 1997. SH has one of the largest groups of gastroenterologists in West Michigan, treating a variety of GI diseases including CD and UC. Data was collected from an existing database of adults diagnosed with either CD or UC who had also been genotyped for TPMT alleles and phenotyped for enzyme activity, and from the date we determined whether drug toxicity and/or disease exacerbation occurred. The occurrence of exacerbations and toxicity were recorded as a dichotomous variable (Y/N), rather than recording the number of events. A disease exacerbation was defined as a change in drug regime, change in symptoms, or need for surgery.

Definitions

- Genotype: we noted the following alleles, *1, *3C, and *3A.
- Phenotype: phenotype was defined as low, normal, or high enzyme activity. Normal is classified as 15.1-26.4 enzyme units (EU), intermediate as 6.3-15 EU, and low as <6.3 EU.
- Exacerbation: change in drug regime, escalation of symptoms, toxicity, or surgery (see Table 1)

Subjects

Deidentified data was collected from the Spectrum Health digestive disease research database, which consisted of data obtained from donors who consented to be a participant in a registry approved by the Institutional Review Board (IRB). The Spectrum Health IRB protocol

number for this project was 2011-332. The time frame for data collection in the database was 1999-2006. Subjects were adults 18 or older, male or female, with a diagnosis of UC or CD.

Data Collection

The following data was collected for each subject.

- Disease type: UC or CD
- Age: 18-61
- Gender: Male or Female
- Genotype: *1 / *1 (Wild type), *1 / *3A or *1/ *3C (Heterozygous), *3A / *3A (Homozygous)
- Phenotype represented: Normal, intermediate, or low TPMT enzyme activity was noted based on the classifications above (see Definitions)
- Exacerbation: disease exacerbation and/or toxicity

Table 1. *Criteria for diagnosing CD and UC.*

	CD	UC
Clinical	• Frequent small-volume diarrhea with urgency • Predominantly bloody diarrhea	• Diarrhea accompanied by abdominal pain and malnutrition • Abdominal mass • Perianal lesions
Endoscopic/radiological	• Diffuse superficial colonic inflammation • Involvement of rectum, but this can be patchy • Shallow erosions and ulcers • Spontaneous bleeding	• Discontinuous transmural asymmetric lesions • Mainly involving ileum and right-sided colon • Cobblestone appearance • Longitudinal ulcer • Deep fissures
Histological	• Diffuse inflammation in mucosa or submucosa • Crypt architecture distortion	• Granulomatous inflammation • Fissures or aphthous ulcers can be seen; often transmural inflammation

Statistical Analysis

Demographic analysis of age, weight, and gender were completed. The normality and equal variance assumptions were met for both age and weight. Therefore, a two-sample t-test was used to compare means between the groups with wild-type or mutant genotypes for TPMT. Gender between groups was compared using Fisher's Exact Test. For all comparisons between genotype versus phenotype (Aim 1), genotype versus toxicity and/or exacerbations (Aim 2), Fisher's Exact Test was used. Secondary analysis was also completed on genotype versus metabolite levels, and phenotype versus metabolite levels using Fisher's Exact Test. Fisher's Exact Test of independence is a nonparametric test used on small sample sizes. Given that the sample size was 30 or less, and expected cell counts that were less than 5 was greater than 20% for all categorical analyses the Fisher's Exact test was the correct statistical test to use. Numeric data is summarized as mean ± standard deviation and categorical data is summarized as frequency (percent). All analyses were assessed at a significance level of 0.05 and completed using IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.

Chapter 4 Results

A total of 62 subjects with UC and CD were screened. Of the 62, 30 were included in this study. Ten subjects were lost to follow-up post genotype/phenotype results. Twenty-two were excluded due to missing data, including genotype/phenotype, weight, and thiopurine data. Age, gender, and weight were included for each subject. Twenty-two patients were genotyped as TPMT* 1/* 1 WT. Seven were genotyped as TPMT* 1/* 3A and TPMT* 1/* 3C heterozygous for one non-functional TPMT allele. One was genotyped as TPMT* 3A/* 3A homozygous for two non-function TPMT alleles. Given the small sample size for the mutant alleles, both the groups were combined to create the mutant (M) group.

A demographic comparison of those with WT or M genotype indicated that there was no significant difference between gender, mean age, or mean weight between groups ($p = 0.242$, 0.093, and 0.522 respectively) (Table 2). While there was no significant difference, we noted that the WT group tended towards a higher mean age and lower mean weight when compared to the M group (Table 2).

Table 2. *Demographics compared between groups, N=30.*

	WT (n = 22)	**Mutant (n = 8)**	p-value
Gender (% Female)	14 (63.6)	3 (37.5)	0.242
Age	42.3 ± 13.3	33.3 ± 10.2	0.093
Weight (kg)	80.7 ± 19.9	86.4 ± 25.1	0.522

Genotype vs. Phenotype

Recall that genetic variations in TPMT have been associated with adverse drug events such as hepatotoxicity, and TPMT genotyping is now being used as a pharmacogenomic biomarker for these adverse events. However, there may be other factors influencing TPMT enzyme activity (phenotype) and consequently adverse events. Because of these confounding

factors, we wanted to ask whether genotype was indeed a good predictor of phenotypic expression. Our data indicates that these two variables are not independent of each other (p = 0.002) (Table 3), and subjects with a mutant genotype are much more likely to have a low/intermediate phenotype when compared to subjects with a wild-type genotype for TPMT (Table 3).

Table 3. *TPMT genotype versus TPMT enzyme activity, N=30.*

	Mutant (n = 8)	**WT (n = 22)**	**p-value**
Low/Intermediate	8 (100.0)	7 (31.8)	0.002
Normal	0 (0.0)	15 (68.2)	

Metabolite Levels

Thiopurine metabolism produces two major metabolites, 6-MeMPN and 6-TGN (Figure 4). 6-TGN is the metabolite responsible for most adverse events associated with thiopurines, as it is incorporated into DNA to ultimately dampen the inflammatory response and leads to cell death. Because of this, one would expect that those with normal TPMT enzymatic activity (WT) to inactivate thiopurines would have corresponding low levels of metabolite production. Similarly, those with mutations in TPMT would have high levels of metabolites, leading to higher risk of toxicity. We tested the association between genotype and metabolite levels. If genotype was normal, intermediate, or low, then we predicted that metabolite levels would be normal, intermediate, or low respectively in accordance with the likelihood of response for 6-TGN and risk of hepatotoxicity for 6-MeMPN.

To study this relationship between genotype and metabolite levels, a 6-TGN level of 0-230 pmol/8x10^8 RBC was classified as a high response, 230-400 pmol/8x10^8 RBC as a low response, and 400-600 pmol/8x10^8 RBC as a higher risk for leukocytopenia. However, no patients presented with 6-TGN metabolite levels in the higher risk category. The median value

for those with a TPMT mutation was 287 pmol/8x10^8 RBC, which was much higher than the median value for the wild-type group (178 pmol/8x10^8 RBC). There was significant evidence to suggest an association between genotype and 6-TGN levels (p = 0.029) (Table 4). Subjects with a mutant TPMT genotype were also much more likely to respond to thiopurine therapy when compared to patients with a wild-type genotype (Table 4). This was quite interesting, as our initial hypothesis predicted that subjects with one or both non-functional TPMT alleles would have a lower response or higher risk of leukocytopenia respectively. Because all 4 subjects in the mutant group fell into the high response category, while none were in the low response group, it is difficult to answer the question with the available data (Table 4).

Table 4. *Genotype vs. responsiveness to 6-TGN therapy, N=17.*

	Mutant (n = 4)	**WT (n = 13)**	**p-value**
High response	4 (100.0)	4 (30.8)	0.029
Low response	0 (0.0)	9 (69.2)	

6-MeMP metabolites were classified with regard to risk of hepatotoxicity: 0-5700 = lower risk of hepatotoxicity (LRH) and >5700 = high risk of hepatotoxicity (HRH). When comparing genotype to 6-MeMPN metabolite levels, we hypothesized that WT genotypes would have lower risk of hepatotoxicity, because two normal alleles leads to normal enzymatic activity and therefore less 6-MP available for the active pathway to convert to metabolite. The median value for the group with mutant TPMT was 528 pmol/8x10^8 RBC and was much lower than the median value for the wild-type TPMT group (1493 pmol/8x10^8 RBC). However, there was no evidence to suggest a relationship between genotype and 6-MeMPN levels (p = 1.00) (Table 5). This is likely due to the small number of subjects in the mutation group. Like 6-TGN, no subjects with a mutant genotype had a designation of high risk for hepatotoxicity. It was also noted that there were more WT subjects with a lower risk of hepatotoxicity (Table 5). This

agrees with our hypothesis that WT genotypes would have a lower risk of hepatotoxicity, because two normal alleles have normal enzymatic activity, decreasing the amount of 6-MP available for the active pathway. However, there were low numbers and multiple outliers in the wild-type group, which may be another reason for the overall non-significant result.

Table 5. *Genotype vs. risk of hepatotoxicity with 6-MeMPN Levels, N=17.*

	Mutant (n = 4)	**WT (n = 13)**	**p-value**
Low risk hepatotoxicity	4 (100.0)	11 (84.6)	1.00
High risk hepatotoxicity	0 (0.0)	2 (15.4)	

Genotype had a significant correlation with 6-TGN levels but not with 6-MeMPN levels. Genotype is a major influencing factor in the development of phenotype, but it is not the only one. Phenotypic plasticity defines the degree to which phenotype can be predicted by genotype. For example, if a phenotype can reliably be predicted from phenotype, it is said to have little plasticity. However, if confounding factors influence phenotypic expression, this causes high plasticity. It is possible that phenotype is a truer representation of enzyme activity, because it reflects metabolite levels more accurately. To determine whether phenotypic expression would be a better predictor of metabolite levels than genotype, we tested the association between TPMT enzyme activity, and 6-TGN or 6-MeMPN metabolite levels.

The results provide no significant evidence of a relationship between enzyme activity and 6-TGN levels ($p = 0.153$) (Table 6). This is evident in the fact that there no significant difference in the mean 6-TGN levels between the mutant and wild-type group, 243.6 pmol/$8x10^8$ RBC and 166.1 pmol/$8x10^8$ RBC respectively (data not shown). In Table 6, there may be some evidence of a trend that showing normal phenotypes tend to be in the high response group compared to the low/intermediate phenotypes. More testing with subjects should be done to determine if this trend may be present.

Table 6. *Phenotype vs. responsiveness to 6-TGN levels, N=17.*

	Low/Intermediate (n = 9)	Normal (n = 8)	p-value
Low response	6 (66.7)	2 (25.0)	0.153
High response	3 (33.3)	6 (75.0)	

Similarly, there was no significant evidence to suggest that phenotype was a good predictor of 6-MeMPN levels (p = 0.206) (Table 7). In Table 7, all mutant subjects fell into the lower risk of hepatotoxicity compared to 6 in the normal subjects. There were no mutants in the higher risk of hepatotoxicity. This was contradictory to our hypothesis. The fact that all mutants were in the lower risk group and small sample size may be contributing to this non-significant result, since we hypothesized that low/intermediate enzyme activity would result in a higher risk of hepatotoxicity given that they would be unable to convert 6-MP into it's inactive form 6-MeMP. More testing should be done to determine if there is a true association or not.

Table 7. *Phenotype vs risk of hepatotoxicity with 6-MeMPN levels, N=17.*

	Low/Intermediate (n = 9)	Normal (n = 8)	p-value
Low risk hepatotoxicity	9 (100.0)	6 (75.0)	0.206
High risk hepatotoxicity	0 (0.0)	2 (25.0)	

Genotype vs Cytotoxicity

Lastly, we wanted to ask whether the genotype was a good predictor of the presence of toxicity and/or exacerbation. We predicted that subjects with a normal genotype would experience no toxicity/exacerbations because normal enzyme activity would reduce the amount of 6-MP available for the active pathway to convert to 6-TGN, leaving less to cause cytotoxicity. There was as a total of 30 subjects. However, one was missing information about toxicity or exacerbation, and was therefore excluded from the analysis. There was no significant evidence to show there is a relationship between the genotype and the presence toxicity (p = 1.00) (Table 8).

Surprisingly, the WT group had more subjects with toxicity than the mutant group (Table 8). The small size of the mutation group had an impact on achieving significance.

Table 8. *Genotype vs Toxicity/Exacerbation, N=29.*

Toxicity/Exacerbation	Mutant (n = 7)	WT (n = 22)	p-value
No	2 (28.6)	8 (36.4)	1.00
Yes	5 (71.4)	14 (63.6)	

Chapter 5 Discussion and Conclusions

TPMT is the key enzyme in the pathway by which 6-MP gets converted to the inactive metabolite 6-MeMP. This reduces the amount of 6-MP available for HGPRT to convert to 6-TGN in the active pathway. Reduced activity of TPMT can lead to an increase in 6-MP and therefore an increase in 6-TGN, leading to cytotoxicity. However, the TPMT enzyme is not only affected by genotype, but other factors as well, including polypharmacy. Thiopurines and ASAs, especially 5-ASA, are often combined in the treatment of IBD, and studies have shown the possibility of an interaction between these drug classes, that impacts the TPMT enzyme activity. These effects on TPMT may directly or indirectly influence the efficacy of thiopurines, depending on the level of metabolites generated in hematopoietic tissues. In our present study, we tried to determine the extent to which the TPMT genotype impacted the enzyme's activity, the generation of 6-TGN and 6-MeMP and resulting toxicity. We also tried to gain additional insight about whether enzymatic activity might be a better predictor of metabolite levels than genotyping.

We demonstrated a correlation between genotype and phenotype, and it was shown that these two variables were not independent of each other. In other words, subjects with a mutant TPMT genotype were much more likely to have low/intermediate enzymatic activity when compared to subjects with the wild-type TPMT genotype. While the data indicated an association between genotype and 6-TGN, it did not show a significant relationship with 6-MeMP. This may be due to the fact that all mutants, had a higher response when comparing genotype to 6-TGN, indicating an ability to reach therapeutic efficacy, This contradicted the given hypothesis that genotypes with low or intermediate activity would have a lower response to thiopurines or a higher risk of cytotoxicity, respectively. TPMT enzyme activity comparisons showed no significant relationship with 6-TGN levels although there may be some evidence of a trend.

TPMT enzyme activity also showed no significant relationship with 6-MeMP. This could be attributed to having no mutant TPMT subjects with a high risk of hepatotoxicity. This too, did not agree with the initial hypothesis. Lastly, we noted that genotype showed no correlation with toxicity and/or exacerbation. Interestingly, the WT genotypes group actually had more subjects with toxicity than the mutant group. As stated previously, we expected WT genotypes to have two functional alleles, and therefore normal enzymatic activity. This was not the case in the present study.

Even though there were some significant associations, the majority of the comparisons tested were not statistically significant. However, the results obtained in this study had several limitations that were contributing factors. First, there was a small sample size. With only 30 subjects included in this study, we had to resort to using mostly non-parametric testing. Sample size is directly proportional to the confidence level of the study. Therefore, a small sample size reduces the confidence level in the results. This result could result in potentially false significant or false non-significant results. This could compromise the conclusions drawn from the present study. Second, in several comparisons multiple subjects were excluded due to missing data. For example, in metabolite level comparisons, 13 subjects were missing this data in their records. Lastly, we did not have access to patient pharmaceutical lists, which would have allowed us to determine the commonality of drugs between subjects and whether polypharmacy was affecting phenotypic expression or metabolite levels.

Ha et al. (41) observed that although TPMT genotype generally correlated with enzymatic activity, some patients with two normal alleles nevertheless had intermediate/low TPMT activity. They suggested that a TPMT enzymatic assay might be more informative than TPMT genotyping. In addition, Ha et al. thought that ITPase deficiency could cause accumulation of 6-TITP, which activates 6-MP. Inosine monophosphate (IMP) is a central intermediate in purine

metabolism that gets converted to adenine or guanine nucleotides. ITPase converts inosine triphosphate (ITP) back to IMP. Deficiency of ITPase leads to accumulation of ITP. Since ITP can readily substitute for GTP-requiring actions such as thiopurine metabolism, the accumulation in 6-TITP could cause toxicity by inhibiting GTP-mediated pathways (41,68). A retrospective study found that the allele associated with ITPase deficiency was significantly linked to adverse drug reactions to Aza (71).

Several studies have addressed the correlation of 6-TGN concentrations with thiopurine effectiveness but have yielded conflicting results. In a clinical setting, Dubinsky et al. found that the 6-MP response correlated with 6-TGN concentrations above 235 pmol/8 x 10^8 RBCs (69). Similarly, Cuffari et al. found that higher 6-TGN concentrations correlated with higher response to thiopurines and maintenance of inflammation in adults with IBD (70). Other studies, such as Goldenberg et al. (71), have disputed the value of 6-TGN levels in predicting the response to thiopurine therapies, stating less than 40% of the subjects had 6-TGN concentrations over 235 pmol/8 x 10^8 RBCs.

The metabolites are responsible for achieving a therapeutic response in the active pathway of thiopurine metabolism. The main 6-TGN nucleotides incorporated into DNA are 6-thio-GMP, -GDP, and -GTP. Neurath et al. developed assays to measure these three nucleotides separately and found that patients with higher 6-TGN concentrations demonstrated a better response rate (72-73). Of note in their study, subjects with increased 6-thio-GDP levels had a worse outcome (72-73). Prospective studies could focus on manipulating 6-TGN nucleotide concentrations to see if there is an effect on therapeutic response.

In the present study, we focused on genotyping and whether it is a good predictor of phenotype. Multiple studies have focused on dose escalation based on genotype. However, genotyping in these studies only includes the most common alleles and also does not consider

cofounding factors such as polypharmacy, which affects thiopurine metabolism. Allopurinol, a uric acid reducer, has been shown to have effects on metabolite concentrations and thiopurine effectiveness, but Sparrow et al. were unable to demonstrate a precise mechanism of action for this effect (73). As mentioned previously, several studies have focused on ASAs, NSAIDs, and benzoic acid derivatives and the role they play in thiopurine metabolism. Most importantly, they focused on the structural influence these therapies have on thiopurine metabolism. These authors assumed an allosteric inhibition that would decrease TPMT's affinity for 6-MP. However, the site of binding is still unknown.

Several future studies could be done to further contribute to this thiopurine research. A prospective study would be advantageous because it would provide a larger sample size and allow us to follow these effects over time. It would also be helpful to measure enzyme activity at multiple time points to confirm the reliability of the results and to look for trends. In addition, liver function tests should be obtained to provide a numerical representation of cytotoxicity rather than relying on more subjective measures of cytotoxicity noted in the subject's medical records. This would allow comparison between enzyme activity, change in drug therapy, and liver function tests.

Other future studies could focus on the nature of the interaction between TPMT enzyme and other drugs that affect its activity. It is possible that the drugs that influence TPMT activity are acting as allosteric inhibitors of TPMT. However, the specific site for allosteric inhibition is still unknown. Future studies should determine what site, if any, these other drugs are binding to and how it affects both TPMT activity and the enzymes affinity for thiopurines. Lastly, future studies should further look into the metabolite concentrations and their effect on dose response.

In conclusion, implementing thiopurine therapy based on genotype is beneficial, but it does not account for the effects of polypharmacy on enzymatic activity. In addition, the genotype-

phenotype relationship is best viewed from two perspectives, one at the genetic level and one at the phenotypic level. Normal genotype-phenotype testing often does not take into account the combined effect of mutations and environmental factors on true enzyme activity. It is best to view genotype-phenotype as an integration of development involving genes, pleiotropy, epistasis, and environmental factors. Thinking of genotype-phenotype relationships in integrated terms clarifies the comparison between environmental and genetic effects on phenotype and helps us to further understand the connection between genotypes and phenotypes.

References

1. Langholz E. Current trends in inflammatory bowel disease: the natural history. Ther. Adv. Gastroenterol. 2010;3:77–86.

2. Hold GL, Smith M, Grange C, Watt ER, El-Omar EM, Mukhopadhya I. Role of the gut microbiota in inflammatory bowel disease pathogenesis: What have we learnt in the past 10 years? World J. Gastroenterol. WJG. 2014;20:1192–210.

3. Sartor RB. Mechanisms of Disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat. Clin. Pract. Gastroenterol. Hepatol. 2006;3:390–407.

4. Smith, Phillip D. "Inflammatory Bowel Disease." Principles of Mucosal Immunology, Garland Science, 2013, pp. 473–489.

5. Agrawal Dilip, Ranawat MS, Khinchi Mahaveer P, Gupta MK, Sharma Natasha, Avlani Gunjan. Diagnosis and Treatment of Colorectal Cancer: A Review.

6. Jess T, Rungoe C, Peyrin–Biroulet L. Risk of colorectal cancer in patients with ulcerative colitis: A meta-analysis of population-based cohort studies. Clin. Gastroenterol. Hepatol. 2012;10:639–45.

7. Triantafillidis JK, Nasioulas G, Kosmidis PA. Colorectal cancer and inflammatory bowel disease: Epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies. Anticancer Res. 2009;29:2727–37.

8. Katz S, Lichtenstein GR, Safdi MA. 5-ASA Dose-response. Gastroenterol. Hepatol. 2010;6:1–16.

9. Caprilli, Renzo, et al. "Mechanisms of action of 5-ASA. Mesalazine inhibits many inflammatory mediators." Science Direct, Elsevier B.V., ars.els-cdn.com/content/image/1-s2.0-S1873994609000555-gr2.jpg.

10. Caprilli, Renzo, Monica Cesarini, Erika Angelucci, and Giuseppe Frieri. The long journey of salicylates in ulcerative colitis: The past and the future. Journal of Crohn's and Colitis 2009; 3(3): 149–156.

11. "Immunomodulator." Merriam-Webster, Merriam-Webster, www.merriam-webster.com/dictionary/immunomodulator.

12. Park, Sung Chul, and Yoon Tae Jeen. Current and emerging biologics for ulcerative colitis. Gut and Liver 2015; 9(1): 18–27.

13. Shah B, Mayer L. Current status of monoclonal antibody therapy for the treatment of inflammatory bowel disease. Expert Rev. Clin. Immunol. 2010; 6: 607–20.

14. Black AJ, McLeod HL, Capell HA, Powrie RH, Matowe LK, Pritchard SC, et al. Thiopurine methyltransferase genotype predicts therapy-limiting severe toxicity from azathioprine. Ann. Intern. Med. 1998; 129:716–8.

15. Axelrad, Jordan E, Abhik Roy, Garrett Lawlor, Burton Korelitz, and Simon Lichtiger Thiopurines and inflammatory bowel disease: Current evidence and a historical perspective. World Journal of Gastroenterology 2016; 22(46): 10103–10117.

16. Bär, Florian, Christian Sina, and Klaus Fellermann. Thiopurines in inflammatory bowel disease revisited. World Journal of Gastroenterology : WJG. 2013; 19(11): 1699–1706.

17. Cuffari C, Dassopoulos T, Turnbough L, Thompson RE, Bayless TM. Thiopurine methyltransferase activity influences clinical response to azathioprine in inflammatory bowel disease. Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc. 2004; 2:410–7.

18. Nielsen OH, Vainer B, Rask-Madsen J. Review article: the treatment of inflammatory bowel disease with 6-mercaptopurine or azathioprine. Aliment. Pharmacol. Ther. 2001; 15:1699–708.

19. Tremaine WJ. Refractory IBD: medical management. Neth. J. Med. 1997; 50:S12-14.

20. Nguyen GC, Chong CA, Chong RY. National estimates of the burden of inflammatory bowel disease among racial and ethnic groups in the United States. J Crohns Colitis. 2014; 8:288–95.

21. Lennard L, Gibson BE, Nicole T, Lilleyman JS. Congenital thiopurine methyltransferase deficiency and 6-mercaptopurine toxicity during treatment for acute lymphoblastic leukaemia. Arch. Dis. Child. 1993; 69:577–9.

22. Lennard L, Van Loon JA, Weinshilboum RM. Pharmacogenetics of acute azathioprine toxicity: relationship to thiopurine methyltransferase genetic polymorphism. Clin. Pharmacol. Ther. 1989; 46:149–54.

23. Yatscoff RW, Aspeslet LJ. The monitoring of immunosuppressive drugs: a pharmacodynamic approach. Ther. Drug Monit. 1998; 20:459–63.

24. Lennard L. Assay of 6-thioinosinic acid and 6-thioguanine nucleotides, active metabolites of 6-mercaptopurine, in human red blood cells. J. Chromatogr. 1987; 423:169–78.

25. Sandborn WJ. A review of immune modifier therapy for inflammatory bowel disease: azathioprine, 6-mercaptopurine, cyclosporine, and methotrexate. Am. J. Gastroenterol. 1996; 91:423–33.

26. Lennard L. The clinical pharmacology of 6-mercaptopurine. Eur. J. Clin. Pharmacol. 1992; 43:329–39.

27. Dubinsky MC, Reyes E, Ofman J, Chiou C-F, Wade S, Sandborn WJ. A cost-effectiveness analysis of alternative disease management strategies in patients with Crohn's disease treated with azathioprine or 6-mercaptopurine. Am. J. Gastroenterol. 2005; 100:2239–47.

28. Relling M, Gardner E, Sandborn W, Schmiegelow K, Pui C-H, Yee S, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. Clin. Pharmacol. Ther. 2011; 89:387–91.

29. Foram Mehta, MS, RPh. Report: Economic implications of inflammatory bowel disease and its management (Internet). AJMC. (cited 2016 Jun 22). Available from: http://www.ajmc.com/journals/supplement/2016/importance_of_selecting_appropriate_therapy_inflammatory_bowel_disease_managed_care_environment/importance_of_selecting_appropriate_therapy_inflammatory_bowel_disease_managed_care_environment_report_economic_implications_ibd/P-3

30. Park KT, Colletti RB, Rubin DT, Sharma BK, Thompson A, Krueger A. Health insurance paid costs and drivers of costs for patients with Crohn's Disease in the United States. Am. J. Gastroenterol. 2016; 111:15–23.

31. K.T. Park MD, Dorsey Bass MD. Inflammatory bowel disease-attributable costs and cost-effective strategies in the United States: A review. Inflamm. Bowel Dis. 2011; 17:1603–9.

32. Xu X-R, Liu C-Q, Feng B-S, Liu Z-J. Dysregulation of mucosal immune response in pathogenesis of inflammatory bowel disease. World J Gastroenterol. 2014; 20:3255–64.

33. Lane ER, Zisman TL, Suskind DL. The microbiota in inflammatory bowel disease: current and therapeutic insights. J Inflamm Res. 2017; 10:63–73.

34. Hold GL, Smith M, Grange C, Watt ER, El-Omar EM, Mukhopadhya I. Role of the gut microbiota in inflammatory bowel disease pathogenesis: What have we learnt in the past 10 years? World J Gastroenterol. 2014; 20:1192–210.

35. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004; 118:229–241.

36. Abraham C, Medzhitov R. Interactions between the host innate immune system and microbes in inflammatory bowel disease. Gastroenterology. 2011; 140:1729–1737

37. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. Nature. 2011; 474:307–17.

38. Azathioprine metabolism and TPMT: Overview, clinical implications of the genetic mutation, testing for the genetic mutation. 2016 (cited 2017 Jan 3); Available from: http://emedicine.medscape.com/article/1829596-overview

39. Chemical structures of prodrug azathioprine and its active moiety. www.researchgate.net/profile/Rafik_Karaman/publication/272565621/figure/fig9/AS:391823290847270@1470429360485/Figure-17-Chemical-structures-of-the-prodrug-azathioprine-and-its-active-moiety.png.

40. Nielsen OH, Vainer B, Rask-Madsen J. The treatment of inflammatory bowel disease with 6-mercaptopurine or azathioprine. Aliment. Pharmacol. Ther. 2001; 15:1699–708.

41. Ha, Christina, and Themistocles Dassopoulos. Thiopurine therapy in inflammatory bowel disease. Expert Review of Gastroenterology & Hepatology; London 2010; 4(5): 575–88.

42. Meijer, Berrie, Joany E Kreijne, Sofia A W van Moorsel, et al. 6-Methylmercaptopurine induced leukocytopenia during thiopurine therapy in IBD patients. Journal of Gastroenterology and Hepatology: 2016. n/a.

43. Leong, Rupert & Gearry, Richard & Sparrow, Miles. Thiopurine hepatotoxicity in inflammatory bowel disease: The role for adding allopurinol. Figure 1. Metabolism of 6-MP. (2008); 607-16.

44. Richard MacDermott, MD, Paul Rutgeerts, MD, PhD, FRCP, Shilpa Grover, MD, MPH. 6-mercaptopurine (6-MP) metabolite monitoring and TPMT testing in the treatment of inflammatory bowel disease with 6-MP or Azathioprine. UpToDate (Internet). 2015; Available from: http://www.uptodate.com/contents/6-mercaptopurine-6-mp-metabolite-monitoring-and-tpmt-testing-in-the-treatment-of-inflammatory-bowel-disease-with-6-mp-or-azathioprine#subscribeMessage

45. "Figure 1. Metabolism pathway of azathioprine: Competing pathways result in inactivation by TPMT or XO, or incorporation of cytotoxic nucleotides into DNA." DailyMed, dailymed.nlm.nih.gov/dailymed/fda/fdaDrugXsl.cfm?setid=d65bc662-2ae8-4ddb-acc8-f616e72e6140&type=display.

46. Leong, Rupert & Gearry, Richard & Sparrow, Miles. Thiopurine hepatotoxicity in inflammatory bowel disease: The role for adding allopurinol. 2008: volume and page number

48. Coenen, Marieke J. H., Dirk J. de Jong, Corine J. van Marrewijk, et al. Identification of patients with variants in TPMT and dose reduction reduces hematologic events during thiopurine treatment of inflammatory bowel disease. Gastroenterology 2015; 149(4): 907–917.e7.

47. Cuffari C, Théorêt Y, Latour S, Seidman G. 6-Mercaptopurine metabolism in Crohn's disease: correlation with efficacy and toxicity. Gut. 1996; 39:401–6.

49. Egan LJ. Mechanisms of drug toxicity or intolerance. DDI. 2011; 29:172–6.

50. Stocco G, Cuzzoni E, De Iudicibus S, Favretto D, Malusà N, Martelossi S, et al. Thiopurine metabolites variations during co-treatment with aminosalicylates for inflammatory bowel disease: Effect of N-acetyl transferase polymorphisms. World J Gastroenterol. 2015; 21:3571–8.

51. Szumlanski CL, Weinshilboum RM. Sulphasalazine inhibition of thiopurine methyltransferase: possible mechanism for interaction with 6-mercaptopurine and azathioprine. Br J Clin Pharmacol 1995; 39: 456-459 (PMID: 7640156)

52. Lewis LD, Benin A, Szumlanski CL, Otterness DM, Lennard L, Weinshilboum RM, Nierenberg DW. Olsalazine and 6-mercaptopurine-related bone marrow suppression: a possible drug interaction. Clin Pharmacol Ther 1997; 62: page numbers

53. Hande S, Wilson-Rich N, Bousvaros A, Zholudev A, Maurer R, Banks P, et al. 5-aminosalicylate therapy is associated with higher 6-thioguanine levels in adults and children with inflammatory bowel disease in remission on 6-mercaptopurine or azathioprine. Inflamm Bowel Dis. 2006; 12:251–7.

54. H. Xin, C. Fischer, M. Schwab, U. Klotz. Effects of aminosalicylates on thiopurine S-methyltransferase activity: an ex vivo study in patients with inflammatory bowel disease. Alimentary Pharmacology & Therapeutics. 2005; 1105–9.

55. Lowry PW, Franklin CL, Weaver AL, Szumlanski CL, Mays DC, Loftus EV, et al. Leucopenia resulting from a drug interaction between azathioprine or 6-mercaptopurine and mesalamine, sulphasalazine, or balsalazide. Gut. 2001; 49:656–64.

56. Woodson LC, Ames MM, Selassie CD, Hansch C, Weinshilboum RM. Thiopurine methyltransferase. Aromatic thiol substrates and inhibition by benzoic acid derivatives. Mol Pharmacol. 1983; 24:471–8.

57. Ames MM, Selassie CD, Woodson LC, Van Loon JA, Hansch C, Weinshilboum RM. Thiopurine methyltransferase: structure-activity relationships for benzoic acid inhibitors and thiophenol substrates. J Med Chem. 1986; 29:354–8.

58. Oselin K, Anier K. Inhibition of human thiopurine S-methyltransferase by various nonsteroidal anti-inflammatory drugs in vitro: A mechanism for possible drug interactions. Drug Metab Dispos. 2007; 35:1452–4.

59. “Aspirin-Skeletal.svg.” Cholesterol (Chemical Structure).Svg - Wikimedia Commons, 16 Apr. 2008, commons.wikimedia.org/wiki/File:Aspirin-skeletal.svg.

60. “Illustrated Glossary of Organic Chemistry - Benzoic Acid; Benzoate; Benzoate Group.” How to Use a Rotary Evaporator, www.chem.ucla.edu/~harding/IGOC/B/benzoic_acid.html.

61. “Mesalazine.” Wikipedia, Wikimedia Foundation, 7 May 2018, en.wikipedia.org/wiki/Mesalazine#/media/File:Mesalazine_structure.svg.

62. Wennerstrand P, Mårtensson L-G, Söderhäll S, Zimdahl A, Appell ML. Methotrexate binds to recombinant thiopurine S-methyltransferase and inhibits enzyme activity after high-dose infusions in childhood leukaemia. Eur J Clin Pharmacol. 2013; 69:1641–9.

63. Herfarth HH, Long MD, Isaacs KL. Methotrexate: underused and ignored? Dig Dis. 2012; 30 Suppl 3:112–8.

64. Rajagopalan PTR, Zhang Z, McCourt L, Dwyer M, Benkovic SJ, Hammes GG. Interaction of dihydrofolate reductase with methotrexate: Ensemble and single-molecule kinetics. Proc Natl Acad Sci U S A. 2002; 99:13481–6.

65. Goodsell DS. The molecular perspective: methotrexate. The Oncologist. 1999; 4:340–1.

66. Böhm I. Increased peripheral blood B-cells expressing the CD5 molecules in association to autoantibodies in patients with lupus erythematosus and evidence to selectively down-modulate them. Biomedicine & Pharmacotherapy. 2004; 58:338–43.

67. Wessels J a. M, Huizinga TWJ, Guchelaar H-J. Recent insights in the pharmacological actions of methotrexate in the treatment of rheumatoid arthritis. Rheumatology (Oxford). 2008; 47:249–55.

68. Marinaki AM, Duley JA, Arenas M et al. Mutations in the ITPA gene predicts intolerance to azathioprine. Nucleosides Nucleotides Nucleic Acids 2398-9). 2004; 1393-1397.

69. Dubinsky MC, Lamothe S, Yang HY et al. Pharmacogenomics and metabolite measurement for 6-mercaptopurine therapy in inflammatory bowel disease. Gastroenterology 2000; 118(4), 705-713.

70. Goldenberg BA, Rawsthoren P, Bernstein CN. The utility of 6-thioguanine metabolite levels in managing patients with inflammatory bowel disease. Am. J. Gastroenterol. 2004; 99(9): 1744-1748.

71. Neurath MF, Kiesslich R, Teichgraber U et al. 6-thioguanosine diphosphate and triphosphate levels in red blood cells and response to azathioprine therapy in Crohn's disease. Clin. Gastroenterol. Hepatol. 2005; 3(10): 1007-1014.

72. Kiszka-Kanowitz, Marianne, Klaus Theede, and Anette Mertz-Nielsen. Randomized Clinical Trial: A Pilot Study Comparing Efficacy of Low-Dose Azathioprine and Allopurinol to Azathioprine on Clinical Outcomes in Inflammatory Bowel Disease. Scandinavian Journal of Gastroenterology 2016; 51(12): 1470–1475.

73. Sparrow MP, Hande SA, Friedman S, Cao D, Hanauer SB. Effect of allopurinol on clinical outcomes in inflammatory bowel disease nonreponders to azathioprine or 6-mercaptopurine. Clin. Gastroenterol. Hepatol. 2007; 5(2), 209-214.

74. Klotz U. Clinical pharmacokinetics of sulphasalazine, its metabolites and other prodrugs of 5-aminosalicylic acid. Clin Pharmacokinet. 1985; 10:285–302.

75. McLeod HL, Siva C. The thiopurine S-methyltransferase gene locus – implications for clinical pharmacogenomics. Pharmacogenomics. 2002; 3:89–98.