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TOPIC HIGHLIGHT

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# Pharmacogenetics of azathioprine in inflammatory bowel disease: A role for glutathione-S-transferase?

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## Abstract

Azathioprine is a purine antimetabolite drug commonly used to treat inflammatory bowel disease (IBD). *In vivo* it is active after reaction with reduced glutathione (GSH) and conversion to mercaptopurine. Although this reaction may occur spontaneously, the presence of isoforms M and A of the enzyme glutathione-S-transferase (GST) may increase its speed. Indeed, in pediatric patients with IBD, deletion of GST-M1, which determines reduced enzymatic activity, was recently associated with reduced sensitivity to azathioprine and reduced production of azathioprine active metabolites. In addition to increase the activation of azathioprine to mercaptopurine, GSTs may contribute to azathioprine effects even by modulating GSH consumption, oxidative stress and apoptosis. Therefore, genetic polymorphisms in genes for GSTs may be useful to predict response to azathioprine even if more *in vitro* and clinical validation studies are needed.



**Key words:** Inflammatory bowel disease; Azathioprine; Pharmacogenetics; Glutathione-S-transferase; Pediatric patients

**Core tip:** Polymorphisms of glutathione-S-transferase-M1 may influence azathioprine effects in young patients with inflammatory bowel disease by increasing the drug activation and by modulating oxidative stress and apoptosis.

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## INTRODUCTION

Azathioprine, the 1-methyl-4-nitroimidazol-5-yl derivative of mercaptopurine, is a purine antimetabolite drug commonly used to treat inflammatory bowel disease (IBD). Despite the introduction of effective biological treatments, such as antibodies against tumor necrosis factor-α (TNF-α), azathioprine is still a mainstay for maintenance therapy of severe IBD. Azathioprine is a prodrug and requires complex conversion to active metabolites (Figure 1). The first step in this conversion is the reaction of azathioprine with reduced glutathione (GSH), to yield the prodrug mercaptopurine and a nitroimidazole derivative/conjugate of GSH. Even though this reaction can occur spontaneously[1] the presence of the enzyme glutathione-S-transferase (GST) may increase its speed,

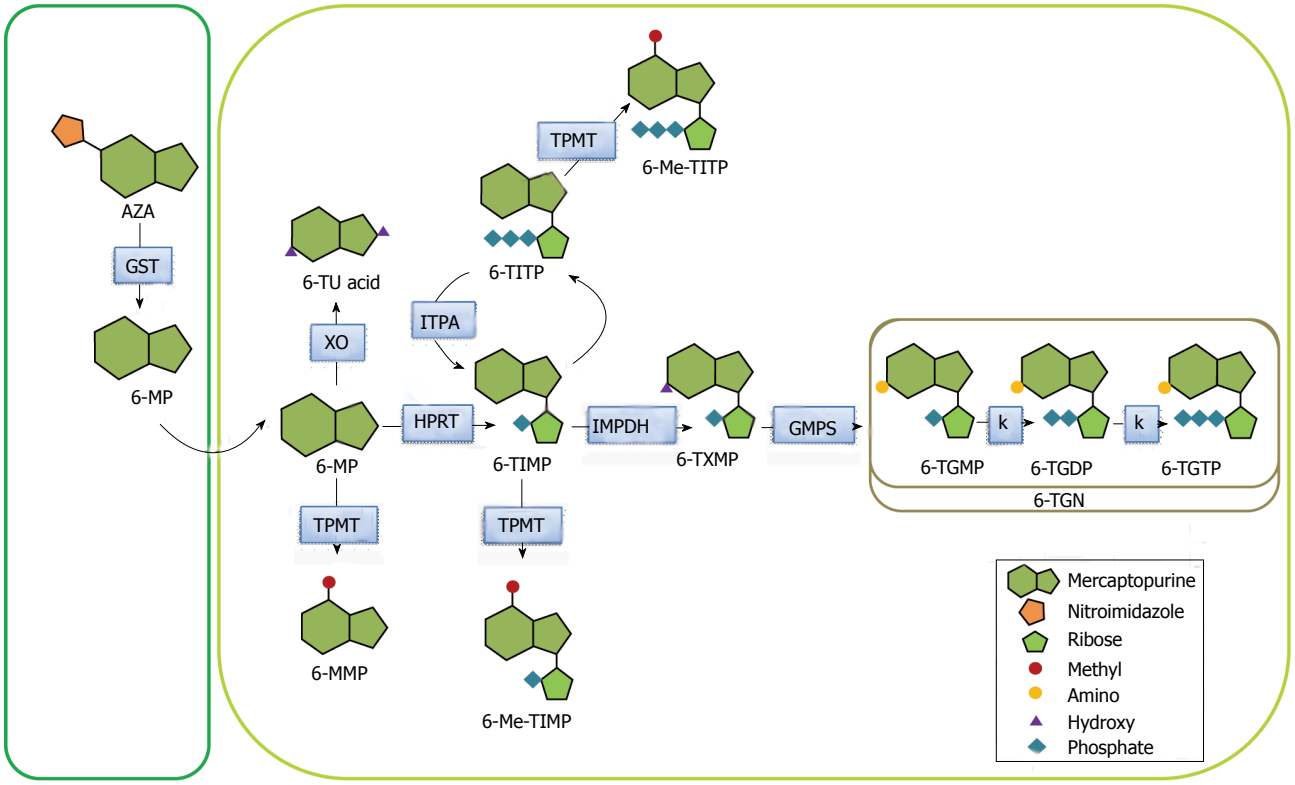


**Figure 1 Metabolism of azathioprine and mercaptopurine.** 6-Me-TIMP: 6-methyl-thioinosine-monophosphate; 6-Me-TITP: 6-methyl-thioinosine-triphosphate; 6-MMP: 6-methyl-mercaptopurine; 6-MP: Mercaptopurine; 6-TGDP: 6-thioguanine-diphosphate; 6-TGMP: 6-thioguanine-monophopsphate; 6-TGN: 6-thioguanine nucleotide; 6-TGTP: 6-thioguanine-triphopshate; 6-TIMP: 6-thioinosine-monophosphate; 6-TITP: 6-thioinosine-triphosphate; 6-TU: 6-thiouric; 6-TXMP: 6-thioxanthosine-monophosphate; AZA: Azathioprine; GMPS: Guanosine-monophosphate-synthase; GST: glutathione-S-transferase; HPRT: Hypoxanthinephosphate-rybosil-transferase; IMPDH: Inosine-monophosphate-dehydrogenase; k: Kinase; TPMT: Thiopurine-S-methyl-transferase; XO: Xanthine-oxidase.

as discussed later. Even mercaptopurine needs metabolic conversion to thioguanine nucleotides (TGNs), catalyzed by the enzymes of the purine salvage pathway. Moreover, mercaptopurine is inactivated in the liver mainly by xanthine oxidase (XO), while in the extra hepatic tissues mercaptopurine catabolism involves predominantly genes that display genetically determined polymorphic activity, such as thiopurine-S-methyltransferase (TPMT) and inosine triphosphate pyrophosphatase (ITPA). After oral administration, intact azathioprine is undetectable in blood because of extensive first pass metabolism[2].

Mercaptopurine's cytotoxic effects are mainly related to incorporation of the active TGNs in the nucleic acids and to the consequent interference with the function of DNA processing enzymes and, to some extent, to inhibition of *de novo* purine synthesis, mainly operated by methylated precursors of TGNs.

While mercaptopurine pharmacokinetics and pharmacodynamics have been characterized extensively, the mechanism of conversion of azathioprine to mercaptopurine and its clinical implications for therapy personalization have not been completely elucidated.

## EFFECTS OF GST POLYMORPHISMS ON AZATHIOPRINE EFFICACY AND METABOLISM IN PATIENTS WITH IBD

The hypothesis that patients with reduced levels of specific GST isoforms, due to genetic polymorphisms, may present decreased sensitivity to azathioprine because of a reduced enzymatic conversion of azathioprine to mercaptopurine, was tested recently by our team in young patients with IBD. The deletion of GST-M1, GST-T1 and the coding non synonymous single nucleotide polymorphism rs1695 in GST-P1 were tested. An initial study considered a cohort of 70 young patients (median age 16.2 years, 36 females) with IBD (41 Crohn's disease, 29 ulcerative colitis). Among these, 15 patients developed adverse events during treatment with azathioprine: in particular, there were three cases of bone marrow suppression, three cases of liver toxicity, seven cases of pancreatic toxicity, one case of neuropathy and one case of arthralgia; all these side effects resolved completely after the reduction or interruption of azathioprine administration: azathioprine was therefore considered the main determinant of the adverse effects. Interestingly, the candidate genetic association analysis in these patients revealed that frequency of GST-M1 deletion was significantly lower in patients that developed an adverse event in comparison to patients that tolerated azathioprine treatment with no adverse event (frequency of deletion respectively 26.7% *vs* 67.3%, $P = 0.0072$). Moreover, the incidence of mild lymphopenia (lymphocytes count under 1000/mm$^3$), that was considered a marker of efficacy during azathioprine treatment, resulted associated with GST-M1 genotype: indeed, among patients tolerating azathioprine treatment

and with lymphopenia, frequency of the deletion was 28.6% in comparison to 72.9% among patients tolerant to azathioprine but that did not present this drug effect ($P = 0.032$)[3]. Taken together, these results are in agreement with a model in which patients with GST-M1 deletion are less sensitive to the effects of azathioprine, putatively because of the contribution of this enzyme on the conversion of azathioprine to mercaptopurine. In a recent study, we evaluated the effects of GST polymorphisms on azathioprine metabolism in a cohort of 75 young patients (median age 15.2 years, 36 females) with IBD (46 Crohn's disease, 29 ulcerative colitis) tolerant to azathioprine therapy (taking azathioprine for more than 3 mo). Azathioprine metabolites were measured on samples collected from these patients using an high performance liquid chromatography assay (HPLC)[4]: 150 measurements of azathioprine metabolites were collected. Patients with the deletion of GST-M1 tolerated a dose of azathioprine significantly higher in comparison to patients with normal GST-M1 (mean dose of azathioprine 2.1 mg/kg per day *vs* 1.8 mg/kg per day, $P = 0.022$). Moreover, the amount of active TGNs generated in patients with the deletion of GST-M1 was significantly decreased in comparison to patients with a normal genotype (mean amount of TGNs metabolites concentration for mg/kg of azathioprine: 252 pmol/8 $\times$ $10^8$ erythrocytes *vs* 164 pmol/8 $\times$ $10^8$ erythrocytes, $P = 0.0030$). Multivariate analysis confirmed that this effect was independent from that of other genes with a significant effect, such as TPMT, the main gene known to influence mercaptopurine metabolism[5]. This study therefore supports a role of GST-M1 on azathioprine efficacy, mediated by an increased conversion of azathioprine to mercaptopurine. The reaction catalyzed by GST-M1 likely occurs after oral administration mainly in the intestine and the liver, modulating the amount of mercaptopurine and TGNs that are released in the main circulation.

These studies considered even the effect of GST-P1 and GST-T1 polymorphisms on azathioprine effects and metabolism but did not detect any significant association. The lack of association may be due to the tissue distribution of GST-P1 and GST-T1, which are not highly expressed in the liver, but even to the lack of specific activity of these enzymes toward the catalysis of the reaction of azathioprine with glutathione[6]. Another study considered GST-M1 genetic polymorphisms as a candidate involved in azathioprine activation[7]: this report considered 51 Asian patients with systemic lupus erythematosus (SLE) and the effect of polymorphisms in the ITPA, TPMT, GST-M1 and GST-T1 genes on the response to a low dose of azathioprine (0.97 mg/kg per day). Response to therapy, evaluated as a change in disease activity index, was associated with ITPA genetic polymorphism but not with the other ones. A clear interpretation of this paper results may be difficult because of the lack of data on azathioprine metabolites concentrations. However, the lack of an effect of GST-M1 on azathioprine efficacy in this study may be due to the very low dose of drug used. This study indicated that in Asian patients with SLE the effect of ITPA might be predominant on those of TPMT and GST-M1 when azathioprine is used at very low doses; indeed, in this study even TPMT genetic polymorphism was not associated with azathioprine efficacy. It is known that in patients with Asian ancestry the frequency of variant TPMT is very low, while that of variant ITPA is increased in comparison to Caucasians[8,9].

## INVOLVEMENT OF GST IN AZATHIOPRINE MOLECULAR AND CELLULAR EFFECTS

GSTs are enzymes responsible for the inactivation of electrophilic substances, both endogenous and exogenous, by catalyzing their reaction with GSH. Human cytosolic GSTs are encoded by 17 genes and the proteins derived can be classified into 7 distinct classes based on their amino acid sequences. The most abundant GSTs in human cells are those of class P, M and A. GST-P1 is the principal isoform in most tissues, such as the small intestine and erythrocytes, but is not detectable in normal liver cells; on the other hand, GSTs M1, A1 and A2 are highly expressed in hepatocytes, while they are not expressed in erythrocytes. GST-T1 is expressed in the liver, intestine and erythrocytes even if its level of expression is lower compared to GSTs of the other classes[6].

All the genes for these GST classes display common genetic polymorphisms that influence the activity of the enzyme in some individuals. For GST-M1 and T1, a common deletion is present in humans, so that about 50% and 20% of caucasians lack activity of these enzymes because of this genetic variant. In patients of African and Asian ancestry, frequency of GST-T1 deletion is higher than Caucasian, reaching around 50%, while frequency of GST-M1 deletion is similar[10]. GST-P1 displays a common coding non-synonymous variant, an A-G transition at base 1578, resulting in the amino acid change I105V in the substrate binding site of the enzyme: the frequency of the variant genotype for this polymorphism in Caucasian and African is similar (13%-15%), while in Asian the percentage is lower (1%-2%)[11,12].

Even expression of GST-A is modulated by genetic polymorphism: GST-A1 -69 C > T results in a reduced GST-A1 expression and related enzyme activity[13,14]. The GST-A1 polymorphism shows different frequencies for the mutated genotype between Caucasian and African (respectively 17% and 11%) and Asian (1%). For GST-A2, a C-T transition at base 328, resulting in the amino acid change P110S in the electrophilic substrate binding site, occurs only in heterozygosis, at a frequency of 11% in Caucasian and 23% in Asian population[15,16]. In the African population, the variant allele to date has not been found (Table 1). Genetic polymorphisms in GSTs, determining the interindividual variability in the activity of these important metabolic enzymes, have been related to the incidence of several pathologies, in particular oncological, and to altered sensitivity to medications, including azathioprine[17-20].

**Table 1 Glutathione-S-transferase polymorphisms and frequencies in different ethnic groups**

| Gene | Polymorphisms | Caucasian | African | Asian |
|---|---|---|---|---|
| *GST-M1* | Deletion | 50% | 50% | 50% |
| *GST-T1* | Deletion | 20% | 50% | 50% |
| *GST-P1* | rs1695 (A > G) | 13% (GG) | 15% (GG) | 1%-2% (GG) |
| *GST-A1* | rs3957357 (C > T) | 17% (TT) | 11% (TT) | 1% (TT) |
| *GST-A2* | rs2234951 (C > T) | 11% (CT) | 0% (CT) | 23% (CT) |

GST: Glutathione-S-transferase.

### Enzymatic conversion

Azathioprine conversion to mercaptopurine can occur spontaneously[1], even if in the presence of specific GST classes and at physiological pH values the reaction catalyzed by the enzyme may be prevalent.

Kaplowitz described an initial report on the enzymatic contribution on the conversion of azathioprine to mercaptopurine in rat liver homogenates. While at relatively high pH levels (*i.e.*, 8.0) the non-enzymatic reaction and the enzymatic one occur in similar proportions; at lower pH levels (*i.e.*, 6.5-7.4), closer to physiological values, the enzymatic reaction prevails[21]. The same reaction has been demonstrated in homogenates of human livers: in these samples, mainly from kidney transplant donors, conversion of azathioprine to mercaptopurine was inhibited by treatment with furosemide, a GST inhibitor[22]. Additional evidence obtained in animal models supports a significant contribution of the enzymatic conversion of azathioprine to mercaptopurine *in vivo*. Indeed, pretreatment of rats with probenecid, a GST inhibitor, determines a greater proportion of unmetabolized azathioprine in the liver and less hepatic GSH depletion. Bilirubin is also a GST inhibitor and, in a model of hyperbilirubinemic rat (Gunn rat), less hepatic GSH depletion was found during exposure to azathioprine[23]. These observations indicate that the conversion of azathioprine to mercaptopurine *in vivo* is mediated enzymatically by the GSTs. After oral administration of azathioprine this reaction likely occurs mostly in the liver: indeed after oral administration azathioprine is undetectable in blood, while mercaptopurine appears after either oral or *iv* azathioprine administration[2]. In addition it has been shown that after *ip* injection of azathioprine in rats, GSH was depleted rapidly in hepatocytes but not in other tissues (*i.e.*, erythrocytes, kidneys and intestine) indicating that after administration of azathioprine, the hepatic contribution to total GSH consumption may be predominant[24].

Eklund *et al*[6] have shown that among 14 GSTs tested, GST-A1, GST-A2 and GST-M1 displayed the highest activity on the catalysis of azathioprine to mercaptopurine; these enzymes are all highly expressed in human hepatocytes and therefore in these cells the uncatalyzed reaction of azathioprine with GSH was estimated to be less than 1% of the GST-catalyzed biotransformation. Interestingly, GST-M1 and GST-A display genetically determined variable expression levels. These differences in GST activity may result in interindividual differences in the conversion of azathioprine to mercaptopurine. The authors suggest that individuals with high levels of GST could be particularly sensitive to azathioprine and potentially more prone to adverse effects during treatment with azathioprine, because of both increased concentrations of free mercaptopurine and of a more pronounced GSH depletion.

### Oxidative damage

Considering the intracellular enzymes involved in thiopurines' metabolism, it is reasonable to suggest that these agents are able to induce oxidative stress. Indeed, it has been demonstrated that thiopurines can generate directly reactive oxygen species (ROS) in cells exposed to ultra violet light[25,26]. Moreover, regarding azathioprine, some evidence suggest an indirect ability to induce oxidative stress, mediated primarily by GSH consumption during the metabolic conversion of azathioprine to mercaptopurine[27,28]. GSTs, influencing the reaction of azathioprine with GSH, may influence therefore the cellular effects of azathioprine mediated by oxidative damage. Moreover, even metabolism of thiopurines by XO may generate ROS, as assessed on primary cultures of rat hepatocytes[28]. XO, which metabolizes mercaptopurine, converting it to thiouric acid, is a well know producer of ROS, such as superoxide anion[29], whose accumulation could worsen the oxidative stress induced by GSH depletion. Allopurinol, a XO inhibitor, has been shown to restore response to thiopurines in patients with IBD unresponsive to thiopurines and with unfavorable metabolic ratio, increasing the concentration of active TGNs and decreasing those of the methylated nucleotides, likely because of inhibition of TPMT[30]. Cellular redox balance is largely determined by GSH. Depletion of such cellular antioxidant defenses allows the accumulation of significant amounts of ROS, as demonstrated in several systems[31,32], which, in turn, have been suggested to act as a signal for apoptosis induction[33]. Regarding azathioprine, exposure to this medication *in vitro* induces a rapid depletion of GSH in hepatocytes before any loss of viability. Addition of exogenous GSH or N-acetylcysteine protected against cell death. Lee *et al*[27] suggested that oxidative stress induced by GSH depletion is able to induce mitochondrial damage, opening of mitochondrial permeability transition pore (MPTP) and rapid consumption of adenosine triphosphate. Ultrastructural analysis showed occurrence of necrosis after azathioprine exposure. The fact that, under the same experimental conditions, mercaptopurine was not able to reduce hepatocyte viability, allows suggesting that the activating steps triggered by GST and the associated GSH depletion could be a crucial step in azathioprine cytotoxicity *in vitro*.

Among the many secondary effects attributed to them, a role for ROS in mediating an anti-proliferative effect has been demonstrated. Indeed, the cellular redox balance fluctuates during cell cycle, so that the redox state modulates cell cycle progression from one phase to the next[34]. In this scenario, significantly higher GSH content

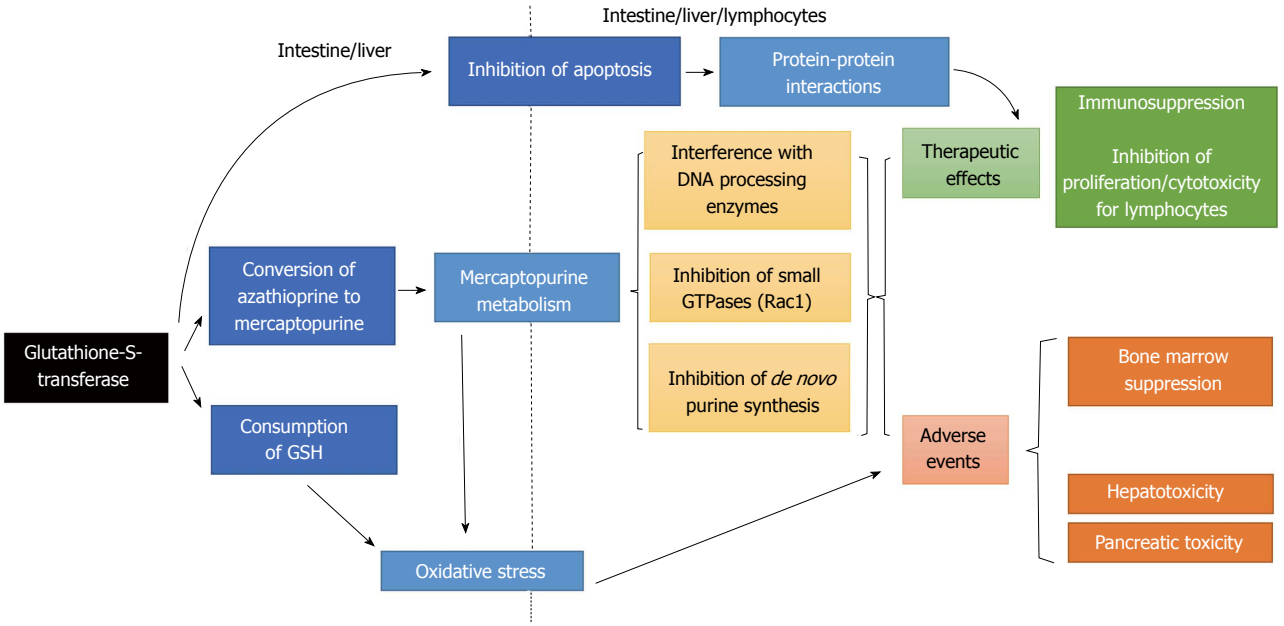


**Figure 2 Schematic representation of different glutathione-S-transferase catalyzed phenomenons (blue boxes) that even through intermediate events (light blue) bring or contibute to the canonical mechanisms of action (yellow), determining the therapeutic effects (green) and/or adverse events (orange).** GSH: Reduced glutathione.

in the $G_2$ and M phases compared with $G_1$ were found[35]. Hence, it is reasonable to hypothesize a role for ROS in affecting the anti-proliferative effect of thiopurines, which are cell cycle phase specific agents and especially azathioprine, which consumes GSH during its conversion to mercaptopurine.

Azathioprine half-life is very short and its therapeutic effects on lymphocytes are likely due to the metabolites produced after first pass metabolism in the intestinal and liver cells. However, azathioprine cytotoxic effects due to GSH consumption are likely to play an important role on the development of cytotoxicity in intestinal and liver cells, supporting a role in the induction of adverse events in these organ systems. However, the fact that on hepatocytes azathioprine is able to modulate a necrotic cell death through a cyclosporine-A sensitive MPTP opening, rise some doubts on the actual role of oxidative stress[27]. Studies are still required to demonstrate if ROS could represent an alternative mechanism of cytotoxicity induced by azathioprine and its clinical relevance.

### Modulation of apoptosis

Azathioprine and mercaptopurine induce apoptosis in activated lymphocytes and these effects should be crucial in determining the efficacy of these medications as immunomodulators in young patients with IBD[36]. GSTs have been shown to modulate apoptosis and the incidence of lymphopenia during treatment of IBD patients and therefore modulation of apoptosis by GSTs may be of significant relevance as a mechanism for the role of these proteins on azathioprine effects.

GST-P1 was the first isoenzyme found to play a role in signaling pathways that control cell survival, and so far is the most important one. This regulation role is achieved independently from the well-known conjugating activity and occurs through a physical protein-protein interaction with c-Jun N-terminal Kinase (JNK), a member of the mitogen-activated protein kinases (MAPK), with the consequent inhibition of the downstream JNK-induced apoptotic pathway. GST-M1 plays a similar role of negative regulator by physically sequestering the apoptosis-signaling regulating kinase 1 (ASK-1), a MAPK kinase kinase that activates both JNK and stress responsive p-38 kinase (another MAPK)[37]. Stress triggers, such as heat shock or the pro-inflammatory cytokines TNF-α and interleukin 1-β, promotes the dissociation of GST-M1 from ASK1, resulting in the activation of ASK-1 and the phosphorylation-dependent activation of p38[38]. The final cell fate (proliferation or apoptosis) depends on the strength and duration of the cellular stress.

GST-A1 has been found to suppress activation of JNK signaling by a pro-inflammatory cytokine and oxidative stress in Caco-2 cells, suggesting also a protective role for GST-A1 in JNK-associated apoptosis[39].

The expression of GSTs of the M and A classes has been reported to drop under apoptotic conditions and their overexpression was able to block apoptosis in rat hepatocytes[40]. GST-M1 has been reported to bind to ASK1 and inhibit apoptosis[37].

Matsumaru *et al*[41] reported that depletion of cytosolic GSH could sensitize murine hepatocytes to apoptosis induced by TNF-α[41,42]; clinical studies have shown that TNF-α protein and mRNA levels are elevated in serum, intestinal tissue and stools of active IBD, in correlation with disease activity[43]. Therefore, even depletion of GSH catalyzed by GST could make cells of patients with IBD particularly sensitive to the cytotoxic effects of thiopurines, potentially leading to an increased incidence

of adverse events.

## CONCLUSION

It would be important that other studies validate the observation of the increased conversion of azathioprine to mercaptopurine in patients with normal GST-M1, resulting in increased sensitivity to the medication in patients with IBD, both clinically and using *in vitro* experiments. Moreover, since GSTs of the A class are highly expressed in the liver, have catalytic activity on the conversion of azathioprine to mercaptopurine and display genetically determined polymorphic activity, it would be important to evaluate the role even of variation in genes for the A class of GST on azathioprine pharmacokinetics and efficacy.

Further insights on the role of genetic polymorphisms of GST and other enzymes on azathioprine pharmacogenetics could come from the use of innovative and more sensitive methods for the measurement of azathioprine metabolites. Indeed, most of the research published so far, including the papers mentioned in this report, have characterized thiopurine metabolites in erythrocytes from patients with IBD, using HPLC methods that group all thionucleosides as TGNs or methylated nucleotides[4,44], without distinguishing the degree of phosphorylation, which may be of relevance for thiopurines' cellular effects[45]. Given the complexity of thiopurines' metabolism, methods with increased sensitivity, allowing to assess the nucleotides degree of phosphorylation and potentially the identification of additional relevant species, such as those based on mass spectrometry are of great interest[46]. Moreover, these methods with increased sensitivity allow the use of very small volumes of patients' samples and this is particularly relevant for pediatric patients[47,48].

The increasing complexity[49] of thiopurines' pharmacogenetics has been consolidating: while TPMT is the strongest determinant of variability in the pharmacokinetics of these medications[50], currently used in several clinical protocols to adjust treatment with thiopurines, even other genes, such as ITPA, have been shown to be of relevance[51]. Based on the clinical and *in vitro* evidence highlighted in this paper (Figure 2), it seems that for azathioprine even GST-M1 genetic polymorphism could enter in a useful multi-locus genotype to predict patients' response to this medication. However, the association of GST-M1 with azathioprine efficacy in patients with IBD still needs to be supported mechanistically by *in vitro* studies and validated by adequately sized prospective clinical trials.

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