

Review

## Non-TPMT determinants of azathioprine toxicity in inflammatory bowel disease

K. Katsanos, E.V. Tsianos

### SUMMARY

Azathioprine (AZA) follows three metabolic routes: the first is the route to 6-thioguanine (TGN) catalyzed by thiopurine methyltransferase (TPMT) and the other two routes are S-methylation to methylmercaptopurine catalyzed also by TPMT or oxidation to thiouric acid via the enzyme xanthine oxidase. Bone marrow toxicity (BMT) mainly in the form of leucopenia represents a major adverse event during AZA therapy in inflammatory bowel disease (IBD). Single nucleotide polymorphisms (SNPs) in the TPMT gene locus affecting 6-TGN intracellular accumulation play a significant role in the occurrence of side effects including BMT. Conflicting data exist regarding the role of TPMT genotyping or TPMT enzyme activity in predicting AZA toxicity. Although some BMT cases can be explained by TPMT genotyping or enzyme activity in the majority of cases BMT remains unexplained. These limitations in TPMT testing pointed out to other genes involved in AZA metabolism. Many non-TPMT genes were investigated but their clinical importance is controversial. To explore the applicability of TPMT and non-TPMT genotyping for AZA toxicity monitoring, large prospective studies are needed. Until the results of such studies are available, the dose adjustments of AZA should be guided primarily by clinical response and peripheral blood counts.

**Key words:** inflammatory bowel disease, azathioprine, toxicity, efficacy, TPMT, non-TPMT genes, single nucleotide polymorphisms.

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### 1. AZATHIOPRINE METABOLIZATION AND MECHANISM OF ACTION

Azathioprine (AZA) or 6-(1-Methyl-4-nitroimidazol-5-ylthio) purine [Prepn: Hitchings, Elion G., U.S patent 3,056,785 (1962)] after its ingestion can follow three competitive metabolic routes: the first is the route to 6-TGN catalyzed by TPMT and the other two routes are S-methylation to methylmercaptopurine (6-MMP pathway) catalyzed also by TPMT or oxidation to thiouric acid via the enzyme xanthine oxidase (XO). The route of aldehyde oxidase (AOX) is also regarded as an additional metabolism route (Figure 1).<sup>1</sup>

Azathioprine is metabolized via 6-MP and 6-TGN into 6-Thio-GTP. 6-Thio-GTP binds to the small GTPase Rac1. GTPases seem to coordinate many of the steps in the chemotactic response of leukocytes (Figure 2).<sup>2</sup> Upon hydrolysis, 6-Thio-GDP bound to Rac1 inhibits Vav guanine exchange activity leading to accumulation of 6-Thio-GDP-

#### Abbreviations:

AOX=aldehyde oxidase

AZA=azathioprine

BMT=bone marrow toxicity

CD=Crohn's disease

GTP=guanine-thophosphate

HPRT= hypoxanthine phosphoribosyltransferase

IBD=Inflammatory Bowel Disease

ITPA=inosine triphosphatase

6-MP=6-mercaptopurine

MTHFR=methylene-tetrahydrofolate-reductase

SNP(s)=Single nucleotide polymorphism(s)

TPMT=thiopurine methyl transferase (gene)

TGN=thioguanine

UC=Ulcerative Colitis

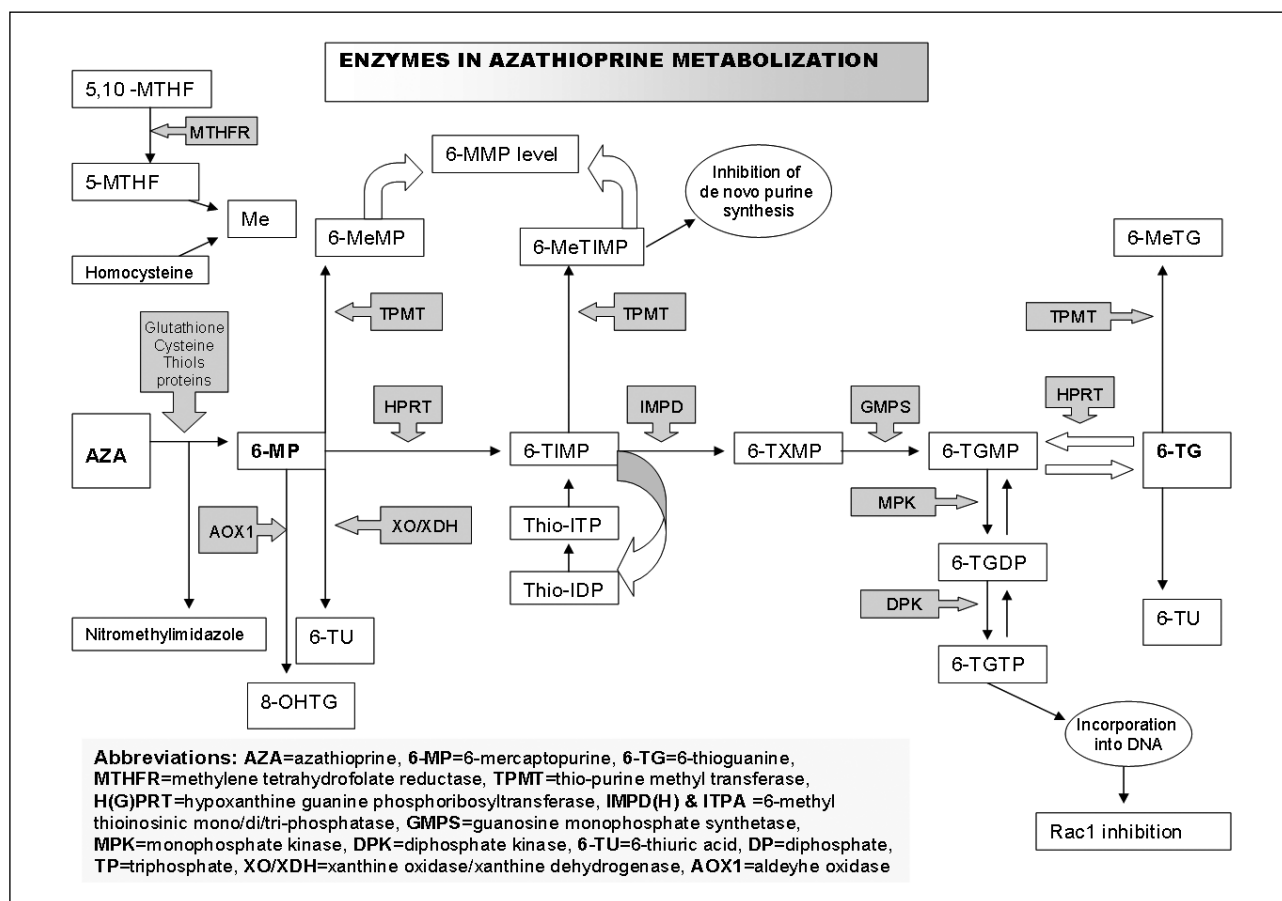
XO/XDH=xanthine oxidase/xanthine dehydrogenase

TPMT alleles:

TPMT \*1=G238C

TPMT \*3A=A719G and G460A

TPMT \*3C=A719G



**Figure 1.** Enzymes involved in azathioprine/6-MP/6-TGN metabolization steps (Katsanos K, 2006).

bound inactive Rac1 molecules, blockade of GTP incorporation into Rac1 and, consecutively, suppression of Rac1 functions on T cell (Figure 3).<sup>3</sup> Data is lacking regarding the route and mechanisms of AZA intracellular accumulation after oral ingestion.

## 2. AZATHIOPRINE-RELATED BONE MARROW TOXICITY

Azathioprine is an effective drug for maintenance of remission in inflammatory bowel disease (IBD), however it is associated with a number of side effects (Table 1). The incidence in the published series of AZA-related adverse events ranges from 5-25%.<sup>4</sup>

Bone marrow toxicity (BMT) mainly in the form of leucopenia represents a major adverse event during AZA therapy in inflammatory bowel disease (IBD). Bone marrow toxicity (BMT) is defined as the occurrence of one at least of the following: leucopenia, anemia.<sup>5</sup>

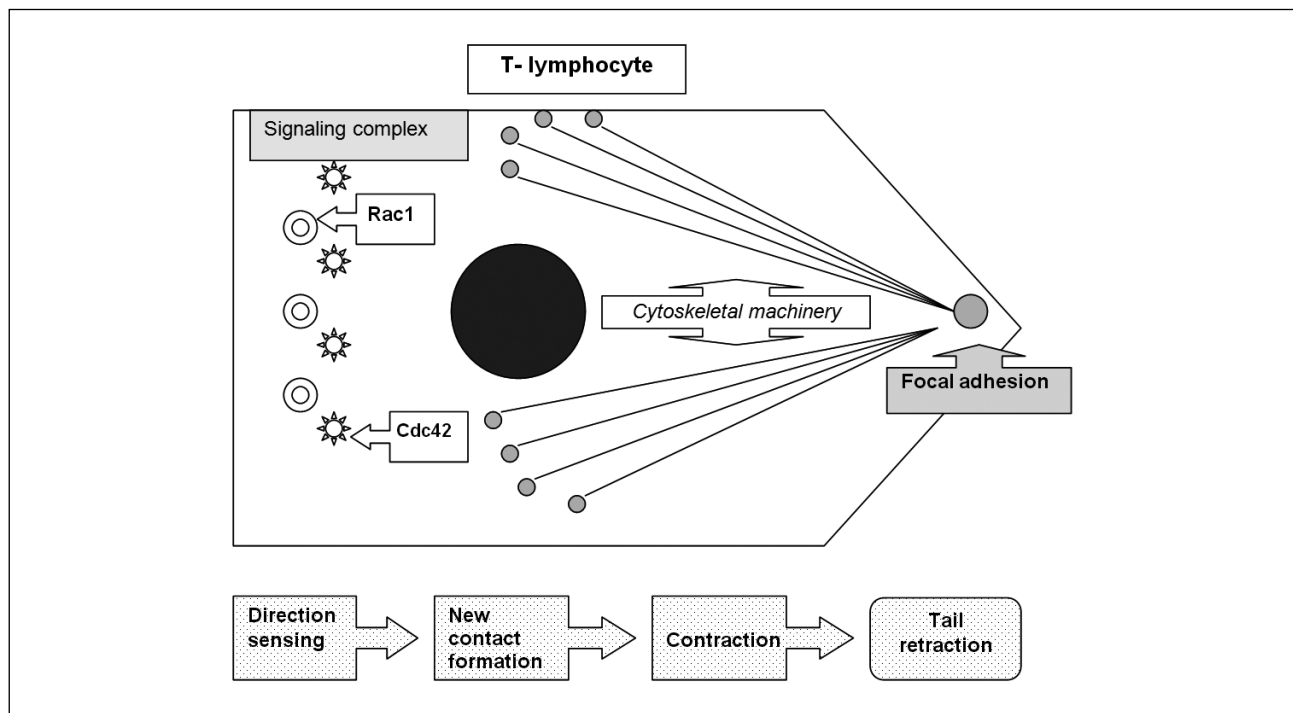
The frequency of leucopenia has been reported up to

10% in AZA-treated IBD patients and nine retrospective studies<sup>6-14</sup> have reported an overall frequency of leucopenia of 3.2% in IBD patients treated with AZA or 6-MP. It is noteworthy that the definition of leucopenia varies among studies (Table 2).

Leucopenia is usually reversible after AZA dose reduction but in some patients AZA has to be withdrawn. Leucopenia occurs when 6-thioguanine (6-TGN), the active product of AZA, is highly accumulated in tissues, including bone marrow tissue. According to the largest study on AZA therapy in IBD patients, BMT may occur at any time during the treatment (range 2 weeks-11 years after starting the drug) either suddenly or over several months.<sup>10</sup> Furthermore BMT is associated with significant co-morbidity mainly in the form of viral infections<sup>15</sup> (Table 3).

## 3. VALUE OF TPMT GENE AND TPMT ENZYME ACTIVITY

The human TPMT gene, consisting of 10 exons, is located on chromosome 6p22.3. A pseudogene for this lo-



**Figure 2.** Regulation of chemotaxis of T-lymphocytes by Rho GTPases. Shown is the coordination of the many steps in the chemotactic response of leukocytes. Cdc42 (and perhaps Rac1) sense the chemotactic factor gradient and polarize the cytoskeletal machinery (Katsanos K. 2007, modified from Bokoch G. *Trends in Cell Biology* 2005;15:165)

cus is located on chromosome 18q. The hereditary nature of the TPMT deficiency in humans was initially identified in a study of TPMT activity in red blood cells.<sup>1</sup>

Clinically sound pharmacogenetic studies over the last two decades have shown that single nucleotide polymorphisms (SNPs) in the TPMT gene locus affecting TGN intracellular accumulation play a significant role in the occurrence of various side effects including BMT.

The allele TPMT\*3B has only the G460A mutation and leads to a ninefold reduction in catalytic activity. TPMT\*3C only has the A719G mutation, which is associated with a 1.4 –fold reduction in activity. The presence of both G460A and A719G, for example TPMT\*3A, leads to complete loss of TPMT activity. Another allele TPMT\*2 (G238C) results from transversion, leading to 100-fold reduction in catalytic activity.<sup>16</sup>

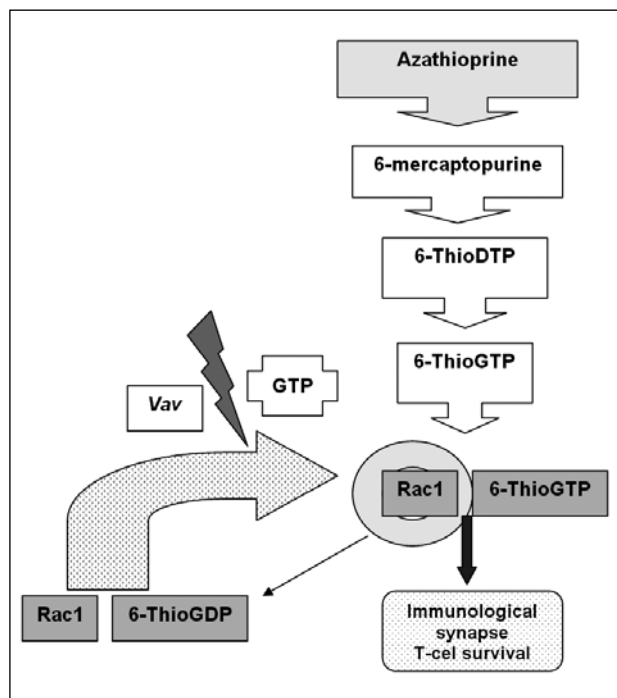
Because hematopoietic tissues have low or undetectable xanthine oxidase activity, TPMT is the only remaining inactivation pathway for thiopurines in these tissues. In fact, individual differences in TGN accumulation after drug therapy have been shown to be associated with bone marrow toxicity. The cellular accumulation of TGN nucleotides is inversely proportional to TPMT activity, since

high TPMT activity results in more drugs to the methylation pathway, leaving less for activation to cytotoxic TGNs. Conversely, TPMT-deficient patients accumulate very high TGN concentrations in tissues, including red blood cells. Subsequently the use of standard doses of thiopurine drugs in patients with complete TPMT deficiency could be hazardous or even fatal due to bone marrow toxicity.

#### 4. TPMT TESTING LIMITATIONS

Conflicting data exist as to whether TPMT genotype or TPMT activity are useful in predicting common adverse events to AZA. There are many studies in favour<sup>16-25</sup> or against<sup>7,9,26-29</sup> the clinical importance of TPMT genotyping and TPMT enzyme activity measurement in AZA-treated patients.

Although some BMT cases can be explained by TPMT genotyping or TPMT enzyme activity, measurements in the majority of cases leucopenia cannot be attributed to usual TPMT variants (TPMT\*2, TPMT\*3A and TPMT\*3C) which account for 80-95% of intermediate or low TPMT enzyme activity cases.<sup>6,15</sup> Of interest, the frequency of pattern of mutant TPMT alleles is different among various ethnic populations.



**Figure 3.** Model of azathioprine-mediated immunosuppression. Azathioprine is metabolized via 6-MP and 6-TGN into 6-Thio-GTP. 6-Thio-GTP binds to the small GTPase Rac1. Upon hydrolysis, 6-Thio-GDP bound to Rac1 inhibits Vav guanosine exchange activity leading to accumulation of 6-Thio-GDP-bound inactive Rac1 molecules, blockade of GTP incorporation into Rac1 and, consecutively, suppression of Rac1 functions on T cell (Katsanos K. 2007, modified from Poppe D, et al. *J Immunol* 2006;176:649).

In addition to this, it seems that there is a high degree of variability in TPMT activity within both the homozygous wild type and heterozygous groups, some individuals with a heterozygous genotype exhibit high activity whereas some homozygous wild type subjects exhibit an

**Table 1.** Reasons for azathioprine/6-MP discontinuation in inflammatory bowel disease (reference No 15).

Reasons for azathioprine discontinuation	No patients (n=229)	%
Ineffectiveness/non-response	56	24.4
Gastrointestinal/general intolerance	40	17.5
Pancreatic toxicity	24	10.5
Hepatotoxicity	22	9.7
Bone marrow toxicity	16	7.0
Severe infection	14	6.1
Pregnancy	14	6.1
Flu-like symptoms	13	5.7
Long-term disease remission*	12	5.2
Skin allergy	11	4.8
Cancer/precancerous/lymphoma**	4	1.7
Rare causes***	2	0.9
Death from other than IBD cause	1	0.4

\* More than 5 years of complete disease remission.

\*\* One patient with breast cancer, one with cerebral hemangioblastoma one with intracranial B-cell lymphoma and one with colorectal cancer.

\*\*\*One patient with increase of creatinine kinase and one with multiple sclerosis.

intermediate phenotype; attention has to be paid also to transfused individuals.<sup>30</sup>

The induction of TPMT activity after commencement of AZA therapy remains controversial.<sup>17, 31-32</sup> After initiation of thiopurine therapy by a fixed dosing schedule, no general induction of TPMT enzyme activity occurred, however, TPMT gene expression decreased. In addition, TPMT activity and the concentration of thioguanine nucleotides were higher in children than in adults.<sup>33</sup>

A recent study could not demonstrate a clear relationship between 6-TGN concentrations on one hand and toxicity and efficacy on the other, as exist in AZA and 6-MP-treated patients. No relationship between 6-TGN con-

**Table 2.** Frequency of leucopenia in IBD patients treated with thiopurine drugs (reference 74 modified).

Author	No patients	Number of leucopenia requiring drug withdrawal (%)	Definition of leucopenia	Deaths
Ansari <sup>8</sup>	106	2 (1.9)	N<2.0	0
Schwab <sup>9</sup>	93	4 (4.3)	WCC<3.0	0
Connel <sup>10</sup>	739	28 (3.8)	WCC<3.0	2
Fraser <sup>11</sup>	622	21(3.4)	WCC<3.0	0
Present <sup>12</sup>	396	8 (2)	WCC<2.5	0
Bouhnik <sup>13</sup>	157	3 (1.9)	WCC<3.0	0
Qasim <sup>14</sup>	110	5 (5.5)	WCC<3.0	0
Katsanos <sup>15</sup>	740	14 (6.1)	WCC<3.9	0
<b>Total</b>	<b>2223</b>	<b>71(3.2)</b>		<b>2</b>

**Table 3.** Infections in IBD patients during AZA-related bone marrow toxicity. (reference No 15)

No	IBD	Sex	Age	No of infections	Severe infections (hospitalized)	Non severe infections (outpatient)
1	UC	M	38	1		bronchitis
2	CD	F	41	1		sinusitis
3	CD	F	48	1	HSV(+) bilateral bronchiolitis*	
4	CD	F	25	1		sinusitis
5	CD	M	33	1		bronchitis
6	CD	M	48	2		sinusitis & bronchiolitis
7	UC	M	39	1		grippal syndrome
8	CD	F	21	1		bronchiolitis
9	CD	M	10	1	infected cyst in front head	
10	CD	F	22	1		gastroenteritis
11	UC	F	37	1		grippal syndrome
12	CD	M	28	1		grippal syndrome
13	CD	M	19	1	EBV(+) upper respiratory tract infection	
14	CD	F	45	1		grippal syndrome
15	CD	M	42	1		grippal syndrome
16	CD	F	38	1		pharyngitis
17	CD	M	54	2		bronchitis twice
18	CD	M	46	1		grippal syndrome**
19	CD	F	53	1	HSV(+) infection	
20	UC	M	14	1		recurrent skin infections
21	CD	M	20	1		toe infection (local surgery)
22	CD	F	28	2	Mycoplasma** pneumonia	pharyngitis
23	CD	F	23	1	microbial pneumonia	
24	CD	F	33	2		sinusitis & grippal syndrome
25	CD	F	10	1		viral enteritis
26	CD	F	30	1	herpes fabialis	
27	CD	F	21	1	severe sepsis	

\*combined azathioprine-infliximab therapy, \*\*combined azathioprine-steroids therapy

centrations and efficacy or toxicity of AZA was demonstrated.<sup>34</sup>

## 5. GENETIC NON-TPMT DETERMINANTS OF AZA TOXICITY

These limitations of TPMT testing lead investigators to other genes and their variants involved in AZA metabolization steps which could probably alter the 6-TGN flow to target cells. These enzymes were investigated in the hope of explaining many of the unexplained cases of BMT in patients with inflammatory bowel disease treated with azathioprine. Many genes have been so far investigated but their importance still remains controversial

### *MDR1 gene (multi-drug resistance)*

The membrane-associated protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC

genes are divided into seven distinct subfamilies. This protein is a member of the MDR/TAP subfamily. Members of the MDR/TAP subfamily are involved in multidrug resistance. The protein encoded by this gene is an ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity. It is responsible for decreased drug accumulation in multidrug-resistant cells and often mediates the development of resistance to anticancer drugs. This protein also functions as a transporter in the blood-brain barrier.<sup>35</sup> Recently MDR1 polymorphisms have been associated with inflammatory bowel disease<sup>36</sup> and according to one study MDR1 G2677T SNP has been associated to gastrointestinal intolerance to azathioprine.<sup>37</sup>

### *ITPA gene (inosine triphosphatase)*

ITPA gene has 8 exons. The protein encoded by this gene hydrolyzes inosine triphosphate and deoxyinosine triphosphate to the monophosphate nucleotide and diphosphate. The encoded protein, which is a member of the HAM1 NTPase protein family, is found in the cytoplasm

and acts as a homodimer. Defects in the encoded protein can result in inosine triphosphate pyrophosphorylase deficiency. Two transcript variants encoding two different isoforms have been found for this gene. Also, at least two other transcript variants have been identified which are probably regulatory rather than protein-coding.

ITPase (inosine triphosphatase) deficiency is not associated with any defined pathology other than the characteristic and abnormal accumulation of ITP in red blood cells. Nevertheless, ITPase deficiency may have pharmacogenomic implications, and the abnormal metabolism of 6-MP in ITPase deficient patients may lead to thiopurine drug toxicity.

The 94C>A transversion in exon 2 results in a Pro32-to-Thr (P32T) substitution. This SNP has a varying frequency in other ethnic groups.<sup>38-39</sup> The frequency of this polymorphism is higher in Japanese, Chinese and East Indian origin populations compared to Caucasians

Homozygous deficient individuals have complete deficient erythrocyte ITPase activity accompanied by accumulation of ITP in red blood cells. In addition ITPase deficient heterozygotes showed a 22.5% ITPase activity of the control value, consistent of a dimeric structure of ITPase. There are studies in favour<sup>40-42</sup> or against<sup>28, 43</sup> the value of this SNP to predict toxicity or even BMT in AZA-treated IBD patients.

Regarding the IVS2+21A/C SNP the activities of IVS2+21A/C heterozygotes and 94C/A-IVS2A/C compound heterozygotes were 60% and 10% respectively, of the normal control mean suggesting that the intron mutation affects enzyme activity.<sup>44</sup> However, it has been suggested that subjects with complete deficiency of ITPase activity have elevated ITP concentrations in erythrocytes but no obvious clinical abnormalities.<sup>45-46</sup>

### ***Rac1 gene***

Rac1 is a member of the Rho family of small GTPases involved in signal transduction pathways that control proliferation, adhesion and migration of cells during embryonic development and invasiveness of tumor cells. The Rac1 gene comprises 7 exons over a length of 29kb and is localized to chromosome 7p22.<sup>47</sup>

The protein encoded by this gene is a GTPase which belongs to the RAS superfamily of small GTP-binding proteins. Members of this superfamily appear to regulate a diverse array of cellular events, including the control of cell growth, cytoskeletal reorganization<sup>48-49</sup> and the activation of protein kinases.<sup>50</sup> Several alternatively spliced transcript variants of this gene have been described, but

the full-length nature of some of these variants has not been determined.

### ***MTHFR gene (methylenetetrahydrofolate reductase)***

Methylenetetrahydrofolate reductase (EC 1.5.1.20) catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for homocysteine remethylation to methionine.

Methylenetetrahydrofolate reductase (MTHFR) plays a central role in the metabolism of folate. Deficiency of MTHFR leads to homocysteinemia. Fourteen rare mutations of MTHFR have been associated with severe MTHFR deficiency, hyperhomocysteinemia, homocystinuria with many vascular and neurologic defects.<sup>51</sup>

Genetic polymorphisms that decrease MTHFR activity result in the depletion of 5-methylenetetrahydrofolate for homocysteine remethylation and the accumulation of 5,10-methylenetetrahydrofolate, the precursor for thymidylate and purine synthesis.<sup>52</sup> A decrease in activity due to genetic polymorphisms would thus tend to favour DNA synthesis over DNA methylation pathways when folate intake is adequate. MTHFR merits further study whether a true protective effect, perhaps mediated through limiting production of methylated metabolites, might exist. A study in liver transplants under AZA therapy MTHFR genotypes (677C>T and 1298A>C) did not predict adverse drug reactions, including bone marrow toxicity.<sup>53</sup>

### ***AOX gene (aldehyde oxidase)***

Aldehyde oxidase produces hydrogen peroxide and, under certain conditions, can catalyze the formation of superoxide. Up to date there is no study on the impact of AOX SNPs in AZA toxicity.

### ***XO/XDH gene (xanthine oxidase /xanthine dehydrogenase)***

The xanthine oxidase /xanthine dehydrogenase enzyme system (XO/XDH) belongs to the group of molybdenum-containing hydroxylases and plays an important role in purine metabolism, iron uptake and transport as well as in the defence against microbial agents. XDH/XO catalyzes the oxidation of hypoxanthine to xanthine, and subsequently to uric acid.<sup>54</sup>

The enzyme is a homodimer. Xanthine dehydrogenase can be converted to xanthine oxidase by reversible sulfhydryl oxidation or by irreversible proteolytic modification. The XO/XDH gene contains 36 exons and allelic variants of these gene have been described in patients with

xanthinuria type I and may contribute to adult respiratory stress syndrome, and may potentiate influenza infection through an oxygen metabolite-dependent mechanism.<sup>55</sup> Up to date there is no study on the impact of XO/XDH SNPs in AZA toxicity.

### ***HPRT1 gene (hypoxanthine phosphoribosyltransferase type 1)***

HPRT maps to Xq26-q27.2 and consists of nine exons. HPRT enzyme activity is required for the phosphorylation of hypoxanthine and guanine, salvaging them for nucleic acid biosynthesis. It also phosphoribosylates purine analogues which is a necessary step for their cytotoxicity. Mutations have been identified in males with brothers with hyperuricemia and nephrolithiasis, gout and Lesch-Nyan syndrome while high frequency of deletions at the HPRT locus has been described in an ataxia-telangiectasia lymphoblastoid cell line irradiated with gamma-rays and in paroxysmal nocturnal hemoglobinuria.<sup>56-57</sup>

Interestingly, the resistance of cells to 6-TG is an indicator of HPRT mutations. In vivo mutations in T cells are now used to monitor humans exposed to environmental mutagens with analyses of molecular mutational spectra serving as adjuncts for determining causation. Most recently HPRT is finding use in studies of in vivo selection for in vivo mutations arising in either somatic or germinal cells.

Resistance to purine analogues provides a highly efficient selective system for HPRT mutant cells allowing them to grow while wild-type cells are killed. The selection is phenotypic, cells with a non-functioning or poorly functioning enzyme will be resistant to the toxic effects of 6-TG or AZA. Conversely, HPRT mutant cells, lacking the salvage pathway are dependent on de novo purine biosynthesis for synthesis of nucleic acids.<sup>56</sup>

Transition mutations at CpG dinucleotides are the most frequent in vivo spontaneous single-base substitution mutation in the human HPRT gene. The rate of increase in mutant frequency is greater in children than in adults, consistent with the higher level of T-cell proliferation in children.<sup>58</sup>

Variants in HPRT have been described to be correlated with sunlight levels and that are induced during exposure to electromagnetic fields, while more than 1,000 variants have been so far described and are available in a special database.<sup>59</sup>

### ***IMPD(H)1 gene (inosine-5-prime-monophosphate dehydrogenase type 1)***

Inosine-5-prime-monophosphate dehydrogenase type 1 [IMPD(H)1] catalyzes the formation of xanthine mo-

nophosphate from inosine-monophosphate (IMP). In the purine de novo synthetic pathway, IMP dehydrogenase is positioned at the branch point in the synthesis of adenine and guanine nucleotides and is thus the rate-limiting enzyme in the de novo synthesis of guanine nucleotides. Among 3 families with autosomal dominant retinitis pigmentosa linked to 7q chromosome, a G to A transition at codon 226 of the IMPDH1 gene, substituting an asparagine for an aspartic acid has been described suggesting that this mutation may be highly deleterious.<sup>60</sup>

## **6. NON-TPMT NON-GENETIC DETERMINANTS OF AZA TOXICITY**

Concomitant to AZA therapy has been also suggest to affect 6-TGN concentrations and by consequence to predict BMT. In vitro studies have suggested that 5-ASA regimens could be potential TPMT inhibitors.<sup>61-63</sup> Clinically higher thioguanine levels have been seen in patients concomitantly taking certain 5-ASAs along with AZA/6-MP.<sup>64-65</sup> However this was not observed in all studies<sup>17,27,66</sup> The administration of allopurinol affects AZA metabolism by inhibiting XO/XDH enzyme and thus increasing the flow towards 6-TGN,<sup>67</sup> furosemide increases BMT by TPMT inhibition while angiotensin converting enzyme inhibitors can increase BMT during AZA treatment by a still unknown mechanism.<sup>68</sup> A study showed that 6-TGN levels were significantly higher and WBC significantly lower within 1-3 weeks after IFX infusion<sup>69</sup> while the prolonged use of trimethoprim-sulfamethoxazole may result in life-threatening hematotoxicity.<sup>70</sup>

## **7. FUTURE PERSPECTIVES IN RESEARCH**

Other factors possibly explaining BMT in our patients could be rare TPMT variants. For example, recently, TPMT\*20, \*21, \*22 variants have been associated with intermediate red blood cell TPMT activity.<sup>71</sup> We should also appreciate individual differences in TGN accumulation<sup>14,30,72</sup> as well as other regulators of TPMT activity such as promoter polymorphisms, viral infections, patient age<sup>33</sup> or other still unknown environmental factors phenotype.<sup>31</sup> Other SNPs in other genes may be proving of importance in explaining many of the unexplained cases of BMT in IBD patients treated with thiopurine drugs.

The discovery and characterization of the TPMT polymorphism grew directly out of pharmacogenomic studies of catechol-O-methyltransferase (COMPT).<sup>73</sup>

## 8. CONCLUSIONS FOR CLINICAL PRACTICE

Although it seems that the TPMT testing cannot safely predict myelotoxicity cases it has the potential for early warning of early severe leucopenia in TPMT homozygous recessive patients as well as of identifying patients who might benefit from higher AZA doses.<sup>4,61,74</sup> We suggest that patients homozygous for one or more TPMT variants are at a very high risk of early severe leucopenia and thus, they have to avoid AZA.

In the future, pharmacogenetic studies will need to focus not only on drug metabolism (pharmacokinetics) but also on drug targets (pharmacodynamics) or both.

To explore the applicability of TPMT and non-TPMT genotyping, 6-MMP and 6-TGN levels for therapeutic drug monitoring, large prospective studies are needed. Until the results of such studies are available, the dose adjustments of AZA should be guided primarily by clinical response and peripheral blood counts.

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