Review (Invited), Expert Opinion On Drug Metabolism and Toxicology

Understanding thiopurine methyltransferase polymorphisms for the targeted treatment of hematologic malignancies.

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ABSTRACT

Introduction

Thiopurine methyltransferase (TPMT) catalyses the S-methylation of thiopurines, such as mercaptopurine and tioguanine (TG), fundamental chemotherapeutic agents used in the treatment of acute lymphoblastic leukemia (ALL). Polymorphisms in *TPMT* gene encode diminished activity enzyme, thus enhancing accumulation of active metabolites, and partially explaining the inter-individual differences in patients' clinical response.

Areas covered

This review gives an overview on *TPMT* gene and function, and discusses the well-established pharmacogenomic implications of *TPMT* variants in the prevention of severe thiopurine-induced hematological toxicities and the less known implication on TG-induced sinusoidal obstruction syndrome. Additional genetic and non-genetic factors impairing TPMT activity are considered. Literature search was done in PubMed for English articles published between 1990 and June 2021, and on PharmGKB for thiopurine drugs.

Expert opinion

In order to titrate thiopurines safely and effectively, achieve the right degree of lymphotoxic effect and avoid excessive myelosuppression, the optimal management will combine a pre-emptive *TPMT* genotyping to establish a safe initial dose to a close phenotypic monitoring of the TPMT activity and/or of the active metabolites during the long-term treatment. Compared to current ALL protocols, replacement of TG by MP during reinduction phase in *TPMT* heterozygotes as well as novel individualized TG regimens in maintenance for *TPMT wild type* subjects could be investigated to further improve outcomes while avoiding the risk of severe hepatotoxicity.

Keywords: acute lymphoblastic leukemia, genetic polymorphisms, myelosuppression, sinusoidal obstruction syndrome, thiopurines, TPMT.

Article highlights box:

- Thiopurine S-methyltransferase (TPMT) is an important phase II enzyme, known for its role in the metabolic transformation of thiopurine drugs (mercaptopurine (MP) and tioguanine (TG)); the TPMT activity is inversely related to the cytoplasmic accumulation of active thiopurine metabolites.
- *TPMT* is a polymorphic gene; the majority of identified single nucleotide polymorphism encode for a diminished activity enzyme
- *TPMT* pharmacogenetic guidelines to prevent severe thiopurine-induced myelosuppression are currently available; prescribing recommendations apply primarily to starting doses.
- Measurement of thiopurine metabolites in patients' red blood cells is generally considered a surrogate marker for thiopurines efficacy and toxicity during a long-term therapy; however, in hematological diseases, clinical applicability of this therapeutic drug monitoring is still limited.
- TG, but not MP, is associated with an enhanced incidence of sinusoidal obstruction syndrome (SOS) in patients affected by acute lymphoblastic leukemia (ALL). The role of *TPMT* polymorphisms or

thiopurine metabolites' levels in the prediction of TG-induced SOS in ALL patients needs to be better characterized.

1. INTRODUCTION

Thiopurine S-methyltransferase (TPMT, EC2.1.1.67) is an important phase II enzyme, known for its role in the metabolic transformation of thiopurine drugs. Thiopurines, such as mercaptopurine (MP) and tioguanine (TG), are antineoplastic agents with immunosuppressant properties commonly used in the clinics for the treatment of acute lymphoblastic leukemia (ALL), the most common hematological malignancy diagnosed in children. MP is a cornerstone drug in long-lasting ALL therapy, being extensively employed to induce, consolidate and maintain remission, whereas the use of TG is generally limited to short periods of therapy re-intensification (e.g.: clinical trial NCT03643276 www.clinicaltrial.gov). Azathioprine (AZA), the MP prodrug, is not used in ALL but has a valuable role to maintain remission in non-malignant conditions that require prolonged immunosuppression such as Crohn's disease and ulcerative colitis, the two major forms of inflammatory bowel diseases (IBD) [1]. In IBD patients, AZA and MP are interchangeable, whereas TG is an alternative for those patients failing to tolerate or respond to AZA/MP, but with safety concerns due to its disadvantageous toxicity profile. Other purine analogues such as cladribine, fludarabine phosphate, nelarabine and clofarabine are employed in hematological malignancies; however, their metabolism is TPMT-independent and therefore they are not taken into consideration here.

This review will first give a general overview on *TPMT* gene and function, and then will discuss in detail the pharmacogenomic implication of *TPMT* variants affecting the enzymatic function in the optimization of thiopurines in ALL. Additional genetic and non-genetic factors impairing TPMT activity will be considered.

1.1 TPMT gene and polymorphisms

TPMT is encoded by *TPMT*, a 34 kb long gene composed of 10 exons and 9 introns that encodes for a 245 amino acid long protein and is located in the short arm of chromosome 6 (6p22.3) [2]; a pseudogene is also located on the chromosome 18 [3,4]. TPMT is a polymorphic gene; currently, more than 40 single nucleotide polymorphisms (SNPs) have been described, and are listed in public available updated databases (https://liu.se/en/research/tpmt-nomenclature-committee; https://api.pharmgkb.org/v1/download/file/attachment/TPMT_allele_definition_table.xlsx). *TPMT* variant alleles present mostly non-synonymous (40 out of 43, e.g.: *TPMT*2, *3A, *3B, *3C*), leading to amino acid substitutions; other variant alleles contain SNPs that lead to the formation of a premature stop codon (e.g.: *TPMT*42*) or to destruction of a splice site (e.g.: *TPMT*4 and TPMT*15*). Most of these variants exhibit low or intermediate enzymatic activity toward thiopurine substrates [5]. Four of these variants (*TPMT*2, *3A, *3B, *3C*) account for 80-95% of cases with decreased TPMT activity in Caucasians, whereas the rest of the variant alleles are very rare [4]. The *TPMT*2* allele (SNP rs1800462) is defined by the 238G>C transversion in exon 5 and the p.Ala80Pro amino acid substitution, whereas the *TPMT*3* alleles are defined by the SNPs 460G>A in exon 7 (rs1800460, p.Ala154Thr, **3B*) and 719A>G in exon 10 (rs1142345, p.Tyr240Cys, **3C*). *TPMT*3A* is the haplotype characterized by both 460G>A and 719A>G transitions, and is the most common variant present in Europeans (frequency approximately 5%) [6,7]. *TPMT*3C* is the most common allele in African-American and East Asian populations (frequency approximately 2%). In Africans there is also another common allele with reduced TPMT activity, *TPMT*8*, identified by the SNP rs56161402 (p.Arg215His, frequency of approximately 2%) [8].

TPMT levels in human tissues are affected by these genetic polymorphisms. Approximately 1 in 300 individuals show a full deficiency in TPMT and 6–11% a partial impairment of the TPMT activity *versus* 89–94% who have a normal/high measurable enzymatic function [9]. This trimodal distribution of phenotype can be traced back to genotypes that influence enzyme levels: subjects carrying homozygous variant alleles are those lacking the TPMT function, heterozygous individuals show decreased but not completely abolished enzyme activity, and homozygous *wild-type* have normal TPMT phenotype [4]. It was demonstrated *in vitro* that variant *TPMT* is associated to post-translational modifications. Heterologous expression of human cDNAs in cells demonstrated that *TPMT*2* and *TPMT*3A* mRNA levels were comparable to *wild type*, although markedly lower TPMT protein levels and catalytic activity were measured. Indeed, variant and wild type TPMT proteins were synthesized at similar rates, however, the formers were less stable over time and more susceptible to proteolysis [10].

In addition to the variability caused by the alleles just described, the variability at the promoter region of the *TPMT* gene needs also to be considered. Within the *TPMT* promoter, there is a variable number tandem repeat (VNTR) region, particularly enriched in G/C, whose function and mechanism of action are still unknown. This DNA region has a three-element architecture in its internal structure, named A, B and C with two of them being variable in their copy number (AmBnC) [11]. The overall length of repeats ranges from three to nine motifs. To date, 19 different alleles have been described and the most frequent VNTR alleles in Caucasians are *VNTR*4a* (A2BC), *VNTR*5a* (A2B2C) and *VNTR*6a* (A2B3C) with an overall frequency of more than 90% [12,13]. In terms of enzymatic activity, a reduced TPMT function was associated to the presence of VNTR genotypes containing more than five repeats in total, although the exact number of A or B motif has not been specifically defined [14]. Recently, Kotur and collaborators have negatively correlated the number of A motifs in VNTR with the transcriptional rate of *TPMT*, suggesting an important aspect for clinical application in which *TPMT* gene promoter VNTR architecture can be used as a pharmacogenomic marker [15]. Urbancic and collaborators have also investigated the association of VNTR architecture with TPMT activity and its connection to common clinically relevant TPMT polymorphisms (*TPMT*3* allele); VNTR pattern AB2C is associated with *TPMT*3C*, while ABnC patterns containing more than 3 B element are associated with *TPMT*3A* allele, suggesting these VNTR sequences as indirect pharmacogenomic markers [13].

1.2 TPMT enzyme and function

TPMT is a 28 kDa cytoplasmic transmethylase expressed ubiquitously in cells, including kidney, liver and red blood cells (RBC). TPMT catalyzes the S-methylation of aromatic and heterocyclic nucleophilic sulfhydryl compounds; the enzyme substrate specificity is low and binding of smaller as well as bulkier molecules is allowed [16]. In its active site, TPMT can accommodate simultaneously both the thio-substrate and the methyl donor S-adenosylmethionine (SAM), which participates in methyl group transfer [17]. During the TPMT-mediated reaction, SAM is converted to S-adenosyl-L-homocysteine (SAH) which is released together with the methylated derivate. Despite the well-characterized function of TPMT on thiopurine xenobiotics, the endogenous substrates of TPMT are not clearly identified yet, and the physiological role of TPMT still needs to be defined. Lack of TPMT enzyme has not been correlated with no disease, so far. Genotype-phenotype relationships could not be established in animal models. Indeed, without thiopurine exposure, no apparent phenotype was observed in homozygous deficient *Tpmt* (*Tpmt*−/−) mice: life span and reproductive capacity

were indistinguishable compared to their wild-type Tpmt (*Tpmt*+/+) or heterozygous Tpmt (*Tpmt*+/−) littermates, as was the histology of liver, lung, kidney, stomach, duodenum, small and large intestine, spleen, thymus, lymph nodes, heart, adrenals, reproductive organs, bone marrow, and brain [18]. Only recently, in 2018, a report provided for the first time experimental and computational evidences of a direct interaction between TPMT and a natural-occurring ligand present in biological systems, the selenocysteine (Sec) [19]. Sec is an analogue of the more common amino acid cysteine, with the essential trace element selenium in place of the sulfur, and is now considered as the 21st "naturally occurring" amino acid encoded by the a non-canonical translation of the UGA codon (as revised by Labunskyy VM and collaborators) [20]. By investigating *in vitro* the activity of recombinant TPMT on selenium-containing amino acids through saturation transfer difference and ^{77}Se nuclear magnetic resonance spectroscopy, fluorescence measurements, as well as computational molecular docking simulations, Urbančič and coworkers demonstrated that methylation of Sec occurred in a SAM-dependent manner. A direct interaction between Sec and SAM in the active site of recombinant TPMT was observed, and both methylated-Sec and SAH were detected as reaction products [19]. Sec is utilized for the selenoprotein synthesis in organs. The human proteome contains 25 known selenoprotein genes [21], including glutathione peroxidase, iodothyronine deiodinase and thioredoxin reductase, methionine-R-sulfoxide reductase, selenophosphate synthetase 2 and various selenoproteins of unknown function [20,22]. With few exceptions, Sec is located in active sites of these enzymes and is involved in catalyzing the redox reactions, because of the highly the nucleophilic nature of the selenol group [23]. Presumably, TPMT could be an important player in regulating selenoprotein activity. Other studies supported the role of TPMT in the methylation of selenium-containing compounds. Deninger and collaborators conducted a systematic research on purine analogs, identifying markedly increased methylation kinetics of selenopurines in the reaction with purified human kidney TPMT [24]. In environmental studies, methylation of selenium compounds has been correlated with the presence of bacterial strains containing highly active TPMT orthologue [25-27]. Fukumoto and collaborators has demonstrated that TPMT acts in a concerted reaction with indolethylamine N-methyltransferase for the formation of trimethylselenonium ions, which are formed and excreted into urine when animals ingest a toxic amount of selenium beyond the nutritional level [28].

2. TPMT AND THIOPURINES: TARGETED TREATMENT OF HEMATOLOGIC MALIGNANCIES.

2.1 TPMT and thiopurine pathway

Thiopurines are a group of agents that structurally resemble naturally occurring purine bases; in particular, MP is a thiolic analogue of hypoxanthine and TG of guanine. They act as antimetabolites, replacing purine nucleotides in nucleic acids and inhibiting *de novo* purine synthesis. MP and TG are inactive prodrugs that require intracellular activation, catalyzed by multiple enzymes, to exert their cytotoxic action after being converted to thioguanine nucleotides. A summary of the activation pathway is provided in Figure 1.

MP and TG enter the cells via sodium-coupled nucleoside transporters (SLC28A2, SLC28A3, SLC29A1 and SLC29A2). AZA is converted into MP by glutathione S-transferases (GSTs) and spontaneously after reaction with thiols (e.g., glutathione) [29]. After the uptake, MP is converted into thioinosine monophosphate (TIMP) by the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT1) and subsequently into thioguanosine monophosphate (TGMP) in two enzymatic steps by inositol monophosphate dehydrogenase

(IMPDH) and guanosine monophosphate synthetase (GMPS) [30]. Finally, TGMP can be converted into active metabolites including thioguanosine di-, tri-phosphate (TGDP, TGTP) and deoxythioguanosine mono-, di-, tri-phosphate (tdGMP, tdGDP, tdGTP). Tri-phosphate thionucleotides (referred to as TGN in this review) compete with the incorporation of dGTP/GTP into nucleic acids (DNA, RNA) and determine the inhibition of DNA-processing enzymes, such as topoisomerase and DNA ligase, which maintain base-pair stability and DNA dynamics, causing cell cycle arrest and apoptosis [31]. Moreover, thioguanosine triphosphate (TGTP) inhibits the activity of the GTPase Rac1, which regulates T-lymphocyte proliferation, and represses immune responses [32]. In contrast to AZA and MP, TG is directly metabolized to TGN: HPRT converts TG into TGMP and, by subsequent kinase activity, TGDP and TGTP are produced [33]. Thiopurines mechanisms of action could be different between ALL and IBD therapy (e.g.: Rac-1 modulation in IBD context) [34]; explaining these differences in details is beyond the purpose of this review.

In addition to anabolic pathways described above, thiopurines undergo catabolic routes mediated by the enzyme xantine oxidase/dehydrogenase (XO/XDH) and by TPMT [4]. The oxidation and methylation of thiopurines and their derivates are competing events with the formation of TGN, as they lead to the production of secondary metabolites. XO/XDH catalyzes the conversion of thiopurines into thiouric acid, which is then excreted by the kidneys [35]. TPMT converts free MP into an inactive metabolite, methylmercaptopurine (MMP), thanks to the methylation of the thiol group performed by the enzyme. The TPMT enzyme also acts on reaction intermediates such as TIMP and TGMP leading to the formation of methyl thioinosine monophosphate (meTIMP) and methyl thioguanine monophosphate (meTGMP), respectively. Methylmercaptopurine is not converted to nucleotides, as it is are a poor HPRT substrate and have no antileukemic activity [30]. Nonetheless, methylmercaptopurine nucleotides, mainly meTIMP, could inhibit the purine *de novo* biosynthesis pathway, thus indirectly contributing to antiproliferative effects. [31,36]. Indeed, the balance between TGNs and MMPNs has been related to thiopurines response and cytotoxicity [37].

Inherited variations in TPMT activity are an important factor in patients' inter-individual differences in clinical efficacy and toxicity during thiopurine therapy. As stated above, TPMT enzyme activity is inversely related to the cytoplasmic accumulation of active TGNs. Thus, when patients with lower TPMT enzyme activity are treated with standard thiopurine doses, they exhibit moderate to high concentrations of TGN metabolites and low to missing concentrations of MMPR, becoming more prone to severe thiopurines-induced side effects. Among these, severe hematologic toxicity can be life-threatening and can lead to early drug withdrawal. [4].

2.2 TPMT pharmacogenetic guidelines to prevent thiopurine-induced myelosuppression

The role of *TPMT* polymorphisms on severe hematological toxicity during therapy with thiopurines is clearly established in the clinics. All the most important protocols for pediatric ALL therapy, such as those released by the European and Canadian-US clinical societies (e.g.: Associazione Italiana Emato-Oncologia Pediatrica Berlin Frankfurt Munster (AIEOP-BFM) Group, Nordic Society of Paediatric Haematology and Oncology (NOPHO), Children's Oncology Group (COG) or the Saint Jude Children research Hospital (SJCRH)) are concordant on the need of pre-emptive *TPMT* genotyping before initiation of MP and TG pharmacotherapy in order to tailor thiopurine doses to the *TPMT* genetic status of the patients. So far, *TPMT*2* and *TPMT*3* are the most clinically relevant variants of interest for thiopurine metabolism and represent a well-validated example of pharmacogenomic research and clinical translation of pharmacogenetic data [4]. Biochemical,

functional as well as clinical evidences based on several criteria (including replication of the association in independent cohorts, statistical parameters and population size) were collected over years with coherent results, so that the *TPMT* variants - thiopurine drugs combination was assigned as level 1A (highest strength) by PharmGKB (www.pharmgkb.org/page/clinAnnLevels), one of the most comprehensive online resources established to collect, curate, and disseminate knowledge about the impact of human genetic variation on drug responses [38]. A strong gene-drug association stands at the basis of development of clinical guidelines, and is necessary, but not enough, to release a prescribing recommendation. For clinical translation into practice, the gene-drug combination should be actionable, meaning that other factors need to be considered such as the drug therapeutic index, the severity of the disease, the consequence of suboptimal prescribing, the availability of genetic testing and the availability of alternative drugs [39]. The Clinical Pharmacogenetics Implementation Consortium (CPIC) developed and classifies guidelines, accordingly. In level A CPIC guidelines, the gene-drug considered is actionable, recommendations for a gene-based prescribing action are moderate-to-strong, and safe and efficient alternative drugs are available. In level B guidelines, recommendations become "optional" whereas in level C and D, no recommendation is given, however CPIC level C and D can also be useful to the clinicians (https://cpicpgx.org/guidelines). Guidelines to correctly interpret *TPMT* genetic tests and guide genotype-based dosing of thiopurines were first published by the CPIC in 2011 [40], and then updated in 2013 [41] and 2018 [42]. At first, focus was mainly on the *TPMT* gene alone, in particular on the most frequent **2, *3A, *3B, *3C* and **4* alleles, and on the assignment of the correct genotype-phenotype correlation. Patient diplotype (*TPMT* wild type, heterozygous and homozygous variant) were translated into a predicted TPMT metabolizer phenotype (normal (NM), intermediate (IM) or low/deficient (PM), respectively) which was linked to a clinical recommendation. With respect to an exemplificative standard MP dose of 75 mg/m^2/day for malignant (pediatric ALL) conditions, only NM TPMT subjects should receive the full dose. A 30–70% reduction was instead suggested for IM patients and a drastic reduction of both dose (10-fold or MP 10 mg/m^2/d) and frequency (thrice weekly instead of daily) for PM individuals; similar adjustments were proposed for TG, although TG effectiveness is less affected by TPMT activity. A similar approach should be followed for IBD patients, as highlighted by the TOPIC-trial, a prospective study to determine whether dose selection based on the results of preemptive *TPMT* genotype analysis affects the outcome of patients with IBD. In the intervention group, heterozygous carriers received 50% of the standard dose of AZA or MP, whereas homozygous variant carriers received 0%-10% of the full dose. Although incidence of hematological complications was similar between controls and intervention group after 20 weeks of treatment, a smaller proportion of carriers of the *TPMT* variants (2.6%) developed hematologic adverse effects in the intervention group compared to those in the control group (22.9%, relative risk, 0.11; 95% confidence interval, 0.01-0.85) [43]. Prescribing recommendations applied primarily to starting doses and were not meant to substitute the clinical monitoring of myelosuppression or disease-specific guidelines generally used for optimizing thiopurines dosing during the long-term therapy. In the 2013 version, CPIC recommendations did not change; however, further studies were provided in support of the *TPMT*–based thiopurine dosing scheme and the applicability of these guidelines in both the adult and pediatric populations was highlighted. Novel genetic evidences on thiopurine tolerance arose in 2014 by a genome-wide association study (GWAS) on susceptibility to thiopurine-induced leukopenia in IBD patients [44]. Then, a GWAS on two independent cohorts of 657 and 371 ALL children confirmed rs116855232 (C>T, p.Arg139Cys) in the gene *nudix (nucleoside diphosphate linked moiety X)-type motif 15 (NUDT15)* as a *locus* associated with MP tolerance during maintenance therapy, finding that

TT carriers received 8.3% of the planned MP dose on average, compared to 63% of the TC individuals and 83.5% of CC [45]. Subsequent studies confirmed the role of this new candidate gene in thiopurine tolerance [46-49] and/or toxicity [47,48,50-52]. NUDT15 is a nucleoside diphosphatase and degrades cytotoxic tGTP metabolites into less toxic tGMP; thus, even a partially impaired NUDT15 function results in an enhanced accumulation of tGTP into the hematopoietic cells and in their increased incorporation into DNA, thus prompting severe myelosuppression at standard full dosages [46,47]. Pre-clinical data demonstrate the efficacy of NUDT15-guided doses of thiopurines decreases on murine leukemias [53, 54]. Moreover, patients harboring *NUDT15* variants demonstrated similar DNA-TGN concentrations even at low doses of MP [55], supporting prospective dose adjustments of MP in the context of *NUDT15* genetic variations to reduce adverse effects without compromising efficacy also in patients with ALL. In the latest published guideline, CPIC defined the *NUDT15* genotype-phenotype conversion and provided *NUDT15*-based recommendations that parallel those of *TPMT*. Full starting doses of thiopurines can be given to NM NUDT15 subjects whereas the thiopurine dose should be reduced to a varying extent in NUDT15 IM, particularly when starting doses of thiopurines are high (e.g., 30-80% of MP 75 mg/m^2/day). Strongly reduced dose or the use of alternative agents should be considered for NUDT15 PM. Dosing recommendation for TPMT phenotypes were thus changed according to the presence of *NUDT15* loss-of function alleles. In particular, dose reductions could be considered also for TPMT NM/ NUDT15 IM or PM and should be recommended for TPMT IM, particularly in those who are NUDT15 PM. Pre-emptive genotyping testing for *TPMT* and *NUDT15* is now recommended also by the Royal Dutch Pharmacists Association - Pharmacogenetics Working Group (DPWG, May 2020 update Annotation of DPWG Guideline for MP and TPMT (pharmgkb.org)), another leading scientific consortium for the pharmacogenetic implementation in clinics.

2.3 TPMT polymorphism and thioguanine-induced sinusoidal obstruction syndrome

Sinusoidal obstruction syndrome (SOS, previously known as hepatic veno-occlusive disease) is a rare but potentially life-threating condition in which sinusoids in the liver become obstructed, leading progressively to tender enlargement of the liver, abdominal pain and swelling, ascites, weight gain, increased liver enzymes, hyperbilirubinemia and jaundice, portal hypertension and, in most severe cases, multi-organ failure. SOS occurs almost exclusively after excessive exposure to hepatotoxic drugs and *stimuli* and/or after high-dose chemotherapy given before a bone marrow transplant. The sinusoidal fibrosis and necrosis of hepatocytes observed in SOS are consequences of the oxidative, pro-inflammatory and pro-coagulant processes triggered by these damaging exposures [56].

TG, but not MP, is associated with an enhanced incidence of SOS in ALL patients [57-59]; there are also strong indications that SOS and nodular regenerative hyperplasia (NRH) of the liver are TG dose or TGN-level dependent phenomena in IBD patients [60]. *In vitro* studies have suggested several mechanisms underneath the different impact of TG and MP on hepatocytes and endothelial cells. TG interferes with bilirubin excretion pathway and failure to excrete bilirubin leads to jaundice and liver toxicity. Oxidative metabolites of TG are potent inhibitors of UDP-glucose dehydrogenase, an enzyme that is responsible for the formation of UDP-glucuronic acid, an essential substrate used in the liver detoxification processes [61]. In contrast to TG, MP showed specific angiogenetic and angioprotective properties, favoring endothelial integrity and attenuating monocytes attraction/adhesion/activation on vascular cells [62,63]. Mouse model demonstrated that thionucleotides stand at the basis of TG-induced SOS, since hypoxanthine-phosphoribosyl transferase-

deficient mice were protected from SOS after exposure to lethal doses of TG; SOS is a dose-dependent consequence of TG administration since splitting the daily dose of TG markedly attenuated SOS [64]. In patients, TG doses at or below 12 mg/m^2/day are rarely associated with severe hepatotoxicity [65]. In IBD, the use of TG at low doses (~20 mg/ m^2/day) for many years was proven to be safe, in particular if guided by therapeutic drug monitoring [60]. In ALL patients, higher doses of thiopurines (~40-60 mg/ m^2/day) for prolonged periods are needed to be effective, and acute hepatotoxicity in the form of SOS occurred in 9-25% of patients whereas long-term hepatotoxicity in the form of NRH was reported in ~2-3% [65]. All together, the *in vitro and in vivo* evidences limit the use of TG to short periods to consolidate remission in ALL therapy (generally in the reinduction phase lasting few months) and support the choice of MP for continued use in association with methotrexate (MTX) during the long-term maintenance (generally lasting between one and two years) [58,66]. Nonetheless, the risk of SOS remains also in short-term setting of TG administration, with an incidence of mild episodes ranging from 0.43% to 0.57%, as reported recently by Stanulla and coworkers in the context of AIEOP-BFM ALL 2000 and 2009 protocols (clinical trials: NCT00430118 and NCT01117441, respectively) [67]. Of note, severe manifestations of SOS could also occur, although very rarely, after a 14-days course of TG, even with fatal outcome [68,69]. Stanulla and coworkers confirmed in large cohorts (3983 patients enrolled in the AIEOP-BFM ALL 2000, 1566 in AIEOP-BFM ALL 2009) that SOS occurred more frequently in TG-containing regimens compared to other treatment phases, and was virtually absent when MP was used instead of TG. Moreover, authors reported that risk of SOS was increased in heterozygous *TPMT* patients compared to *wild-type* subjects (AIEOP-BFM ALL 2000, 813 patients with *TPMT* genotype information available: 22.4-fold, 95% confidence interval 7.1–70.7; $P \leq 0.0001$; AIEOP-BFM ALL 2009 1566 patients with *TPMT* genotype information available: 6.73-fold, 95% confidence interval 1.71–26.53; $P = 0.007$), highlighting the genetic contribution of *TPMT* in the TG-induced SOS [67]. Impaired TPMT activity could result in the accumulation of active TGN metabolites in hepatocytes, triggering their necrosis and pro-inflammation processes. However, in the Stanulla's article, TPMT activity and/or TGN metabolites were not assessed, so it can not be elucidated whether patients developing SOS were those with higher TGN levels (regardless of *TPMT* genetic status). Previous studies showed controversial results on the association between *TPMT* genotypes or TPMT activity/TGN levels and SOS in ALL therapy, but smaller cohorts of patients were analyzed and SOS definition criteria were not uniformly assessed across studies [59,70]. Clinicians should be aware that besides guiding the thiopurine-dose adjustment to avoid severe myelosuppression, replacement of TG by MP during reinduction phase as well as a closer monitoring for early signs of hepatotoxicity when TG is given in *TPMT* heterozygotes, could represent a strategy to reduce SOS incidence in ALL protocols.

2.4 TPMT enzymatic activity and thiopurine effects: other contributing factors

Enzymatic activity of TPMT is inherited as an autosomal monogenic codominant trait, as highlighted by genome-wide association studies, in which only variants in *TPMT* gene were associated with TPMT activity measured in RBC of healthy individuals and pediatric ALL patients [71,72]. In another GWAS study conducted on HapMap human lymphoblastoid cell lines, the intronic SNP rs2413739 (-511C>T) in the gene *PACSIN2* (C kinase protein and casein kinase protein 2) was identified as an additional modulator of TPMT enzymatic activity [73]. The mechanisms by which PACSIN2 influences TPMT are still unclear. Members of the PACSIN family generally regulate intracellular vesicular transit and biological processes such as endocytosis and

autophagy, through protein-protein interactions. Indeed, in *NALM-6* leukemic cell lines, the knock-down of *PACSIN2* affects autophagy and reduces the expression of the heterologous mRNA of *TPMT*1* (wild type), resulting in a reduction in measurable enzymatic activity, while having no influence on heterologous expression and the functionality of the *TPMT*3A*. The role of *PACSIN2* SNP rs2413739 was investigated in independent cohorts of ALL patients enrolled in SJCRH Total XXXIIIB, AIEOP-BFM ALL 2000 and AIEOP-BFM ALL 2009 protocols. Homozygous variant TT individuals showed consistently a reduced TPMT activity compared to CC subjects; however, when TGN and methylmercaptopurine ribonucleotides (MMPR's) were measured in patients' erythrocytes, no significant variation was observed according to genotypes [73,74]. Interestingly, TT carriers experienced an increased incidence of severe gastrointestinal adverse effects (grade III-IV mucositis and diarrhea) during consolidation therapy across all protocols analyzed [73,74]. Other authors found that the TT genotype increased the risk of MP-induced hematotoxicity in ALL pediatric patients presenting wild type *TPMT* genotype [75].Therefore, *PACSIN2* SNP rs2413739 could represent an example of a low penetrance genetic factor affecting TPMT activity, with a potential clinical significance as biomarker of adverse drug reactions, in particular on gastrointestinal tissues. Further studies are needed to validate these results, to understand the mechanism beneath and clarify the potential impact of *PACSIN2* SNP rs2413739 in the optimization of target therapy with thiopurines.

In the treatment of leukemia, MTX is often used alongside MP [76]. A synergistic effect between MTX and MP has been shown *in vitro* and *in vivo*, and is believed to be associated to the inhibitory effect of MTX on purine *de novo* synthesis and pyrimidine synthesis [77,78]. As folate analogue, MTX and its polyglutamate metabolites (MTX-PG) interfere with enzymes of the folate pathway, which are interconnected to many other synthetic pathways. The main intracellular target is the dihydrofolate reductase (DHFR) enzyme, which converts dihydrofolate (DHF) into tetrahydrofolate (THF). DHF is generated by thymidylate synthetase (TYMS) in a reaction coupled with the formation of dTMP from dUMP The enzyme TYMS is also inhibited by MTX and MTX-PG, leading to the impairment of the pyrimidine synthesis. This mechanism of action synergizes with that of thiopurines and prompts the desired antileukemic effects. THF is essential for *de novo* purine synthesis; its active form, 5-methyltetrahydrofolate (5-CH_3-THF), is an important cofactor in one-carbon metabolism. Indeed, impairment of the folate cycle results in a decreased availability of 5-CH_3-THF, among other intermediates. 5-CH_3-THF is required by methionine synthase to catalyze the conversion of homocysteine to methionine that, in turn, is required to generate SAM, the TPMT cofactor (Figure 2). Milek and collaborators provided evidences that SAM is involved in the post-translational stabilization of TPMT in live cells, and that erythrocyte SAM levels impact basal TPMT activity in healthy wild-type individuals [79]. To authors' knowledge, SAM availability had never been measured in ALL patients undergoing polychemotherapy with MTX and thiopurines. It is reasonable to hypothesize that fluctuations in MTX dosages, particularly those occurring during the long-term phase of maintenance, when immunosuppressive ALL therapy is continuously adjusted to target white blood cells (WBC) counts of 2.0-3.0 x 10^9/L (clinical trials: NCT01117441 and NCT03643276), could affect SAM levels, thus altering TPMT activity in hematopoietic cells. Whether such oscillations are of clinical interest for thiopurines management remains to be demonstrated.

Despite these pharmacogenetic-based guidelines, thiopurines severe hematological toxicity and/or poor tolerance may still occur in patients because of other genetic contribution unrelated to *TPMT* or to *NUDT15*.

Genes encoding for inosine triphosphatase *(ITPA)* and the ATP binding cassette subfamily C member 4 (*ABCC4)* received great attention. Both proteins are involved in the thiopurines pharmacokinetic pathway: ITPA degrades tITP into tIMP, regenerating an intermediate of reaction required for synthesizing TGNs; ABCC4 is a nucleoside transporter, extruding thiopurines and their metabolites from the cell. Candidate SNPs in these genes (e.g.: *ITPA* rs1127354 (94C>A, p. Pro32Thr) [37,47,80,81], and *ABCC4* rs3765534 (C>T, p. Glu757Lys) [82-84], were shown to influence thiopurine metabolism, dose, efficacy and toxicity; however, results across studies have been contradictory and level of evidence is still low (https://www.pharmgkb.org/pathway/PA2040). Moreover, genetic profiles might favor the skewing of the thiopurine into a methylation way metabolism, producing more MMPR at the expense of TGN. Only a small percentage of patients (~3%) was found to have ultrahigh TPMT enzyme activity; high TPMT enzyme activity is not the major cause of preferential MMPR production [85]. Recently a novel gain-of-function noncoding variant in *TPMT* associated with increased MP tolerance (rs12199316) was described [86]. Variation in genes other than *TPMT* may contribute to the preferential MMPR metabolizer status. Roberts and collaborators performed a whole-exome sequencing (WES) in a cohort of IBD patients refractory to thiopurine therapy, finding that non-synonymous variants rs747629729 and rs61750370 in *GMPS*, leading to GMPS impaired activity, were associated to MMPR/TGN ratios >100 [87]. In the gastrointestinal practice, higher levels of MMPR are associated to increased risk of hepatotoxicity and higher MMPR/TGN ratio to ineffectiveness of thiopurine therapy. In ALL, such associations were not established.

2.5 TPMT genotypes versus TPMT enzymatic activity: Pros-and-cons

So far, *TPMT*2* and *TPMT*3* are the most clinically relevant variants of interest for thiopurines metabolism, and are those routinely assessed by the majority of medical laboratories, through cost-effective low-throughput polymerase chain reaction (PCR)-based techniques. Concordance between genotyping for common *TPMT* alleles and phenotyping is ~95% in Caucasians, as previously reported [7,88], and recently confirmed in a large Swedish cohort of 12,663 patients sampled before or during thiopurine treatment [89]. Although there is a good degree of concordance, genotyping the most common *TPMT* variants could be misleading in a minority of patients who would be wrongly identified as *wild type/* TPMT NM and thus will be prescribed with a full dose of thiopurine. To diagnose TPMT deficiency, another method is the measurement of TPMT activity in erythrocytes by high performance liquid chromatography (HPLC) [90]. Clear advantage is the direct assessment of the TPMT phenotype (NM; IM, PM), regardless the genetic and non-genetic factors affecting it. However, a number of limitations can be acknowledged in this method compared to *TPMT* SNP genotyping. Concerns are both technical and practical. HPLC assays are less standardized and more time-consuming compared to standard genotyping assays [91]. Moreover, time-sampling is relevant for results, in particular in leukemic patients. Clinicians should be aware that sufficient time must be allowed to elapse in patients who have received blood transfusions for correctly measuring TPMT in patient erythrocytes and not in donor RBC. Moreover, TPMT enzymatic activity could vary with patient age (although this association is controversial in the literature [92,93] and could vary during ALL therapy according to RBC age, as younger erythrocytes developed after early stages of chemotherapy (i.e.: induction and consolidation of remission, when stronger immunosuppression is achieved), could have a higher TPMT activity [94-96]. Then, in contrast to *TPMT* genetic data that provide physicians with a therapy-long reliable information on the patient, TPMT phenotype assessment could be more punctual, and conditioned by the contingencies that are currently happening. While

preemptive *TPMT* genotyping could be useful for recommending initial dosage decreases, ultimate phenotype in patients' erythrocytes is more useful to manage correctly thiopurines during long-term treatment [30].

3. EXPERT OPINION

The role of *TPMT* polymorphisms on severe hematological toxicity during MP therapy is clearly established in the clinics. Nowadays, there is widespread confidence among healthcare professionals about the usefulness of preemptive *TPMT* genotyping to identify patients at risk of severe hematological adverse effects for sparing them from excessive exposure to thiopurines. Although treatment goal and pharmacodynamics of thiopurine use differ between haematological and gastrointestinal practice, awareness has greatly improved the safety and effectiveness of thiopurine treatment, in both conditions. Pharmacogenetic pre-therapeutic diagnostic genotyping assay on patient clinical outcome are mostly limited to the investigation of common candidate *TPMT*2* and *TPMT*3* SNP, thus losing the therapeutic implication given by additional uncharacterized *TPMT* variants. The upcoming use of next generation sequencing (NGS) approaches will likely overcome this challenge in the future. By analyzing thousands of individuals, NGS techniques can identify both rare mutations predicted to cause functional alterations and genes strongly enriched in rare variants, thus prioritizing genes that need to be sequenced in detail for boosting precision therapy [97]. For *TPMT* gene, 154 variant alleles with MAF less than 3% were identified by whole-genome sequencing (WGS) and WES data from 138,842 individuals across eight different populations [98]. Only for 26 (17%) of them, the impact on methyl-transferase activity was demonstrated *in vitro* (23 with deleterious impact); functional assessment of the other alleles was only predicted by computational tools with no experimental characterization in support [98]. It is interesting to note how few variants of *TPMT* gene are being found; this suggests that the endogenous, still unknown, physiological process(es) in which TPMT is involved need to be tightly regulated. Consequently, although the potential role that rare genetic variation can play in explaining the missing heritability in drug response is now recognized [99,100] and although NGS services are becoming more and more feasible, rare variants in preemptive setting remains of difficult interpretation for clinicians. Moreover, NGS techniques may not be accessible to all healthcare providers, in particular in low-income countries, because of the associated costs (i.e.: purchase of facilities, time-consuming technologies, personnel specialization required to conduct and interpret data). Policies of reimbursement by insurance companies and/or National Health System represent also a limiting issue to the clinical implementation of pharmacogenetics. In the near future, candidate pharmacogenetic panel tests will likely remain the preferential option in most medical centers. When these panels need to be chosen, attention should be paid on SNPs of interest, in *TPMT* or other genes, according to patients' ancestry. In particular, initial dosages of thiopurines should be assessed on *NUDT15* risk alleles rather than *TPMT* in Asiatic patients. Importantly, clinicians should be also aware that some of *TPMT* heterozygous ALL patients could tolerate conventional standard doses without severe myelosuppression [101] and that in these cases, thiopurine undertreatment for prolonged periods would be unnecessary and harmful for the final outcome. Thus, the optimal management of thiopurines will combine a pre-emptive *TPMT* genotyping to a therapeutic drug monitoring (TDM) of TGN/MMPR metabolites in order to titrate dosages to the right degree. Although commonly assumed as surrogate marker for therapeutic efficacy and toxicity, measurement of TGN levels in RBC are not entirely representative of pharmacodynamic effects of thiopurines in target cells (leucocytes). Indeed, the range of RBC-TGNs is higher in patients treated with TG compared to those treated with MP (~7 times, at equipotent doses [102]); nonetheless, TGNs accumulate at comparable

levels in target WBC [103]. Depending on the method used, TDM assays might not be measuring all the relevant metabolites; RBC-TGN values should be interpreted with caution and caution should be adopted comparing results of different laboratories. In the IBD context, there is general consensus on the reference TGN range to be achieved (i.e.: 250-450 pmol/8×10^8 RBC). TGN above 450 pmol/8×10^8 RBC were associated with an increased risk of myelotoxicity, while MMPR levels above 5700 pmol/8 × 10^8 RBC were related to a higher risk of hepatotoxicity [104]. To authors' knowledge, effective target ranges of TGN/ MMPR metabolites are not well defined in ALL therapy. It is generally acknowledged that TGN values above 1000 pmol/8×10^8 RBC correlate with a greater incidence of hematological adverse events [37], but MMPR limiting hepatotoxicity has not been reported so far in hemato-oncological diseases.

Recent studies of the Children's Oncology Group (COG) and of the Nordic Society of Pediatric Hematology and Oncology (NOPHO) demonstrated that a higher therapeutic success in childhood ALL is associated to an adequate and constant thiopurine exposure during maintenance [105,106] and that increased thionucleotides incorporated per leucocyte DNA (DNA-TGN) reduced the risk of relapse [107]**.** On the basis of these novel findings on the need of a stable exposure to TGN over time, the question arises whether it's possible to redesign clinical applicable strategies based on the use of TG for prolonged treatment. Attempts to obtain higher DNA-TGN by implementing MP doses would generally be difficult, because of the complex pharmacokinetics of MP and the increase risk of serious toxicities. Due to its direct intracellular activation, TG could be more advantageous than MP in terms of predictable TGN levels but might not yield sufficient DNA-TGN because of the lack of inhibition of purine *de-novo* synthesis: the presence of natural purine nucleotides compete with TGN for DNA incorporation. Individualized TG regimens in maintenance, with the addition of very low and titratable doses of TG to conventional MP have been investigated [108], and could be an option particularly for *TPMT wild type* subjects to further improve outcome while avoiding the risk of severe hepatotoxicity. Dedicated randomized studies will be needed to establish the validity of such therapeutic approach or the superiority of standard maintenance therapy of daily MP combined to weekly low-doses of MTX. A comprehensive evaluation of end-points of interest such as the thiopurine metabolites (TGN and MMPR) and their intra-individual variation in RBC over time, the DNA-TGN concentration and survival, should be undertaken.

Nowadays, the role of *TPMT* polymorphisms or TGN levels in the prediction of TG-induced SOS needs to be better characterized. Compared to current ALL protocols, replacement of TG by MP during reinduction phase in *TPMT* heterozygotes as well as novel individualized TG regimens in maintenance for *TPMT wild type* subjects could be investigated to further improve outcomes while avoiding the risk of severe hepatotoxicity.

Figure Legend

Figure 1: Intracellular conversion pathway of thiopurines. Orange hexagons represent drugs, yellow circles represent derivatives from catabolic pathway; blue diamonds represent derivates from anabolic pathway. Red square boxes are used for enzymes whose genetic polymorphisms are used in pharmacogenomic-based dosing guidelines. AO: aldehyde oxidase; AZA: azathioprine; dTGDP: deoxythioguanosine diphosphate; dTGMP: deoxythioguanosine monophosphate; dTGTP:

deoxythioguanosine triphosphate; GMPS: GMP synthase; GST: glutathione-transferase; HPRT1: hypoxanthine guanine phosphoribosyl transferase; IMPDH: Inosine-5′-monophosphate dehydrogenase; MP: mercaptopurine; MMP: methyl-mercaptopurine; MeTIMP: methyl-thioinosine monophosphate; MeTGNs: 6-methyl thioguanine nucleotides; NUDT15: nucleotide triphosphate diphosphatase gene; TIMP: thioinosine monophosphate; TG: tioguanine; TGDP: thioguanine diphosphate; TGMP: thioguanine monophosphate; TGTP: thioguanine triphosphate; TGNs: thioguanine nucleotides; 2; TPMT: thiopurine methyltransferase; XO: xanthine oxidase; XDH: xanthine dehydrogenase; 8-OHTG: 8-hydroxythioguanine.

Figure 2: Schematic representation of mercaptopurine and methotrexate SAM-mediated interplay. Additional synergic mechanisms of action at the level of inhibition of *de novo* purine synthesis and pyrimidine synthesis are possible, but not highlighted in this figure. Hcy: homocysteine; Met: L-methionine; MP: mercaptopurine; MMP: methylmercaptopurine; MS: methionine synthase; MTX: methotrexate; SAH: S-adenosyl-L-homocysteine; SAM: S-adenosylL-methionine; TPMT: thiopurine S-methyltransferase, 5-CH3-THF: 5-methyltetrahydrofolate; dUMP: deoxyuridine monophosphate; dTMP: deoxythymidine monophosphate; dTTP: deoxythymidine triphosphate; TYMS: thymidylate synthase.

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