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RIIN TAMM

In-depth analysis of factors affecting variability in thiopurine methyltransferase activity

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Institute of Molecular and Cell Biology, University of Tartu, Estonia

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Ref. I **Tamm R**, Oselin K, Kallassalu K, Magi R, Anier K, Remm M, Metspalu A. (2008) Thiopurine S-methyltransferase (TPMT) pharmacogenetics: three new mutations and haplotype analysis in the Estonian population. Clin Chem Lab Med. 46:974–9. doi: 10.1515/CCLM.2008.187

Ref. II Karas-Kuželički N, Smid A, **Tamm R**, Metspalu A, Mlinarič-Raščan I. (2014) From pharmacogenetics to pharmacometabolomics: SAM modulates TPM activity. Pharmacogenomics. 15:1437–49. doi: 10.2217/pgs.14.84

Ref. III **Tamm R**, Mägi R, Tremmel R, Winter S, Mihailov E, Smid A, Möricke A, Klein K, Schrappe M, Stanulla M, Houlston R, Weinshilboum R, Mlinarič Raščan I, Metspalu A, Milani L, Schwab M, Schaeffeler E. (2016) Polymorphic variation in TPMT is the principal determinant of TPMT phenotype: a meta-analysis of three genome-wide association studies. Clin Pharmacol Ther. doi: 10.1002/cpt.540.

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My contributions to the above listed original publications:

Ref. I The author designed the study, collected the samples, conducted the genetic experiments (DNA extraction, PCR, and Sanger sequencing), participated in data analysis and was responsible for drafting the manuscript.

Ref. II The author participated in study design, conducted the genetic experiments (PCR, TaqMan genotyping and Sanger sequencing), participated in data analysis, and wrote the manuscript.

Ref. III The author participated in the study design and data analysis and was responsible for drafting the manuscript.

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ABBREVIATIONS

6-MP	6-mercaptopurine
6-MMP	6-methylmercaptopurine
6-TG	6- thioguanine
6-TGN	6- thioguanine nucleotide
6-TIMP	6-thioinosine-5'-monophosphate
6-TITP	6-thioinosine triphosphate
ABCC4	ATP-binding cassette, sub-family C member 4
ADR	Adverse drug reactions
ABC	ATP-binding cassette
ALL	Acute lymphoblastic leukemia
AZA	Azathioprine
CYP450	Cytochrome P450
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6
CPIC	Clinical Pharmacogenetics Implementation Consortium
DNPS	*de novo* purine synthesis
DME	Drug metabolizing enzymes
FDA	Food and Drug Administration
GST	Glutathione *S*-transferase
GWAS	Genome-wide association study
H1-H4	Haplotypes 1-4
HGPRT	Hypoxanthine guanine phosphoribosyltransferase
HPLC	High-performance liquid chromatography
IBD	Inflammatory bowel disease
IMPDH	Inosine monophosphate dehydrogenase
ITPA	Inosine triphosphatase
meTIMP	methyl-tioinosine monophosphate
MTHFR	5,10-methylene tetrahydrofolate reductase
ORF	Open reading frame
PACSIN2	Protein kinase C and casein kinase substrate in neurons 2
PD	Pharmacodynamics
PGx	pharmacogenomics
PK	Pharmacokinetics
Rac1	Ras-related C3 botulinum toxin substrate 1
RBC	Red blood cell

SAM	S-adenosyl-methionine
SLC	Solute carrier
SLC28A3	Solute carrier family 28 member 3
SLC29A2	Solute carrier family 29 member 2
SLCO1B1	Solute carrier organic anion transporter gene
TDM	Therapeutic drug monitoring
TPMT	Thiopurine methyltransferase
TPMT*1-*39	TPMT alleles 1-39
VNTR	Variable number tandem repeat
VNTR*3 –*9	Variable number tandem repeat 3–9
XO	Xantine oxidase

INTRODUCTION

Since the first working human genome draft was published in 2001, there has been rapid development of high-throughput sequencing technologies. The resultant mass of genomic datasets contains information about inter-individual genome variance of great interest for personalized medicine, which has become a major focus of human genomics, with the goal of providing comprehensive and reliable data regarding complex genetic phenotypes and, ultimately, implementing this knowledge into clinical practice such that individuals' genomic data can be used to assess individualized risk, including expected drug responsivity. Pharmacogenetics/genomics – the practice of administering treatments based on the individual's genomic make-up – has started to become integrated into everyday clinical practice, informing treatment decisions and allowing for more accurate and efficient selection of therapies that are best suited for specific patients.

The extensively researched enzyme thiopurine methyltransferase (TPMT) is a notable example in personalized cancer and immunotherapy. TPMT is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds, including thiopurine drugs such as 6-mecaptopurine (6-MP), azathioprine (AZA), and 6-thioguanine (6-TG). These drugs are used to treat acute lymphoblastic leukemia (ALL) and autoimmune diseases as well as to prevent rejection of transplanted organs. Individuals with low or deficient TPMT activity are at risk of developing severe reactions to drugs methylated by TPMT. TPMT activity level correlates inversely with the accumulation of cytotoxic metabolites (6-thioguanine nucleotides), such that low enzyme activity results in high metabolite levels and *vice versa.* It has been shown in population studies that TPMT activity is trimodally distributed, with the three dominant modes observed being deficient/low, intermediate, and normal. Among people of European descent, approximately 0.3%, 11%, and 89% have deficient/low, intermediate, and normal TPMT activity, respectively, indicating that some 11% of individuals in this population may be prone to adverse drug events. A small subgroup of individuals with ultra-high TPMT activity has also been described. One study showed a relationship between trinucleotide repeats in the *TPMT* promoter region and ultra-high TPMT activity.

Several *TPMT* polymorphisms and mutations have been identified that alter (usually decrease) the encoded enzyme's activity. Insufficient metabolism of standard-dosage thiopurine drugs by TPMT results in myelosuppression, a severe adverse event wherein bone marrow does not produce sufficient blood cells. Meanwhile, standard thiopurine doses are ineffective in patients with ultra-high TPMT activity because the drugs are eliminated rapidly. Aside from identifiable inactivating variants of *TPMT*, there are additional geno- and phenotype variances, especially among individuals with intermediate TPMT activity, indicating that there are factors other than *TPMT* genotype influencing TPMT activity.

In the first phase of this study, we determined TPMT activity in 253 healthy Estonian subjects, sequenced *TPMT's* coding region, and determined the allele frequencies of *TPMT* genetic markers in the population. Previously undescribed mutations were detected. Next, we examined genotype-phenotype variance. The second phase of the TPMT study was conducted with a large cohort of randomly selected individuals from the Estonian Genome Center. The effect of S-adenosyl-methionine (SAM) on TPMT activity was studied. In addition, a genome-wide association study (GWAS) and meta-analysis were performed with an Estonian cohort, German pediatric ALL patients, and liver samples with the aim of finding new genetic markers of TPMT activity.

1. REVIEW OF THE LITERATURE

1.1. The main aspects of pharmacogenetics/-genomics

It has long been known that individuals respond differently to drugs due to many factors such as age, gender, liver and kidney function, environmental/ lifestyle factors, and drug-drug and drug-disease interactions. It has since become very clear that one's genetic profile can have a big impact on drug sensitivity and efficacy (Meyer, 2004; Meyer, 2012).

Pharmacogenetics was defined over half a century ago as the study of variable drug responses due to inherited characteristics (Vogel, 1959). Owing to the rapid development of genomics, molecular pharmacology, and genome analysis methods, we are now heading towards a situation where every patient will get prescribed a dosage according to their genetic make-up, thereby reducing adverse drug reactions (ADRs) and increasing the treatment efficiency (Johnson, 2003; Meyer, 2012; Squassina et al., 2010; Weinshilboum and Wang, 2004). Frequently, the terms pharmacogenetics and -genomics are used interchangeably. However, in pharmaco*genetic* studies, particular candidate genes are investigated, whereas in pharmaco*genomics* (PGx) studies, the whole genome is scanned at the level of DNA and/or RNA. PGx studies may include various "-omics" methods, such as metabolomics, proteomics, and epigenomics, among others (Pinto and Dolan, 2011).

There are two fundamental areas of PGx research, namely studies of the genetic underpinnings of pharmacokinetics (PK) and pharmacodynamics (PD) (Fig. 1). PK describes the course of drug and metabolite levels and the rate of drug metabolism in different tissues. It incorporates data describing drug absorption, distribution, metabolism, and elimination, which are referred to in conglomerate by the acronym ADME. PD describes the pharmacological effects of a drug on the body, desired or not. Thus, PD can be viewed as what the drug does to the body, whereas PK is what the body does to a drug. Variance in both mechanisms leads to differences in drug efficacy and toxicity (Johnson, 2003; Schwarz et al., 2016).

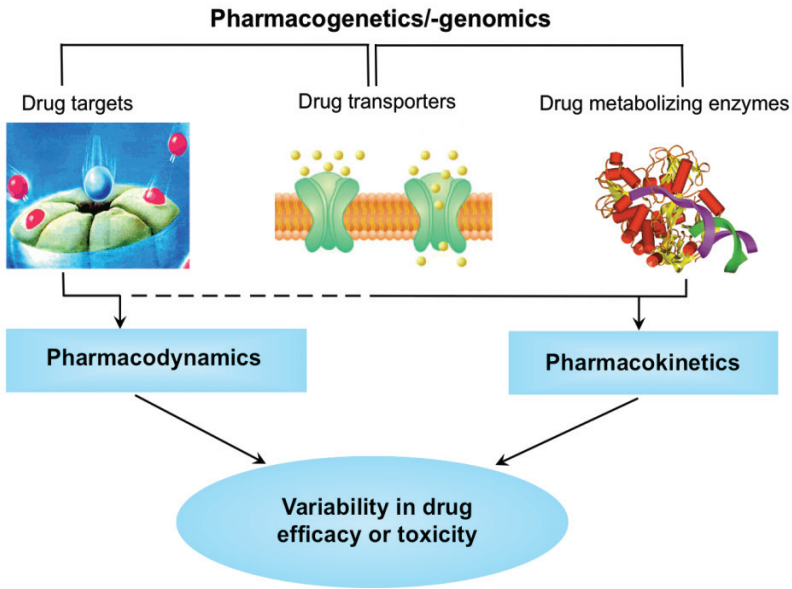


Figure 1. Pharmacogenetics/-genomics is comprised of different components underlying the mechanisms of drug actions. See the text for more details. Figure adapted from (Johnson, 2003).

1.1.1. Drug metabolizing enzymes

The biotransformation, metabolism, and/or detoxification of xenobiotics (i.e., exogenous compounds), including drugs, are mediated largely by enzymes known as xenobiotic metabolizing enzymes or drug metabolizing enzymes (DMEs). Once in the body, xenobiotics can affect a great variety of processes, including cell differentiation, cell division, apoptosis, and necrosis. The body manages xenobiotic effects with diverse phase I and phase II DMEs expressed in various tissues (Fig. 2); DMEs may be present in abundance basally or upregulated after exposure (Pasipoularides, 2016; Rushmore and Kong, 2002). The ultimate goal of the reactions mediated by these enzymes is to convert lipophilic drugs into hydrophilic metabolites amenable to excretion (Brunton et al., 2005; Schwarz et al., 2016).

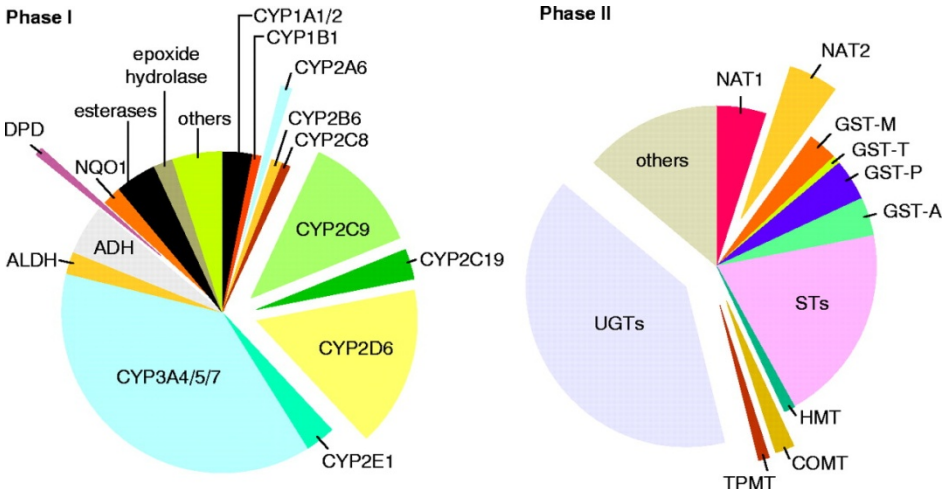


Figure 2. Phase I and phase II enzymes. The relative size of each sector represents how much the indicated enzyme type contributes to drug metabolism. Those enzymes, were polymorphisms are known to affect drug efficacy are separated from the corresponding pie charts. Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; DPD, dihydropyrimidine dehydrogenase; NQO1, NADPH:quinone oxidoreductase or DT diaphorase; COMT, catechol *O*-methyltransferase; GST, glutathione *S*-transferase; HMT, histamine methyltransferase; NAT, *N*-acetyltransferase; STs, sulfotransferases; TPMT, thiopurine methyltransferase; UGTs, uridine 59-triphosphate glucuronosyltransferases. Figure adapted from (Evans and Relling, 1999).

The phase I enzyme group includes mostly cytochrome P450 (CYP450) family enzymes. Generally, they modify the functional groups of xenobiotics/pro-drugs, converting them into active compounds. In some cases, Phase I enzymes may inactivate drugs. Subsequently, drug detoxification is carried out by Phase II enzymes (e.g. methyl-, sulfotransferases, quinone oxidoreductases, N-acetyl-transferases). Phase II enzymes alter chemical structures by adding groups that make compounds easier to excrete in urine (Evans and Relling, 1999; Schwarz et al., 2016).

The Clinical Pharmacogenetics Implementation Consortium (CPIC) established consensus terms for phenotyping individuals according to the activity of drug metabolizing enzymes (e.g. CYP2C19, CYP2D6, CYP3A5, CYP2C9, TPMT, DPYD, UGT1A1). Patients are classified as poor, intermediate, normal, rapid, or ultra-rapid metabolizers of a target drug. This diversity in metabolism is related mainly to the polymorphic genes encoding phase I and II DMEs. Polymorphism of DME genes may be the result of gene copy number variations (e.g. gene deletions and duplications), small insertions/deletions (indels), and single nucleotide polymorphisms (SNPs). Non- and hypofunctional alleles yield reduced drug metabolism and thus elevated risk for ADRs (Ingelman-Sundberg et al., 1999; Norton, 2001; Schwarz et al., 2016; Zhou et al., 2008); patients with

two such alleles for the same key gene should be administered lower doses. Intermediate metabolizers, who have one normal allele and one non-or hypo-functional allele, may also suffer ADRs to standard doses. Standard doses of the drugs are suitable and expected to be therapeutically effective in patients with normal enzyme activity (usually two wild-type alleles). Conversely, the problem of faster than normal drug metabolism, due to the presence of one (rapid metabolism) or two (ultra-rapid metabolism) alleles is critical for drug efficacy. Such patients require higher doses to achieve drug efficacy because the drug is eliminated quickly (Ahmed et al., 2016; Caudle et al., 2016; Kirchheiner and Seeringer, 2007; Schwarz et al., 2016).

In the case of genes that encode enzymes that activate pro-drugs, the opposite pattern occurs, wherein individuals with poor and intermediate enzyme activity may not reach therapeutic efficacy, while rapid and ultra-rapid metabolizers are at risk of ADRs due to excessive concentrations of active metabolites being produced. For example, the cytochrome P450 family isoenzyme CYP2D6 is the main metabolizer of codeine and tamoxifen, from which it produces morphine and endoxifen, respectively. Low CYP2D6 activity is due to loss of function alleles, whereas rapid/ultra-rapid CYP2D6 activity has been attributed to duplication of the functional gene. There have been several reported cases of routinely recommended codeine doses being lethal. For example, there was a case reported of a fatal opioid overdose in a breastfeeding neonate due to the mother who was taking codeine being a rapid metabolizer of CYP2D6 and, therefore, having excessive accumulation of morphine. Postmortem examination of the infant revealed significantly elevated serum morphine concentrations (Ahmed et al., 2016; Madadi et al., 2007).

1.1.2. Drug transporters

Translocation of drugs across biological membranes was long assumed to be mediated by passive transport. However, it is now very clear that the main critical modulators of drug absorption, tissue distribution, and elimination are transporters mainly in the intestines, liver, kidney, and blood-brain barrier. Two types of transporters have been discovered (Fig. 3): uptake and efflux.

Uptake transporters facilitate drug translocation into the cells. Their driving force is mainly the exchange or cotransport of ions (e.g. Na^+, H^+). Notable examples of uptake transporters include several solute carrier (SLC) superfamily members, namely organic anion transporting polypeptides, organic anion transporters, and organic cation transporters (DeGorter et al., 2012).

By contrast, efflux transporters expel compounds from the intracellular space into the extracellular milieu, thereby preventing the cellular accumulation of their substrates. Their driving force is ATP hydrolysis, which enables them to pump their substrates against steep concentration gradients. Efflux transporters include ATP-binding cassette (ABC) superfamily members, such as multidrug resistance-related proteins, and multidrug resistance proteins (DeGorter et al., 2012).

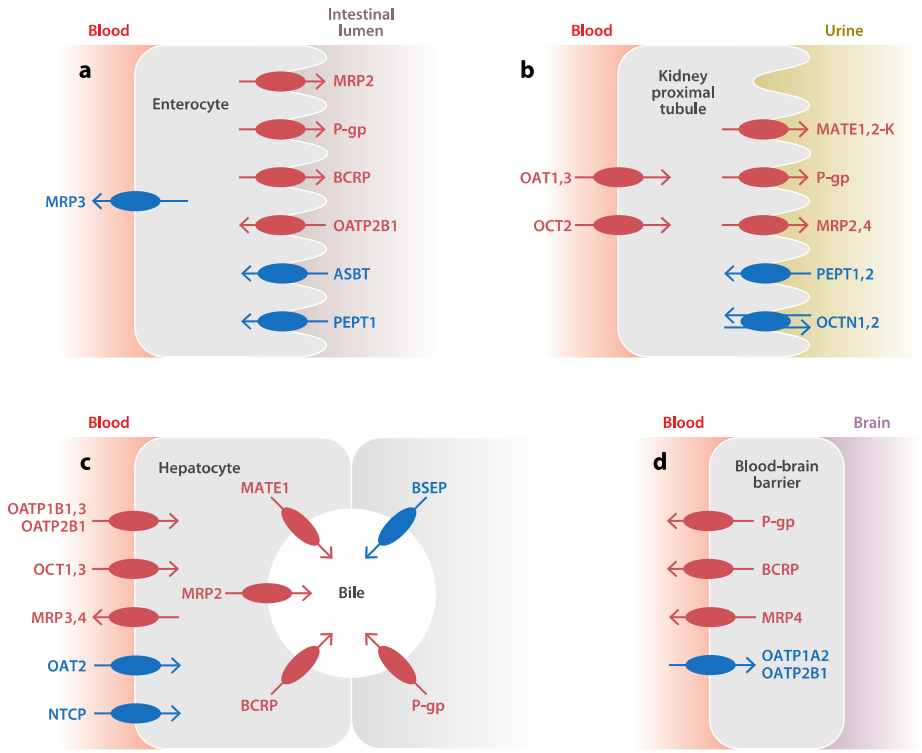


Figure 3. Expression of drug transporters in (*a*) human intestinal epithelia, (*b)* kidney proximal tubule epithelia, (*c*) hepatocytes, and (*d*) brain capillary endothelial cells. Transporters with major roles in drug efficacy or toxicity are colored red. Sodium-dependent taurocholate co-transporting polypeptide (NTCP), apical sodium-dependent bile acid cotransporter (ASBT), and bile-salt export pump (BSEP) are bile acid transporters. Peptide transporter 1 and 2 (PEPT1 and PEPT2) are transport small peptide fragments. Organic cation/carnitine transporter 1 and 2 (OCTN1 and OCTN2) transport organic cations and carnitine. Abbreviations: BCRP, breast cancer resistance protein; MATE, multidrug and toxin extrusion; MRP, multidrug resistance-associated protein; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PEPT, peptide transporter; P-gp, P-glycoprotein. Figure adapted from (DeGorter et al., 2012).

Interindividual variability in activity among transporters is determined by several factors, including polymorphisms in drug transporter genes. A well-known example of transporter gene polymorphism effect is the case of the transporter gene *SLCO1B1*, which encodes the solute carrier organic anion transporter 1B1 (OATP1B1). *SLCO1B1* has a causative SNP (rs4149056) that reduces hepatic transport and increases plasma concentrations of simvastatins, resulting in myopathy (Link et al., 2008). Several clinically impactful SLC and ABC transporter polymorphisms have also been described (Ahmed et al., 2016; Cascorbi, 2011; Chinn and Kroetz, 2007; Sissung et al., 2010; Srimaroeng et al., 2008; Yee et al., 2010; Zhou et al., 2016).

1.1.3. Drug targets

Although studies of drug metabolism pharmacogenetics have been underway since the 1950s, the literature on drug target pharmacogenetics is much more recent, having emerged in the mid-1990s. Drug targets in the body can be direct or indirect. Direct drug targets may be receptors (nuclear or cell surface), enzymes, metabolites, ion channels, or transport proteins (Fig. 4). Indirect targets include proteins involved in the pharmacologic response, such as signal transduction proteins, downstream proteins, and proteins associated with disease risk or pathogenesis (Fig. 4).

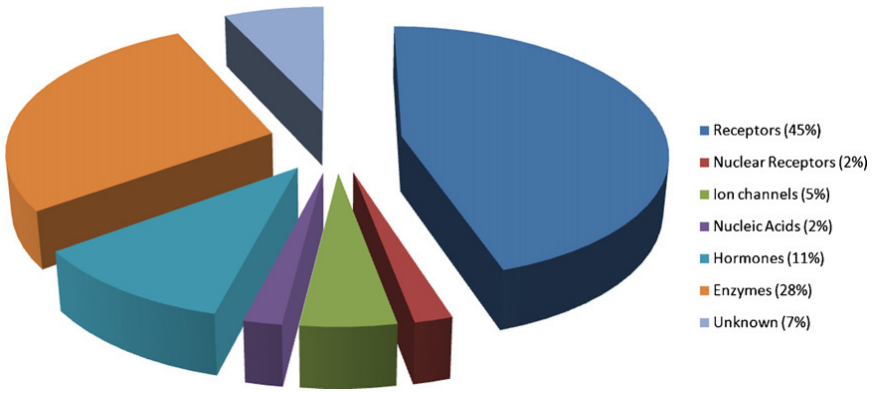


Figure 4. Percentage of current drug target classes. About half of all known drug targets are membrane proteins, however their structures are largely unresolved, remaining a bottle-neck in the drug-development pipeline. Figure adapted from (Adams et al., 2012).

Many drugs have more than one specific target. Drug responses can be divided into primary and secondary drug responses. Primary responses occur when a drug binds to its target; secondary responses can be followed as functional changes in a tissue, organ, or whole organism (Armstrong, 2008). Drug target PGx research is focused on identifying the inheritance basis of interindividual variability in drug response and toxicity (Johnson, 2001). The human epidermal growth factor receptor 2 (HER2) and human epidermal growth factor (EGF) receptors are well-established examples of how drug target pharmacogenetics can be used to predict anti-cancer drug responses. The drug trastuzumab is administered selectively for breast cancers associated with overexpression of HER2 due to gene duplication (Sim and Ingelman-Sundberg, 2011). In recent years, several new therapeutics targeting specific oncology biomarkers, on-label Food and Drug Administration (FDA) drug uses, and companion diagnostics have been accepted (Patel, 2016).

1.2. Thiopurine drugs

In the early 1950s, Gertrude B. Elion and George H. Hitchings synthesized a group of compounds known as thiopurines, for which they received the Nobel Prize in 1988 (Elion, 1951; 1986; 1989). The thiopurine drug 6-mercaptopurine (6-MP), its pro-drug azathioprine (AZA), and 6-thioguanine (6-TG), have been in common use for more than four decades as antineoplastic and immunosuppressive agents (Coulthard and Hogarth, 2005).

1.2.1. Clinical use of thiopurine drugs

6-MP, AZA, and 6-TG are used to treat malignancies, rheumatic diseases, dermatologic conditions, and inflammatory bowel disease (IBD), and to prevent solid organ transplant rejection. Thiopurines have a narrow therapeutic index and may cause life-threatening ADRs, including myelosuppression. These drugs are antagonists for endogenous purines that are essential components of DNA and RNA. 6-MP, which was approved by the FDA in 1953 (Burchenal et al., 1953; Elion, 1986; Veerman et al., 1996), is used mainly in combination with methotrexate to induce and maintain remission in childhood ALL (Cheok et al., 2009; Fotoohi et al., 2010). AZA was introduced in the 1960s as an immunosuppressant for organ transplant recipients (Murray et al., 1963). Nowadays, it is used to treat IBDs, such as Crohn's disease and ulcerative colitis (Blaker et al., 2012; Cosnes et al., 2005).

1.2.2. Metabolism of thiopurine drugs

1.2.2.1. Pharmacokinetics of thiopurines

The common oral daily dose for 6-MP in ALL maintenance therapy is 1.5–2.5 mg/kg/d, with plasma concentrations peaking within, on average, 2.2 h. The bioavailability of oral 6-MP is in the range of 5–37%, with a half-life of 21 min in children (Blaker et al., 2012; Fotoohi et al., 2010). Renal transplant patients receive oral AZA (55% 6-MP by molecular weight) at a dosage of 2 mg/kg/d; its bioavailability range is 27–83% and, once absorbed, ~90% of AZA is converted immediately to 6-MP. The half-life of AZA is quite short at <2 h (Blaker et al., 2012; Chan et al., 1990). AZA is currently indicated for IBD, with dosing that is based on trial results (Axelrad et al., 2016). When single-agent chemotherapy with 6-TG is determined to be appropriate, the usual initial oral dosage is approximately 2 mg/kg/d, which results in peak plasma levels within 2–4 h (Brox et al., 1981). The bioavailability of 6-TG is relatively low and variable at 14–46% (LePage and Whitecar, 1971) with a plasma half-life of 90 min (Konits et al., 1982).

1.2.2.2. Metabolism pathways of thiopurines

Before exerting their cytotoxic and clinical effects, thiopurines are metabolized through a multi-enzyme pathway (Fig. 5). Once absorbed, AZA is transformed extensively into 6-MP by glutathione S-transferase (GST) via a non-enzymatic reaction (Blaker et al., 2012; Eklund et al., 2006; Kurtovic et al., 2008). Subsequently, it is taken up by cells via several transporters (e.g., SLC28A3 and SLC29A2) (Fotoohi et al., 2006; Peng et al., 2008; Zaza et al., 2005). There are three competitive enzymatic pathways by which 6-MP is metabolized: xantine oxidase (XO), hypoxanthine guanine phosphoribosyltransferase (HGPRT), and TPMT. In cells, both 6-MP and 6-TG are metabolized by XO into inactive metabolite-thiouric acid, which is excreted renally. XO can also metabolize 6-TG after prior conversion by guanase. TPMT catalyzes SAM-dependent S-methylation of 6-MP, 6-TG, and their metabolites into methyl metabolites, 6-methylmercaptopurine (6-MMP), 6-methyltioguanine (6-MTG), 6-methyl-thioinosine monophosphate (meTIMP), and 6-methyl-thioguanine nucleotides (6-MTGN) (Al Hadithy et al., 2005; Blaker et al., 2012; Derijks et al., 2006; Moon and Loftus, 2016; Sahasranaman et al., 2008).

Whereas TPMT is expressed ubiquitously in human tissues [e.g. liver, intestine, red blood cells (RBCs), white blood cells], XO is not expressed in hematopoietic tissue, making TPMT-dependent inactivation of drugs critical in white blood cells (Lennard et al., 1987). HGPRT is responsible for the bioactivation of the thiopurines. 6-MP metabolism is mediated step-wise by a series of enzymes. After HGPRT, conversion by inosine monophosphate dehydrogenase (IMPDH), guanosine monophosphate synthase (GMPS), and several kinases, active cytotoxic metabolites known as thioguanine nucleotides (6-TGNs) are formed (Al Hadithy et al., 2005; Blaker et al., 2012; Derijks et al., 2006; Moon and Loftus, 2016; Sahasranaman et al., 2008). Conversion of 6-TG into active 6-TGNs is more direct, involving only HGPRT. The 6-TGNs include 6-thioguanosine 5'-monophosphate (TGMP), -5'-diphosphate (TGDP), and -5'-triphosphate (TGTP) (Elion, 1993).

The therapeutic response to thiopurines is related to 6-TGN production and accumulation (Moon and Loftus, 2016; Zimm et al., 1983). *In vivo*, a 6-TGN steady state is achieved in 4–5 weeks of therapy, with a highly variant half-life of 3–13 days (Chouchana et al., 2012). For most patients with Crohn's disease, signs of efficacy are apparent 12–17 weeks after initiation of therapy (Prefontaine et al., 2010).

Clinical studies have found that the cellular accumulation of TGN nucleotides is inversely proportional to TPMT activity because high TPMT activity shunts more molecules down the methylation pathway, leaving less for activation into cytotoxic TGNs (Krynetski et al., 1996; McLeod et al., 2000; McLeod et al., 1994). Conversely, TPMT-deficient patients accumulate very high TGN concentrations, which leads to the severe ADRs when conventional doses are given (Evans et al., 1991; Krynetski et al., 1996; McLeod et al., 1993).

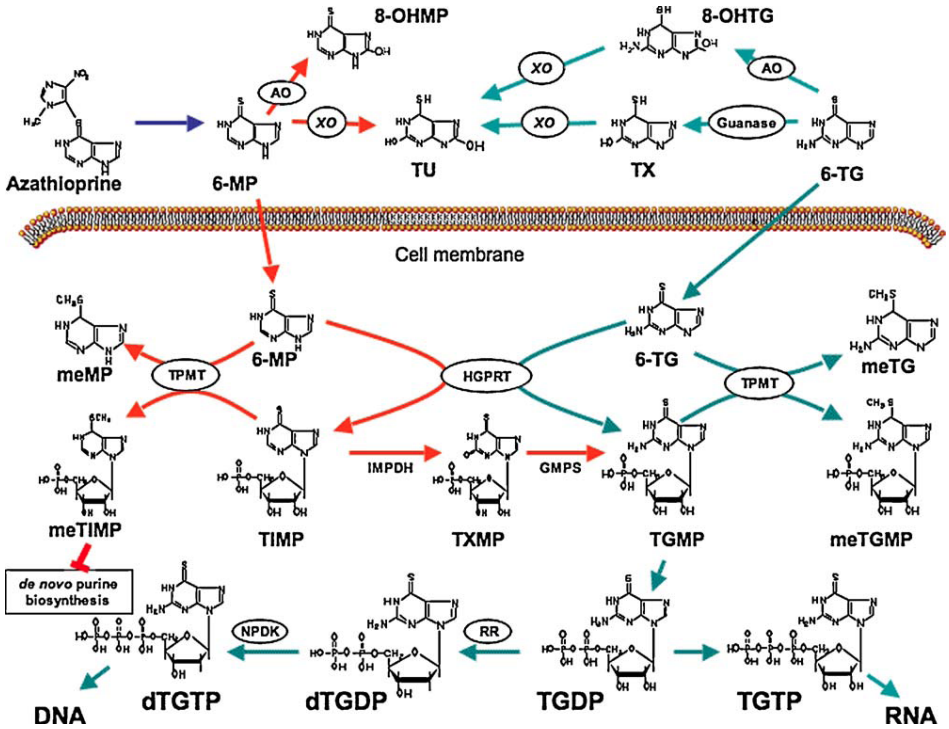


Figure 5. The metabolism of thiopurine drugs. Thiopurines are catabolized by XO, guanase, and aldehyde oxidase (AO) in the extracellular space. When inside the cell, 6-TG is converted directly by HGPRT into TGMP; 6-MP is converted first to 6-thioinosine-5'-monophosphate (TIMP) by HGPRT then to 6-thioxanthine-5'-monophosphate (TXMP) by inosine monophosphate dehydrogenase (IMPDH), and finally to TGMP by guanosine monophosphate synthetase (GMPS). Both 6-MP and 6-TG and their respective monophosphates (TIMP and TGMP) are inactivated extensively inside the cell by thiopurine-S-methyltransferase (TPMT). meTIMP is a strong inhibitor of *de novo* purine synthesis. The remaining TGMP is converted to TGDP, reduced to deoxy-6-thioguanosine-5'-diphosphate (dTGDP) by ribonucleotide reductase (RR), and phosphorylated by nucleoside diphosphate kinase (NDPK) to form dTGTP. Figure adapted from (Fotoohi et al., 2010).

1.2.3. Mechanism of action of thiopurines

As described in the previous section, thiopurines must be bioactivated via a series of non-enzymatic and enzymatic steps before they can exert their therapeutic effects. 6-TGNs are bioactive metabolites with cytotoxic and immunosuppressant properties. Following their formation, 6-TGNs are incorporated into DNA and RNA, thereby inhibiting replication, DNA repair mechanisms, and protein synthesis (Blaker et al., 2012; Moon and Loftus, 2016; Somerville et al., 2003; Swann et al., 1996). 6-TGN cytotoxicity occurs selectively in the S-phase of the cell cycle (Inamochi et al., 1999). It has been shown that one possible

action of AZA and 6-MP may be increasing apoptosis of activated T-lymphocytes. Thiopurine therapy results in accumulation of 6-TGNs in lymphocytes. In immunologically driven diseases, T-cell activation occurs and 6-TGNs block the expression of Tumor necrosis factor-related apoptosis-inducing ligand, tumor necrosis factor receptor-S7, and α4-integrin, effects that reduce inflammation (Blaker et al., 2012; Moon and Loftus, 2016; Thomas et al., 2005).

The effects of AZA and its metabolites on T-cell apoptosis have been attributed, at least in part, to modulation of Rac1 activation upon CD28 and CD3 co-stimulation. The small GTPase Rac1 is involved in cell growth modulation, cytoskeletal organization, and protein kinase activation. Specific blockade of Rac1 activation is achieved by AZA-generated 6-TGTP binding Rac1 instead of GTP. Consequently, AZA suppresses activation of Rac1 target genes, resulting in a pro-apoptotic influence on T-lymphocytes. AZA thus converts a co-stimulatory signal into an apoptotic signal by modulating Rac1 activity (Blaker et al., 2012; Moon and Loftus, 2016; Tiede et al., 2003).

As shown in the Figure 5 above, several other metabolites in this pathway are substrates for TPMT. For example, meTIMP is a strong inhibitor of *de novo* purine synthesis (DNPS), which contributes to the cytotoxic action of 6-MP (Erb et al., 1998; Evans et al., 1991). Inhibition of DNPS is immunosuppressive and block proliferation of various lymphocyte lines. TPMT activity level is expected to influence the production of meTIMP, and consequently, affect DNPS (Hanauer et al., 2001).

1.2.4. ADRs associated with thiopurines

There are generally two types of ADRs described: dose dependent and dose-independent. Dose-dependent toxicity is associated with intra-cellular concentrations of active metabolites, which may evolve months or years after initiation of the therapy. Bone marrow and liver toxicities are particularly worrisome. Dose-independent reactions include myalgia, flu-like symptoms, rash, acute pancreatitis, and gastric intolerance (Moon and Loftus, 2016). In clinical trials, up to 25% of patients treated with purine antimetabolites experience dose-independent side effects (Marinaki et al., 2004).

Thiopurines often have hematologic toxicity, most commonly leucopenia (white blood cell count $< 3 \times 10^9$/L) and neutropenia (absolute neutrophil count $< 1.5 \times 10^9$/L). These ADRs can occur any time during the therapy, but most often occurs early in the course of therapy; they can be reversed by dose reduction or treatment discontinuation (Connell et al., 1993; Moon and Loftus, 2016; Present et al., 1989). Mild leucopenia is the most common hematological ADR to AZA. Drug-induced decreases in white blood cell count, however, has been reported to be associated with an improved clinical outcome (Colonna and Korelitz, 1994). Notwithstanding, severe bone marrow suppression is considered the worst ADR to thiopurines (Connell et al., 1993; Kirschner, 1998). The authors of a recent study concluded that Crohn's disease patients should be

closely monitored, especially during the first three months of the thiopurine therapy when the majority of complications emerge, even if the patient has normal TPMT activity (Benmassaoud et al., 2015). A prospective study of 30 Dutch IBD patients in which a pretreatment TMPT genotype analysis group was compared to a non-genotyped control group indicated that TPMT screening significantly reduced the risk of hematologic ADR in the subgroup of patients with TPMT variants (Coenen et al., 2015).

In ALL patients, a high concentration of 6-TGNs in RBCs correlates with degree of leucopenia and positive drug responsivity, whereas low concentrations may lead to a higher risk of relapse (Bostrom and Erdmann, 1993; Lennard et al., 1997). Hematotoxicity is the only dose-dependent ADR that has been associated with TPMT activity/genotype, thus far. TPMT-deficient patients taking standard doses of thiopurines are at approximately 100% risk of developing severe bone marrow suppression (Ansari et al., 2002; Gardiner et al., 2006; Gisbert and Gomollón, 2008; Moon and Loftus, 2016) due to very high concentrations of 6-TGNs (Lennard et al., 1989). Childhood ALL patients that are TPMT heterozygotes and patients with rheumatological diseases taking standard doses of thiopurines are also at risk of severe hematoxicity (Black et al., 1998; Relling et al., 1999).

IBD patients with intermediate or low TPMT activity have been found to be at risk of developing myelosuppression (Colombel et al., 2000; Moon and Loftus, 2016). A large meta-analysis of 67 studies assessing the risk of myelosuppression among patients taking thiopurines indicated that, compared to patients with normal TPMT activity, those with intermediate TPMT activity were at a higher risk of developing myelosuppression, albeit not at as high of risk as those with low TPMT activity (Higgs et al., 2010). In another recent meta-analysis, the authors found an association of TPMT polymorphisms with overall AZA-induced ADRs, bone marrow toxicity, and gastric intolerance, but not with hepatotoxicity per se (Liu et al., 2015). However, several studies have indicated that hematotoxicity risk in IBD patients may be independent of TPMT status (De Ridder et al., 2006; Gisbert et al., 2006; Moon and Loftus, 2016). Other factors may determine risk, such as drug interactions (allopurinol), other enzymes (e.g. ITPase) and other genes (e.g. IL6ST, MOCOS) or TPMT-dependent metabolites (meTIMP) may be involved (Coelho et al., 2016; Colombel et al., 2000; Hindorf et al., 2006; Venkat et al., 1990; Zabala et al., 2013).

A variety of ADRs, including nausea, vomiting, malaise, myalgia, and arthralgia as well as hepatotoxicity and pancreatitis have been reported, especially early in the course of AZA therapy (Teml et al., 2007). As early as 2000, an association between hepatotoxicity and levels of the TPMT methylation product 6-MMP had been suggested (Dubinsky et al., 2000). Later, the same group confirmed that escalating AZA doses in non-responders lead to a preferential production of 6-MMPs, which was hepatotoxic (Dubinsky et al., 2002), though the underlying mechanism of this phenomenon had not been uncovered. However, these ADRs have not been shown to be associated with empirically established TPMT status (Schwab et al., 2002). Although several

genes and enzymes have been studied searching for mechanisms underlying ADRs, results related to *ITPA* gene polymorphisms have been inconsistent (Gearry et al., 2004; van Dieren et al., 2005; von Ahsen et al., 2005). The development of gastrointestinal intolerance during thiopurine therapy was reported to be associated with TPMT activity by several groups (Hindorf et al., 2006 Marinaki et al., 2004), but the putative association was not confirmed in a subsequent prospective study (Ansari et al., 2008a). Hence, TPMT involvement in gastrointestinal intolerance remains unclear and should be assessed further.

1.3. Thiopurine methyltransferase (TPMT)

1.3.1. *TPMT* gene

TPMT (EC 2.1.1.67) is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds, including thiopurine drugs (REMY, 1963). In 1996, Szumlanski and colleagues mapped the *TPMT* gene to the short arm of chromosome 6 (6p22.3). They determined that *TPMT* is approximately 34 kb long and composed of 10 exons and nine introns (Fig. 6). Due to alternative splicing, exon 2 is present only in subset of mRNAs transcribed from the gene (Szumlanski et al., 1996). A year later, another group described *TPMT* as spanning only 25 kb and containing only 9 exons with 17 additional nucleotides upstream of the transcription start site and a shorter intron 8 (Krynetski et al., 1997). Subsequently, Seki and colleagues determined that *TPMT* spanned 27 kb and contained 9 exons; they did not identify the intron 2 reported by Szumlanski group (Seki et al., 2000; Szumlanski et al., 1996).

Several groups have confirmed the presence of variable number tandem repeats (VNTRs) in the promoter region of the gene. These consist of 17–18-bp repeated sequences that are repeated three to nine times (VNTR*3–*9), most frequently four (*V4) or five (*V5), and contain putative binding sites for transcription factors (Alves et al., 2000; 2001; Marinaki et al., 2003; Spire-Vayron de la Moureyre et al., 1998a; 1999; Yan et al., 2000;). The 5' of *TPMT* is GC rich (71%) with binding sites for several transcription factors (Sp1, NF-κB, AP-2 and KROX-24), but no TATA box or CCAAT element consensus sequences (Fessing et al., 1998; Szumlanski et al., 1996). In the human genome, there is a *TPMT* pseudogene in chromosome 18, which shares 96% identity with the *TPMT* open reading frame (ORF) on chromosome 6 (Lee et al., 1995).

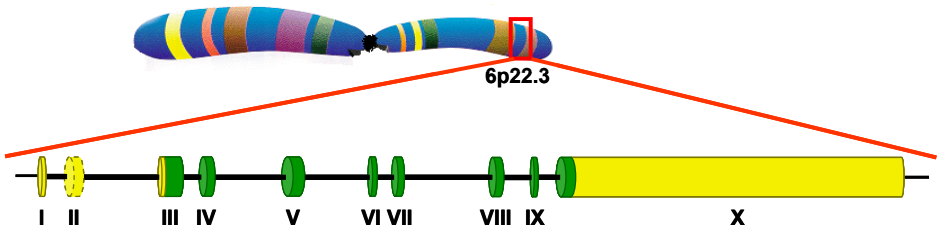


Figure 6. Genetic structure of *TPMT*. The gene consists of 10 exons and 9 introns. Exon 2 is shown with dashed outline because it is spliced out of most transcript forms. The ORF is shown in green. Figure adapted from (Yan et al., 2000).

1.3.2. TPMT protein

TPMT has long been recognized for its major role in the metabolic transformation of exogenous thiopurine drugs. However, an endogenous substrate for TPMT has yet to be identified and TPMT deficiency has not been associated with any pathological condition (Blaker et al., 2012).

The protein encoded by *TPMT* has a molecule mass of 28 kDa and is 245 amino acids long (Honchel et al., 1993; Krynetski et al., 1996; Lee et al., 1995). Partly purified TPMT was characterized by Woodson and Weinshilboum in 1983. The complete structure of TPMT was published in 2007 by Wu and colleagues. However, because the full-length TPMT protein failed to crystallize, the protein's N-terminus was truncated, lacking the first 16 amino acids (Wu et al., 2007). TPMT was found to be a single-domain monomer with a classic Class-I methyltransferase fold. The domain contains a nine-stranded β-sheet flanked on each side by three α-helices (Fig. 7). The β-sheet contains five parallel β-chains with a pair of antiparallel β-hairpins on either end. An additional two helices were identified in the catalytic region of the protein, to which structurally similar S-adenosyl-homocystein, S-adenosyl-L-metionine, and 6-MP bind (Wu et al., 2007).

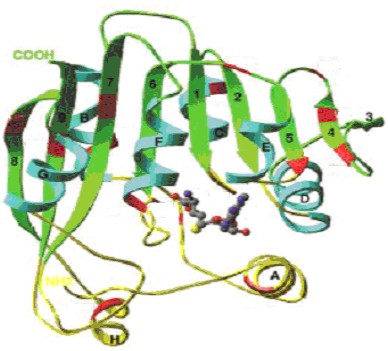


Figure 7. Structure of TPMT in a complex with S-adenosyl-homocysteine. Strands (1-9) are colored green and helices (B-G) cyan. The N-terminal helices A and H (shown in yellow) constitute the catalytic site of the enzyme. Figure adapted from (Wu et al., 2007).

In vitro and computational structural/functional analyses have shown that *TPMT* variants produce proteins of differing stability. Polymorphisms occur throughout the structure, but the amino acids whose alterations have the most influence on function are those involved in intra-molecular stabilizing interactions (Fazel-Najafabadi et al., 2015; Wu et al., 2007).

1.4. TPMT phenotypic and genetic variability

TPMT activity in humans is influenced by variations in the *TPMT* (Weinshilboum and Sladek, 1980). Although TPMT enzyme activity has no apparent influence on individuals' general phenotype, it becomes influential when one is administered a thiopurine drug (Lennard et al., 1993). Allelic differences have an impact on treatment efficacy and ADR risk (Weinshilboum and Sladek, 1980). A series of population studies have shown that TPMT activity is trimodal, with approximately 89% of subjects exhibiting normal activity, 11% exhibiting intermediate activity, and 0.3% having very low or deficient activity. The association between enzyme activity and mutation is best established for individuals with poor or deficient enzyme activity, who are usually mutant homozygotes or compound heterozygotes for inactive alleles (Appell et al., 2010; Colombel et al., 2000; Feng et al., 2010; Garat et al., 2008; Hamdan-Khalil et al., 2003; Hamdan-Khalil et al., 2005; Hon et al., 1999; Kham et al., 2009; Landy et al., 2011; Lee et al., 2012; Lindqvist et al., 2004; Lindqvist et al., 2007; Otterness et al., 1997; Sasaki et al., 2006; Schaeffeler et al., 2006; Schaeffeler et al., 2004; Schaeffeler et al., 2003; Spire-Vayron de la Moureyre et al., 1998b). The correlation between genotype and enzyme activity is mixed in heterozygous subjects because some heterozygotes exhibit normal-range TPMT activity (enzyme activity range, 53–100% of typical) (Alves et al., 2001; Milek et al., 2006; Schaeffeler et al., 2004; Spire-Vayron de la Moureyre et al., 1998a). There is also a subgroup of individuals with rapid or ultra-rapid enzyme activity, which confers therapeutic resistance (Roberts et al., 2008).

Due to the aforementioned population variance in TPMT activity, standard thiopurine doses that are effective in most patients are not suitable for patients with hypo- or hyper-active TPMT. Therefore *TPMT* genotype and/or TPMT enzyme activity should be considered when prescribing a thiopurine, together with other potential factors that may influence thiopurine drug effects (Karas-Kuzelicki and Mlinaric-Rascan, 2009).

1.4.1. Factors that may influence TPMT activity

TPMT genotype is not a perfect predictor of thiopurine therapy efficacy or ADR risk. Indeed, roughly half of all patients who develop leucopenia during thiopurine therapy have normal TPMT activity and a wild-type *TPMT* genotype (Blaker et al., 2012). Hence, there is a need to identify additional markers of

thiopurine responsivity. In the following sections, I will give an overview of the different types of factors that are thought to influence TPMT activity as of the time of writing this thesis.

1.4.1.1. Gender, age, and diseases

A large systematic review summarized the effects of various factors on TPMT activity (Loit et al., 2011). Results regarding the influence of gender and age have been contradictory. Some have found that TPMT activity is significantly higher in males than females (Indjova et al., 2003; Schaeffeler et al., 2004; Tamm et al., 2008), whereas others found no correlation between gender and TPMT activity (Alves et al., 2001; Ganiere-Monteil et al., 2004; Zhang et al., 2007). Schaeffeler and colleagues also showed a statistically significant difference in TPMT activity between smokers and non-smokers, within both male and female groups (Schaeffeler et al., 2004). Children have been reported to have lower TPMT activity than adults, though the distinction was attributed to their juvenile group have a greater portion of intermediate metabolizers (Hindorf et al., 2004). A large study with more than 14,000 patients demonstrated that TPMT activity in groups of patients with IBD, autoimmune hepatitis, multiple sclerosis, myasthenia gravis, pemphigus, and chronic renal failure differed from that in a healthy control population (Gisbert et al., 2007).

1.4.1.2. S-adenosylmethionine (SAM)

SAM is an endogenous universal methyl donor involved in a multitude of reactions catalyzed by numerous SAM-dependent enzymes. It has been described as a modulator of a number of important genes via transcriptional, posttranscriptional, and posttranslational mechanisms. Most notably, SAM provides posttranslational stabilization of catechol O-methyltransferase, a polymorphic enzyme involved in the catabolism of catechol estrogens and catecholamines (Rutherford et al., 2006), as well as cystathionine b-synthase, the rate-limiting enzyme in the trans-sulfuration pathway (Prudova et al., 2006).

It has been shown that SAM stabilizes the conformational structure of TPMT by binding to its active site and protecting it from degradation (Scheuermann et al., 2004). Milek et al. (2012) demonstrated effects of fluctuations in physiological levels of SAM and related metabolites on TPMT activity levels in cell lines and in erythrocytes collected from healthy individuals. In *TMPT* wild-type subjects, TPMT activity was significantly higher in subjects with high SAM concentrations than in those with low SAM levels. Those findings extend a previous study demonstrating that restriction of L-methionine (SAM precursor) in cell growth media decreased TPMT activity and protein levels reversibly, but had no effect on *TPMT* mRNA expression (Milek et al., 2009). Interestingly, in a large cohort of healthy individuals, the influence of SAM availability on TPMT activity was shown to be particularly pronounced in TPMT heterozygotes (Karas-

Kuželički et al., 2014). Thus, the bioavailability of SAM has been shown to influence TPMT activity *in vivo* and to affect the formation of thiopurine metabolites (Blaker et al., 2012).

1.4.1.3. Drugs

It is well known that there are extrinsic factors beyond genetics that can influence TPMT activity. Because patients are usually on polytherapy, it should be kept in mind that interactions between drugs and other factors can occur. This thesis will address those interactions for which there are clear outcome data.

Several drugs are known to inhibit TPMT activity and lead to ADRs when administered together with thiopurines. In the 1980s, benzoic acid derivatives were shown to inhibit TPMT activity strongly (Ames et al., 1986; Blaker et al., 2012). Several studies have shown that TPMT can also be inhibited by aminosalicylates, such as mesalamine, sulphasalazine, olsalazine, and balsalazide (de Graaf et al., 2010; Szumlanski and Weinshilboum, 1995). Notably, patients with Crohn's disease who were co-administered 6-MP and olsalazine were reported to exhibit several episodes of myelosuppression, and olsalazine was demonstrated to be a non-competitive inhibitor of TPMT (Blaker et al., 2012; Lewis et al., 1997). Co-administration of the thiopurine drugs AZA or 6-MP with mesalamine, sulphasalazine, or olsalazine has also been reported result in TPMT inhibition, presenting clinically as leucopenia (Lowry et al., 2001a; 2001b). The same phenomenon was described for IBD patients with different TPMT phenotypes. A strongest TPMT inhibitor examined thus far appears to be sulfasalazine (Xin et al., 2005a).

Diuretics, like furosemide, have also emerged as potential inhibitors of TPMT (Blaker et al., 2012; Xin et al., 2005b). In 2007, one study aimed at analyzing the potential inhibitory effect of nonsteroidal anti-inflammatory drugs on TPMT *in vitro* showed that naproxen, tolfenamic acid, and mefenamic acid were non-competitive inhibitors of TPMT. Also, propionic derivatives (ibuprofen and ketoprofen) have been suggested to have the potential to inhibit TPMT activity in a clinically significant manner (Blaker et al., 2012; Oselin and Anier, 2007). The xanthine oxidase inhibitor allopurinol is not a direct inhibitor of TPMT, but rather an important modulator of thiopurine tolerance; it is prescribed to patients with IBD to yield better efficacy of reduced-dose thiopurine therapy by way of increasing 6-MP bioavailability and augmenting 6-TGN levels (Blaker et al., 2012; Moon and Loftus, 2016; Zimm et al., 1983). Co-administration of these drugs must be monitored carefully to enable timely adjustments to be made as needed. Further clarification of TPMT-related drug interactions should clarify the mechanisms thiopurine intolerance and ADRs.

1.4.2. Known genetic variants of *TPMT*

The *TPMT* gene has a number of known polymorphisms and mutations affecting exons, exon-intron junctions, and the 5'-flanking region. Those sequence variants affecting the ORF of *TPMT* have received the most attention, particularly in studies of TPMT enzyme activity (see references in Table 1). Several allelic variants have been functionally characterized *in vitro* (Ujiie et al., 2008; Wennerstrand et al., 2012) or computationally (Fazel-Najafabadi et al., 2015).

1.4.2.1. TPMT sequence mutations

According to the published literature available at the time of writing this thesis, there are more than 35 non-synonymous sequence variants of *TMPT* that alter TMPT activity (Table 1). *TPMT*1* is the wild-type allele and encodes normal-activity TPMT (Szumlanski et al., 1996). Coding-region variants exhibit accelerated protein degradation or aggregation (Tai et al., 1997; Wang et al., 2005). Meanwhile, exon-intron mutations can alter mRNA splicing in ways that affect the functional status of the protein (Otterness et al., 1997; Salavaggione et al., 2005).

Table 1. *TPMT* alleles confirmed by TPMT nomenclature committee (November 2016).

Allele	rs number	Location	Amino acid change	Reference
*TPMT*1*	rs2842934		Wild-type	
*TPMT*1A*	–	Exon 1	–178C>T, –	Spire-Vayron de la Moureyre et al., 1998b
*TPMT*1S*	rs2842934	Exon 7	474T>C, Ile158Ile	Yates et al., 1997
*TPMT*2*	rs1800462	Exon 5	238G>C, Ala80Pro	Krynetski et al., 1995
	rs1800460	Exon 7	460G>A, Ala154Thr	Tai et al., 1996
*TPMT*3A*	rs1142345	Exon 10	719A>G, Tyr240Cys	
*TPMT*3B*	rs1800460	Exon 7	460G>A, Ala154Thr	
*TPMT*3C*	rs1142345	Exon 10	719A>G, Tyr240Cys	
	rs72552739	Exon 5	292G>T, Glu98stop	Otterness et al., 1997
*TPMT*3D*	rs1800460	Exon 7	460G>A, Ala154Thr	
	rs1142345	Exon 10	719A>G, Tyr240Cys	
*TPMT*3E*	rs3931660	Intron 3	140 +114T>A	Colleoni et al., 2012
	rs12529220	Intron 3	141 –101A>T	
	rs2518463	Intron 4	366+58T>C	
	rs1800460	Exon 7	460G>A, Ala154Thr	
	rs2842934	Exon 7	474T>C, Ile158Ile	
	rs1142345	Exon 10	719A>G, Tyr240Cys	
*TPMT*4*	rs1800584	Intron 9 /exon10	626-1G>A, in splice junction	Otterness et al., 1997
*TPMT*5*	rs72552740	Exon 4	146T>C, Leu49Ser	Otterness et al., 1997

Allele	rs number	Location	Amino acid change	Reference
*TPMT*6*	rs75543815	Exon 8	539A>T, Tyr180Phe	Otterness et al., 1997
*TPMT*7*	rs72552736	Exon 10	681T>G, His227Glu	Spire-Vayron de la Moureyre et al., 1998b
*TPMT*8*	rs56161402	Exon 10	644G>A, Arg215His	Hon et al., 1999
*TPMT*9*	rs151149760	Exon 5	356A>C, Lys119Thr	Schaeffeler et al., 2004
*TPMT*10*	rs72552737	Exon 7	430G>C, Gly144Arg	Colombel et al., 2000
*TPMT*11*	rs72552738	Exon 6	395G>A, Cys132Tyr	Schaeffeler et al., 2003
*TPMT*12*	–	Exon 6	374C>T, Ser125Leu	Hamdan-Khalil et al., 2003
*TPMT*13*	rs72552742	Exon 3	83A>T, Glu28Val	
*TPMT*14*	rs9333569	Exon 3	1A>G, Met1Val	Lindqvist et al., 2004
*TPMT*15*	rs9333570	Intron 7 /exon8	495-1G>A, in splice junction	
*TPMT*16*	rs144041067	Exon 7	488G>A, Arg163His	Schaeffeler et al., 2004
*TPMT*17*	–	Exon 3	124C>G, Gln42Glu	
*TPMT*18*	–	Exon 4	211G>A, Gly71Arg	
*TPMT*19*	–	Exon 5	365A>C, Lys122Thr	Hamdan-Khalil et al., 2005
*TPMT*20*	rs150900439	Exon 10	712A>G, Lys238Glu	Schaeffeler et al., 2006
*TPMT*21*	rs200591577	Exon 4	205C>G, Leu69Val	
*TPMT*22*	–	Exon 7	488G>C, Arg163Pro	
*TPMT*23*	rs74423290	Exon 8	500G>C, Ala167Gly	Lindqvist et al., 2007
*TPMT*24*	rs6921269	Exon 8	537G>T, Gln179His	Garat et al., 2008
*TPMT*25*	–	Exon 10	634T>C, Cys212Arg	
*TPMT*26*	rs72556347	Exon 9	622T>C, Phe208Leu	Kham et al., 2009
*TPMT*27*	–	Exon 5	319T>C, Tyr107Asp	Feng et al., 2010
*TPMT*28*	–	Exon 5	349C>A, Gly117Arg	Landy et al., 2011
*TPMT*29*	rs267607275	Exon 3	2T>C, Met1Thr	Lee et al., 2012
*TPMT*30*	–	Exon 3	106G>A, Gly36Ser	Sasaki et al., 2006
*TPMT*31*	rs79901429	Exon 9	611T>C, Ile204Thr	Appell et al., 2010
*TPMT*32*	rs115106679	Exon 5	340G>A, Glu114Lys	Lennard et al., 2012
*TPMT*33*	rs12339338	Exon 7	487C>T, Arg163Cys	
*TPMT*34*	rs111901354	Exon 5	244C>T, Arg82Trp	
*TPMT*35*	–	Exon 3	200T>C, Phe67Ser	Skrzypczak-Zielinska et al., 2013
*TPMT*36*	–	Exon 8	595G>A, Val199Ile	
*TPMT*37*	rs398122996	Exon 10	648T>A, Cys216Ter	Roberts et al., 2014a
*TPMT*38*	–	Exon 8	514T>C, Ser172Pro	Kim et al., 2015
*TPMT*39*	–	Exon 6	218C>T, Ala78Val	Coelho et al., 2016

*TPMT*2, TPMT*3A*, and *TPMT*3C* are the most frequent (80–95%) alleles causing intermediate and low/deficient enzyme activity (Tai et al., 1996; Yates et al., 1997). *TPMT*2* has a variant in exon 5 (Ala80Pro) (Krynetski et al., 1995). *TPMT*3A*, which occurs frequently in people of European descent, has non-synonymous changes in exon 7 (Ala154Thr) and exon 10 (Tyr240Cys) (Tai et al., 1996), which when they occur alone are referred to as *TPMT*3B* and *TPMT*3C*, respectively (Aarbakke, 1995). The enzyme activity levels of the proteins encoded by *TPMT*2* and *TPMT*3A* are decreased markedly to <1% and <0.5% of that of normal of the wild-type protein, respectively (Fazel-Najafabadi et al., 2015; Tai et al., 1997); *TPMT*3C* has relatively little effect on enzyme activity (Tai et al., 1997). Both *TPMT*2* and *TPMT*3A* are transcribed at normal levels, but people with these alleles carry low levels of the enzyme due to posttranslational modifications that affect secondary and tertiary structure and aggregate formation. The resultant mutant proteins are subjected to degradation in proteasomes and lysosomes (Tai et al., 1997; Wang et al., 2005).

Alleles *TPMT*4–*18* and **20–*39* have been identified in only a few subjects, who carry the heterozygous genotype of their respective variants (Appell et al., 2010; Coelho et al., 2016; Colombel et al., 2000; Feng et al., 2010; Garat et al., 2008; Hamdan-Khalil et al., 2003; Hamdan-Khalil et al., 2005; Hon et al., 1999; Kham et al., 2009; Kim et al., 2015; Lee et al., 2012; Lennard et al., 2012; Otterness et al., 1997; Roberts et al., 2014a; Sasaki et al., 2006; Schaeffeler et al., 2006; Schaeffeler et al., 2004; Skrzypczak-Zielinska et al., 2013; Spire-Vayron de la Moureyre et al., 1998b), though some are compound heterozygotes for different non-wild-type alleles (Landy et al., 2011; Lindqvist et al., 2004; Lindqvist et al., 2007; Otterness et al., 1997; Schaeffeler et al., 2003). The *TPMT* alleles have an ethnically differentiated distribution. *TPMT*6, *26, *27, *29*, and **38* are only found in individuals of Asian origin, whereas *TPMT*8* has only been found in African-Americans (Feng et al., 2010; Hon et al., 1999; Kham et al., 2009; Kim et al., 2015; Lee et al., 2012; Otterness et al., 1997). All other alleles are mostly present in Caucasians, but are very rare with very low frequencies (see references in Table 1). *In vitro* functional analysis have demonstrated that alleles **2–*24, *27, *28* are associated with decreased enzyme activity and accelerated protein degradation compared to the wild-type enzyme (Appell et al., 2010; Feng et al., 2010; Ujiie et al., 2008). *TPMT*19* (exon 5 mutation) was identified in a single patient with Crohn's disease, who exhibited TPMT activity comparable to that seen with the wild-type enzyme (Hamdan-Khalil et al., 2005). Computational analysis indicated diverse effects of missense mutations on TPMT structure and function, with several variants being expected to affect stability, aggregation propensity, and ligand binding (Fazel-Najafabadi et al., 2015).

Phenotype-genotype discrepancies for TPMT variants that defy the typical expectations for sequence variance are abound. Notably, phenotype-genotype correlation is very high for wild-types and mutant homozygotes (93–100%), but much less reliable (53–100%) for *TPMT* heterozygotes (Coelho et al., 2016; Ford et al., 2009; Laróvere et al., 2003; Milek et al., 2006). This divergence from

phenotype-genotype correlation points to the importance of other unidentified factors, including other genetic variations, that have an indirect effect on TPMT activity or otherwise modulate thiopurine metabolism.

1.4.2.2. Variable number tandem repeats

Other sequence variations, besides SNPs, have been identified in the 5'-flanking region of *TPMT*. The contributions of GC-rich VNTRs, which consist of three elements (A, B and C) that differ in sequence and length between individuals, to TPMT enzyme activity have been explored. Spire-Vayron de la Moureyre and colleagues (1998a) identified 17–18-bp units that repeat four to eight times (*V4–*V8). Variable three to nine repeats (**V3–*V9*) have also been found in the promoter with differing nucleotide sequences (Alves et al., 2000; Marinaki et al., 2003; Spire-Vayron de la Moureyre et al., 1998a; 1998b; 1999; Yan et al., 2000). Four-repeat VNTRs are most frequent, followed by five-repeat VNTRs (Spire-Vayron de la Moureyre et al., 1998b).

Because the VNTRs contain binding sites for the transcription factor Sp1, they could potentially have an impact on inter-individual variability in TPMT activity by way of modulating transcription and, consequently, expression levels. However, population studies analyzing the influence of VNTRs on TPMT activity have been contradictory, with some showing only modest effect (Alves et al., 2001; Marinaki et al., 2003; Yan et al., 2000). Repeats composed of 6 or 7 tandem copies have been reported to reduce promoter activity relative to alleles with less tandem copies (Spire-Vayron de la Moureyre et al., 1999). Specific combinations may also be correlated to decreased activity (Alves et al., 2001). The presence of at least one allele with more than five repeat elements has been associated with notable low activity (Yan et al., 2000), albeit a modest reduction relative to that produced by ORF-based variants. One study found no differences in VNTR allele frequencies between British Asians and Caucasians (Marinaki et al., 2003).

The effect of VNTRs on TPMT activity during thiopurine therapy has been investigated. In patients with rheumatoid arthritis taking AZA, enzyme activity levels before and after treatment were not found to be associated with VNTRs (Arenas et al., 2004). In addition, in children with ALL, there was no correlation between the number of VNTRs and 6-MP treatment outcome (Dokmanović et al., 2008). Recently, a long-term study investigating the functional role of VNTR number and type with respect to *TPMT* gene transcription was completed. The researchers demonstrated that both the number and type of VNTRs in the *TPMT* promoter influenced the level of gene transcription observed. The study also showed that the 'A' repeat has a negative effect on *TPMT* transcription and that a positive regulatory element immediately upstream to the VNTR region in the promoter was indispensable for *TPMT* transcription (Zukic et al., 2010). The same group showed later that 6-MP influences *TPMT* transcription in a VNTR-dependent manner mediated by the binding of newly recruited protein complexes

to the promoter upon 6-MP treatment. They also demonstrated that ALL patients carrying different VNTR genotypes respond differently to 6-MP therapy. Indeed, patients with VNTRs that decreased *TPMT* promoter activity were treated with lower doses of the drug and given a longer discontinuation period (Kotur et al., 2012). In 2015, in a study examining *TPMT* expression in childhood ALL patients at diagnosis and during the maintenance therapy, the same group detected a three-fold increase in gene expression during maintenance therapy, with this upregulation being modulated by the architecture of the VNTR region. They concluded that the *TPMT* VNTR region should be considered at the commencement of maintenance therapy for childhood ALL patients because it may serve a pharmacogenomic biomarker of thiopurine therapy responsivity (Kotur et al., 2015). Notwithstanding, larger cohort studies are needed to confirm these findings independently and more functional analysis should be performed to uncover the modulatory mechanism of VNTRs on TPMT activity.

1.4.2.3. Triple repeats

As mentioned above, there are more than *TPMT* variants that are predictive of decreased TPMT enzyme activity. Meanwhile, there is far less information regarding the cause of ultra-high enzyme activity. Thus far, there is only one study published that has shown an association between ultra-high enzyme activity and GCC trinucleotide repeats in the promoter region of *TPMT*. Significantly increased TPMT activity was observed for five and seven repeats, compared to six repeats, which is considered to be wild-type; however the mechanism by which the trinucleotide repeat may affect *TPMT* expression is unknown (Roberts et al., 2008). At the time of the writing of this thesis, this was the only study in the literature addressing the influence of these triple repeats on *TPMT* expression.

1.4.3. Variations in other genes and thiopurine drug response

Although thiopurines are considered to have good clinical efficacy, they are associated with some drawbacks with respect to ADRs as well as drug resistance. The mechanism of thiopurine metabolism is quite well-established with the most important player being the enzyme TPMT, though there have been many cases in which TPMT genetic and enzymatic variability could not explain drug response variance. Therefore, there is a need to identify novel markers of thiopurine drug responsivity, in genetic and metabolite levels. Several studies have investigated the possible role of other genes in thiopurine drug response (Fig. 8). The preliminary results obtained thus far need to be replicated and confirmed in further prospective studies before their clinical applicability is known. In the following section, there are examples of some findings in this regard.

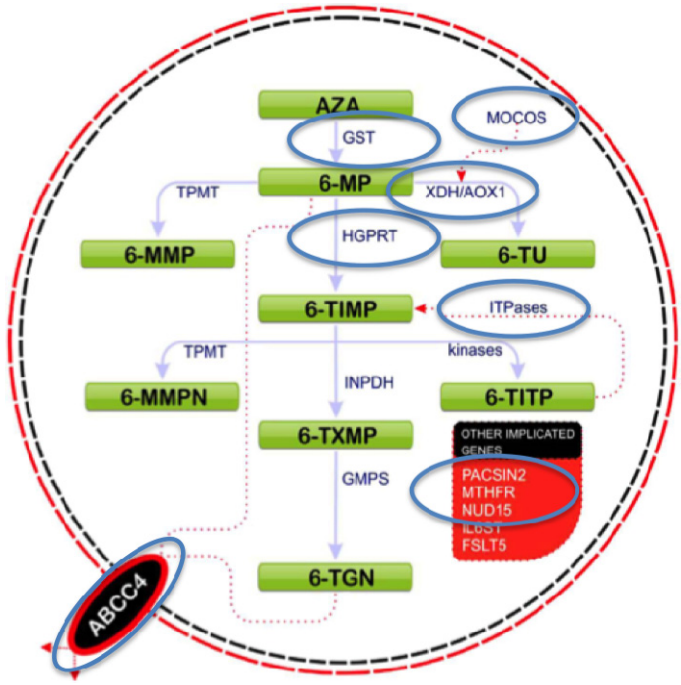


Figure 8. Schematic diagram showing the main steps and metabolites of thiopurine drug metabolism (green boxes) and genes implicated in thiopurine induced toxicity. Genes described in the text are indicated with blue encircling. Details of thiopurine metabolism are in Figure 5 above. Abbreviations: 6-MP, 6-mercaptopurine; 6-MMP, 6-methyl mercaptopurine; 6-MMPN, 6- methyl mercaptopurine nucleotides; 6-TGN, 6-thio-guanines; 6-TIMP, 6-thioinosine monophosphate; 6-TITP, 6-thioinosine triphosphate; 6-TU, 6-thiouracil; 6-TXMP, 6-thioxanthosine monophosphate; ABCC4, ATP-binding cassette, sub-family C (CFTR/MRP), member 4; AOX1, aldehyde oxidase 1; AZA, azathioprine; FSLT5, Follistatin-Like 5; GMPS, guanosine monophosphate synthetase; GST, glutathione S-transferase; HGPRT, hypoxanthine guanine phosphoribosyl-transferase; IL6ST, Interleukin 6 signal transducer; IMPDH, inosine 5-monophosphate dehydrogenase; ITPase, inosine triphosphatase; MOCOS, molybdenum cofactor sulfurase; MTHFR, methyl-enetetrahydrofolate reductase; NUDT15, nudix (nucleoside diphosphate linked moiety X)-type motif 15; PACSIN2, protein kinase C and casein kinase substrate in neurons 2; TPMT, thiopurine methyltransferase; XDH, xanthine dehydrogenase [synonym: xanthine oxidase (XO)]. Figure adapted from (Coelho et al., 2016).

1.4.3.1. Glutathione S-transferase (GST)

GSTs are cytosolic enzymes responsible for the conjugation of several xeno-biotics. Some of them are abundantly expressed in the human liver and are involved in the early metabolism of AZA (Fig. 8). These enzymes catalyze the release of 6-MP from its pro-drug AZA (Blaker et al., 2012; Moon and Loftus, 2016). The GST isoforms A1-1, A2-2, and M1-1 are abundantly expressed in the liver and exhibit robust activity towards AZA in their wild-type forms (Eklund et al., 2006).

Several polymorphisms in *GST A2-2* gene have been detected, with one variant *GST-A2-2*E* showing the three- to four-fold increased activity (Zhang et al., 2010). It has been suggested that excessively high enzyme activity with a high drug dose may lead to glutathione depletion and cause cell damage (Eklund et al., 2006). In one study, *GST-M1* was associated with adverse events (Stocco et al., 2007). The authors posited that high-activity *GST-M1* may lead to a slight, but significant increase in the risk of developing lymphopenia. Therefore, it would be expected that the null genotype (low GST-M1 activity) may protect against adverse events (Moon and Loftus, 2016; Stocco et al., 2007). In an evaluation of the association between *GST-M1* genotype and thiopurine metabolites in IBD patients treated with AZA, *GST-M1* deletion was associated with a lower TGN/dose ratio, higher AZA requirement, and dampened therapeutic response (Al-Judaibi et al., 2016; Stocco et al., 2014a). Because the frequency of the null *GST-M1* genotype is high in Caucasians and present in about half of Asians, there is a pressing need to determine its role in thiopurine drug response (Hamdy et al., 2003). However, there is not enough evidence to explain the relationship between *GST* genotype and other ADRs (Moon and Loftus, 2016). It has been suggested that genetic polymorphisms in genes encoding GSTs may be useful for predicting therapeutic response to AZA, though *in vitro* and clinical validation studies are needed to test this expectation (Stocco et al., 2014b).

1.4.3.2. Xanthine oxidase (XO)

The enzyme XO participates in the early detoxification of thiopurine drugs (Fig. 8). It oxidases 6-MP into inactive thiouric acid, which is excreted in urine, thereby removing up to two-thirds of a delivered dose (Parks and Granger, 1986). XO is a ubiquitous cytoplasmic enzyme found in particularly high levels in the intestine and liver (Huh et al., 1976). XO deficiency is very rare, but when it occurs, it yields severe toxicity following administration of a full dose of AZA (Ansari et al., 2008b; Moon and Loftus, 2016; Serre-Debeauvais et al., 1995). Activity of XO can vary 10-fold between individuals, with ethnic and gender differences having been detected (Ansari et al., 2008b; Relling et al., 1992). A variety of SNPs have been identified and associated with deficient XO activity, which consequently may alter thiopurine metabolite levels (Hawwa et al., 2008; Kudo et al., 2008; Moon and Loftus, 2016). XO activity requires its cofactor molybdenum; thus, molybdenum deficiency affects XO activity (Smith et al., 2009). It is also known that some XO products, namely oxidized purine metabolites, inhibit TPMT, which may increase 6-TGN levels (Deininger et al., 1994). Thus, the potential clinical utility of these findings is unclear. Confirmatory studies are needed to develop firm conclusions regarding the role of XO in characterizing individual patients' drug responsivity profiles.

1.4.3.3. Aldehyde oxidase (AOX)

The enzyme AOX, which is ubiquitously expressed in animals, is involved in early thiopurine metabolism (Fig. 8). It catalyzes 8-hydroxylation of 6-MP and AZA into inactive metabolites (Clarke et al., 1958). SNPs on the *AOX* gene have been associated with a poor clinical response to AZA. When *AOX1 3404G* SNP is combined with TPMT activity data, the likely drug response to AZA can be predicted effectively. Patients with normal TPMT activity and wild-type *AOX1* were found to have 86% likelihood of a favorable clinical response to AZA therapy, whereas those with deficient TPMT activity who were carriers of the *AOX1 3404G* SNP were found to only have a 33% likelihood of exhibiting a favorable response (Smith et al., 2009). However, the frequency of this SNP in different populations is unknown, and further work is needed to explore the clinical implications of these findings (Chouchana et al., 2012; Moon and Loftus, 2016).

1.4.3.4. Hypoxantine guanine phosphoribosyltransferase (HGPRT)

HGPRT catalyzes the first step in the conversion of 6-MP and 6-TG into 6-TGNs and it is the key enzyme in the purine salvage pathway (Fig. 8). Inter-individual variability in HGPRT activity could help to explain the board range of thiopurine therapeutic responses and ADRs observed in IBD patients. Mutations have been identified throughout the *HGPRT* gene, and many of these may influence the enzyme's activity (Moon and Loftus, 2016). However, there are only sparse data on the relationship between HGPRT activity and thiopurine related ADRs. In IBD patients prescribed thiopurines, high HGPRT activity was associated with an increased risk of leucopenia, which correlates with elevated 6-TGN levels (Ding et al., 2012). However, our knowledge is still insufficient to determine the significance of HGPRT for predicting and avoiding thipurine-related ADRs (Blaker et al., 2012; Moon and Loftus, 2016).

1.4.3.5. Inosine triphosphatase (ITPA)

The enzyme ITPA is widely expressed in variety of tissues in the human body, including in leucocytes and erythrocytes (Lin et al., 2001). It converts inosine triphosphate (ITP) back into inosine monophosphate (IMP), which prevents the intracellular accumulation of potentially harmful nucleotides that can be mis-incorporated into nucleic acid molecules (Holmes et al., 1979). In thiopurine metabolism, there is a parallel cycle wherein ITPA hydrolyzes 6-thioinosine triphosphate (6-TITP) back into 6-thioinosine monophosphate (6-TIMP) (Fig. 8). Thus, ITPA deficiency leads to an accumulation of toxic 6-TITPs (Marinaki et al., 2004). To date, five single nucleotide polymorphisms have been identified in the *ITPA* gene, two of which have been associated with enzyme deficiency (Sumi et al., 2002), though contradictory results exist. Some studies have shown

that ITPA deficiency in patients on AZA therapy is associated with greater risk of adverse events like flu-like symptoms, rash, leucopenia, and pancreatitis compared with control subjects (Ansari et al., 2008a; Marinaki et al., 2004; Moon and Loftus, 2016; Zelinkova et al., 2006), while other studies have not shown any significant association (Allorge et al., 2005; Kurzawski et al., 2009; Moon and Loftus, 2016). Large prospective studies and clinical trials are needed to assess the influence of ITPA on clinical responses to thiopurines.

1.4.3.6. Methylene tetrahydrofolate reductase (MTHFR)

As mentioned above, the cofactor SAM acts as an essential methyl donor for TPMT. In this reaction, SAM is converted back to S-adenosyl-L-homocystein (SAH), which is thereafter recycled back into SAM via the folate cycle. 5,10-MTHFR is an enzyme in the folate cycle that may influence TPMT activity indirectly by altering the availability of SAM. Two SNPs that have been associated with decreased MTHFR activity may decrease TPMT activity in this way (Karas-Kuzelicki et al., 2009; Ogino and Wilson, 2003; Schwahn and Rozen, 2001). Additionally, antifolate drugs such as methotrexate or trimethoprim inhibit the folate cycle and may thereby also influence TPMT activity and 6-TGN production (Brouwer et al., 2005; Dervieux et al., 2003).

1.4.3.7. Protein kinase C and casein kinase substrate in neurons 2 (PACSIN2)

PACSIN2 is a member of the 'protein kinase C and casein kinase substrate in neurons' family of proteins that are involved in vesicle formation via interactions with the large GTPase dynamin and N-WASP, which forms a critical part of the actin polymerization machinery (Kessels and Qualmann, 2004). Using genome-wide analysis (GWAS), Stocco and colleagues identified a new marker (rs2413739) in the *PACSIN2* gene that is related to variability in TPMT activity. They showed that *PACSIN2* polymorphism and gene expression information together can be used to predict TPMT activity level in HapMap CEU cell lines, and further found that *PACSIN2* genotype was related to TPMT activity and mercaptopurine-induced ADRs in children with ALL. A significant association was confirmed in both patients with wild-type and patients with variant *TPMT* genotypes, who developed gastrointestinal toxicity during consolidation therapy (Stocco et al., 2012). A recent study reported an interaction between the *TPMT* genotype and *PACSIN2* rs2413739 presence in hematotoxicity risk. Specifically, patients with wild-type *TPMT* and a *PACSIN2* rs2413739 mutant homozygous genotype were at elevated risk of experiencing hematotoxicity compared to patients that were heterozygous for or without the *PACSIN2* rs2413739 allele during ALL maintenance therapy (Smid et al., 2016).

1.4.3.8. Nucleoside diphosphate-linked moiety X-type motif 15 (NUD15)

Although TPMT deficiency is less common in individuals of Asian descent than in individuals of European ancestry, the Asian population appears to be particularly intolerant to a full dose of thiopurines. This finding suggests that there are other genetic variants associated with thiopurine intolerance that may be common in Asian populations (Moon and Loftus, 2016). In 2014, a study in Korean IBD patients, and more recently two studies of pediatric ALL patients in the USA and Japan, identified an association between a novel pharmacogenetic variant in *NUDT15* (rs116855232) and thiopurine ADRs, especially hematopoietic toxicity (Tanaka et al., 2015; Yang et al., 2015; Yang et al., 2014). NUDT15 is a member of the nudix hydrolase enzyme family; it underlies a safeguard mechanism in mammalian cells to minimize DNA damage and thereby avoid subsequent repair and apoptosis. Several studies have confirmed that *NUDT15* variant alleles represent a highly robust toxicity-related locus in Asian populations (Moriyama et al., 2016; Zgheib et al., 2017; Zhu et al., 2016). The risk allele (rs116855232) is most frequent in East Asians (9.8%), followed by Hispanics (3.9%); it is exceedingly rare in Europeans (0.2%) and not observed in Africans (Yang et al., 2015). More research examining the association between *NUDT15* genotype and thiopurine intolerance is needed. Nevertheless, the results obtained thus far suggest that the *NUDT15* risk allele should be considered a factor in leucopenia risk for patients undergoing thiopurine therapy.

1.4.3.9. Molybdenum cofactor sulfurase (MOCOS)

The MOCOS enzyme sulfurates the molybdenum cofactor of XO and AOX, key enzymes involved in the degradation of thiopurines. In a preliminary study still in need of independent confirmation, Kurzawski et al. (2012) found that the *MOCOS* rs594445 polymorphism influenced AZA dose responsivity in a manner similar to *TPMT* heterozygosity in a cohort of kidney transplant recipients on AZA therapy. Furthermore, recently, Coelho et al. (2016) identified an association between the *MOCOS* gene and TPMT activity. However, the mechanism by which MOCOS may influence TPMT function remains to be determined.

1.4.3.10. Drug transporters

Dysfunction of transport proteins specific for thiopurine metabolites may explain some of the variability in thiopurine clinical efficacy and ADRs. It has been shown that down-regulation of SLC28 and SLC29, which mediate the cellular intake of the nucleosides and nucleoside analogues, decreases the uptake of thiopurines and therefore the cellular accumulation of TGNs (Fotoohi et al., 2006; Zaza et al., 2005).

ATP binding cassette subfamily C member 4 (ABCC4) is a nucleotide efflux transporter for purines that is expressed in many cells, including myeloid progenitors. ABCC4 protects cells from the accumulation of cytotoxic metabolites that can modify sensitivity and resistance to thiopurine therapy (Janke et al., 2008; Krishnamurthy et al., 2008; Sampath et al., 2002). *ABCC4* genetic variant that reduces the encoded transporter's activity dramatically and were associated with depressed white blood cell counts were identified in a cohort of Japanese patients undergoing thiopurine therapy for IBD (Ban et al., 2010). It is not yet known whether this phenomenon generalizes to other populations.

1.5. Using TPMT pheno- and genotyping to guide thiopurine therapy

Dosing decisions for specific patients can be informed by a variety of biological markers associated with a drug-disease combination, such as genotype, gene expression profile, protein/enzyme concentration/activity, systemic metabolite/drug levels, and cell/clinical response (Jayachandran et al., 2015). Pharmacogenetic information is included on the labels of more than 180 drugs approved by US FDA and European Medicines Agency. Some include dosing guidelines based on genetic factors, such as gene variants (including *TPMT*), functional deficiencies, expression changes, and chromosomal abnormalities.[1]

There is still ongoing debate as to whether TPMT pheno- or genotyping are reliable enough to be used to determine treatment course and whether such testing would be cost-effective. In 2016, a large meta-analysis of 47 high-quality pharmocogenetic studies, of which 12 involved TPMT testing, yielded mixed results for TPMT. The cost-effectiveness of genotyping prior to the use of 6-MP or AZA was highly variable across studies, casting doubt on whether such testing would be worthwhile (Plumpton et al., 2016). The position of the European Crohn's and Colitis Organization thus far has been that no recommendations can be made according to TPMT geno- and phenotype (Travis et al., 2006), whereas the American Gastroenterological Association included it in their consensus on immunosuppressive therapy (Lichtenstein et al., 2006). Regardless, because TPMT is a well-known and important thiopurine metabolizing enzyme, several assays have been developed to measure TMPT activity and assess *TPMT* genotype.

The first guidelines for thiopurine starting dose for use at therapy initiation based on TPMT pheno- and genotype have been established by the Clinical Pharmacogenetics Implementation Consortium from the US National Institutes of Health (Relling et al., 2013; Relling et al., 2011); the therapeutic algorithm is depicted in Figure 9A and B. Current *TPMT* genotype-based recommendations are as follows: wild-type homozygotes, standard AZA dosage (2.5 mg/kg/d);

[1] https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=4490128b-e73f-4849-9d6e-e8591639d771

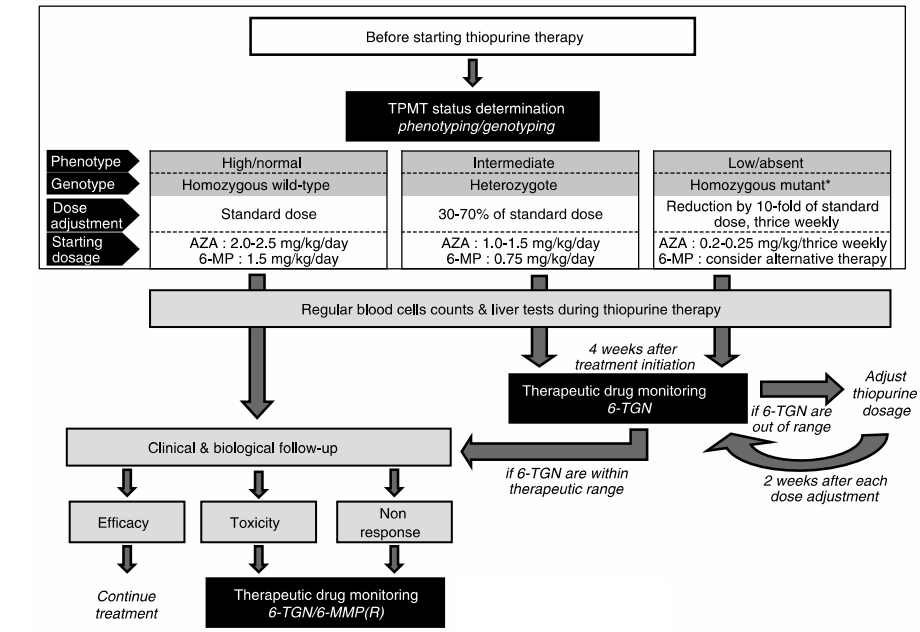
heterozygotes, 30–70% of standard dosage; mutant homozygotes or compound heterozygotes, 10% dosage or switch to alternative drug. For the two latter groups, therapeutic monitoring is important to confirm whether the adjusted dosage is appropriate (Relling et al., 2013; Relling et al., 2011). Notably, because some patients exhibit TPMT geno-/phenotype discrepancies, *in vivo* enzyme activity assays are useful. For patients with IBD who are undergoing thiopurine treatment, therapeutic drug monitoring (TDM) based on thiopurine metabolite levels (6-TGN or 6-MMP) may enable clinicians to optimize the treatment, prevent ADRs, and help elucidate the mechanisms of clinically poor response or drug resistance (Moon and Loftus, 2016; Roblin et al., 2011).

1.5.1. Measurement of TPMT enzyme activity

TPMT is widely expressed in the human body with expression varying between tissues, with notably high TPMT activity levels in the liver and kidneys, and relatively lower activity levels in the brain, lungs, intestine, and placenta (Lee et al., 1995). TPMT tissue-specific distribution differs pre- versus postnatally (Pacifici et al., 1991). Because hepatic TPMT activity correlates well with that in erythrocytes, the phenotypic measurements are usually carried out on whole blood owing to the low invasiveness of drawing blood (Szumlanski et al., 1992; Van Loon and Weinshilboum, 1982).

There are several methods available for assessing TPMT status. TPMT activity is most commonly determined by measurement of 6-MMP formation from 6-MP employing SAM as a methyl donor (Loit et al., 2011). The originally developed radiochemical method has been modified over the years to rely on nonradioactive detection by high performance liquid chromatography (HPLC) (Jacqz-Aigrain et al., 1994; Lennard and Singleton, 1994; Weinshilboum et al., 1978). A method that uses 6-TG as the enzyme substrate instead of 6-MP has also been developed (Kröplin et al., 1998). The choice of dosage threshold criteria is crucial and may be influenced by the patient population as well as the clinical indication for testing (Roberts et al., 2007; Zur et al., 2016). Although TPMT phenotyping provides important information for treatment planning, patients' on thiopurine therapy should be also monitored for toxicity and drug metabolite levels because numerous other factors can influence ADR risk, including blood transfusions, age, renal function, interactions with other drugs, and genetics.

A.



B.

	Group 1	Group 2	Group 3	Group 4	Group 5
TDM	Low/absent 6-TGN and Low/absent 6-MMP(R)	Low 6-TGN and Low 6-MMP(R)	Low 6-TGN and High 6-MMP(R)	High 6-TGN and Low 6-MMP(R)	High 6-TGN and High 6-MMP(R)
Risk	Inefficacy (false resistance)	Inefficacy or poor response	Poor response and/or hepatotoxicity	Myelotoxicity	Myelotoxicity and/or hepatotoxicity
Hypothesis	Poor compliance to treatment	Underdosing	Very high TPMT activity *i.e.* pharmacological resistance to thiopurines	Deficient TPMT activity	Overdose or refractoriness to thiopurines
Action	Therapeutic patient education	Increase thiopurine dosage	Add allopurinol 100mg/day and decrease thiopurine dosage (25-50% of original dose)	Decrease thiopurine dosage according to TPMT phenotype*	Switch to another drug if active disease

Figure 9. A: Before initiating thiopurine therapy, determine recommended starting dose based on TPMT pheno-/genotype. During thiopurine treatment, therapeutic monitoring is critical for patients on adjusted dosages. *or compound heterozygote. B: TDM based on thiopurine metabolite profiles in IBD patients experiencing toxicity or resistance. *low/absent TPMT activity (homozygous mutant or compound heterozygote). Abbreviations: TPMT, thiopurine methyltransferase; AZA, azathiopurine; 6-MP, 6-mercaptopurine; 6-TNG, 6-thioguanine nucleotides; 6-MMP(R), 6-methylmercaptopurine ribonucleotides. Panel A dapted from (Relling et al., 2011); panel B adapted from (Dewit et al., 2010).

1.5.2. Diagnostic genotyping of *TPMT*

Generally, genotyping in routine clinical laboratories targets specific variants, mostly the most common ones. When ethnic background is considered in the selection of target alleles, one may identify up to 95% of individuals with the clinically relevant alleles. However, rare variants or relevant alleles in other genes may still be missed in some cases (Zur et al., 2016). Genotyping methods used in practice include restriction-fragment length polymorphism (Coulthard et al., 1998; Yates et al., 1997), denaturing HLPC (Hall et al., 2001; Schaeffeler et al., 2001), and sequencing (Haglund et al., 2004). The advantage of *TPMT* genotyping over phenotyping is that it never changes, whereas TPMT activity may be influenced by several factors. However, the cost-effectiveness of genotyping of *TPMT* remains in question (Plumpton et al., 2016).

Both genotyping and phenotyping have their limitations. Obviously, phenotyping by measuring RBC TPMT activity is misleading in patients who have received blood transfusions because it reflects the enzyme activity of donor RBCs. On the other hand, we still do not know and understand all of the DNA sequence variations that influence TPMT activity. Considering current state of knowledge, ideally, both pheno- and genotyping should be used with other clinically relevant monitoring applications to guide the best therapy.

Epilogue

Future perspectives of pharmacogenomics

There is substantial interindividual heterogeneity in drug responses with respect to both efficacy and toxicity. The proportion of patients who respond beneficially to the first drug offered in the treatment of a wide range of diseases has reported to be 20–60% (Wilkinson, 2005). For example, on average, 38%, 40%, 43%, 50%, and 75% of patients who have depression, asthma, diabetes, arthritis, and cancer, respectively, show no response to their initial treatments (Spear et al., 2001). Furthermore, between 1990 and 2013, 43 drugs were withdrawn from the market due to severe ADRs (Wei et al., 2012). Approximately 6.5% of admissions to hospitals are related to ADRs. Hence, interindividual drug response variability is an important factor in morbidity and can lead to potentially avoidable strains on limited healthcare resources (Pillans, 2008).

As the cost of whole-genome sequencing is declining, it has been predicted that in the near future, every individual could have their entire genome sequenced in early life with the information being available for clinical use throughout one's lifetime. Thus, we are moving forward from the debate over the need for introducing PGx markers into the clinic towards a discussion about how to preemptively integrate genetic information into everyday clinical practice. More than 180 US FDA-approved drugs have at least one PGx association in their product labeling[2] and pharmaceutical companies are realizing the need to include genomic information in clinical trials.

Clinical drug responses and outcomes are always patient-specific. Such interindividual variation is often a challenge to optimizing dosage. Because different patients respond differently to the same drug and dose, the suggested population-based standard dose can lead to severe ADRs, including death, in some patients or result in therapeutic failure in others. TDM is suggested for drugs with a narrow therapeutic index or known serious ADRs (Ahmed et al., 2016). Many studies have been conducted to identify PGx markers with possible effect on diseases or conditions through drug pharmacokinetics/-dynamics. For example, several drug metabolizing enzymes, transporters, and receptors have been discovered to have potential effect on metabolic pathways of certain anticancer drugs. But for most chemotherapeutics, the association of gene polymorphisms with pharmacokinetics is not well understood (Bertholee et al., 2016).

Several implementation studies and programs have been initiated in recent years to address the barriers that prevent the clinical implementation of PGx. The main aims related to overcoming these hurdles are: implementing drug-gene pairs one at a time and assessing their clinical utility; educating healthcare providers on PGx; implementing existing CPIC and Dutch Pharmacogenomic Working Group guidelines; integrating PGx test results into the electronic health records and clinical decision support systems at the point of care to guide

[2] http://www.fda.gov/drugs/scienceresearch/researchareas/pharmacogenetics/ucm083378.htm

healthcare providers (including the standardization of pharmacogenetic terms for clinical tests); and expanding the field of PGx by making use of next generation sequencing techniques. Notably, to improve the cost-effectiveness, the Ubiquitous Pharmacogenomics Consortium aims to develop a panel of relevant PGx markers for various therapeutic areas for pre-emptive clinical testing rather than advancing single gene-drug pairs (Caudle et al., 2016; Relling and Evans, 2015; van der Wouden et al., 2016).

Analyses of large-scale sequencing efforts, such as the 1000Genomes project and Exome Sequencing Project, have indicated that each individual harbors, on average, more than 100 SNPs, of which 90% are rare non-synonymous variants in pharmacogenetic loci (minor allele frequency < 1%). It was estimated that these variants account for 30–40% of the functional variability in so-called adsorption-distribution-metabolism-excretion pharmacogenes (Kozyra et al., 2017). Thus, beyond the potential clinical utility of identifying such variants, they may have a major impact on drug discovery, particularly with respect to potentially driving stratified drug development. In light of the fact that drug development is very complex, time consuming, and costly, it is important to recognize that PGx studies enable predictions of drug response to be made based on individual variations, identifying patient subgroups that are most likely to respond. The information gained is helpful for designing drugs and drug therapy regimes based on patient profile (Gupta and Jhawat, 2017).

Beyond the research identifying the contributions of genetic variants to differences in drug response and efficacy, other "-OMICS" are emerging with potential applications in PGx. For example, (pharmaco)epigenomics (including microRNAs) and (pharmaco)metagenomics may expand the scope of PGx. Both are dynamic over time and quite susceptible to environmental factors. There is a growing body of evidence indicating that epigenomic alterations, such as methylation, histone modification, and microRNAs, regulate the expression of genes involved in drug metabolism, xenobiotic transport, drug target effects, and downstream signaling molecules, either directly or indirectly (Ingelman-Sundberg and Cascorbi, 2016).

Recognition of the physiological importance of the microbiome of the human gut is growing. Gut microbe genomes encode gene products that extend human metabolism biotransform xenobiotics, including drugs, in direct or indirect ways. Thus far, already more than 50 drugs have been shown to be amenable, *in vitro* or *in vivo*, to being metabolized by gut microbiota via biochemical reduction and/or hydrolysis. Understanding the metabolic contributions of gut microbes may provide information about the energetic demands of the gut microbiota and be useful for predicting how drugs will be modified (Spanogiannopoulos et al., 2016).

Combining PGx with traditional clinical phenotypic variables (e.g., sex and age) alone is unlikely to be sufficient to describe the net effect of the multitude of factors influencing drug responses adequately. Thus, Turner and colleagues have proposed a new interdisciplinary translational field, called systems pharmacology, that aims to parse systematically and comprehensively all of a drug's clinically

relevant activities to explain, simulate, and predict clinical drug responsivity. It is hoped that systems pharmacology will accelerate drug discovery and development by way of facilitating the identification and validation of new targets, elucidating target network responses to drug perturbation, and uncovering drug-response biomarkers. Moreover, the application of systems pharmacology holds additional transformative potential for a deeper parsing of interindividual drug variability, which would facilitate drug stratification (Turner et al., 2015).

AIMS OF THE STUDY

The overall aim of the thesis was to identify causes of variability in TPMT activity in healthy individuals and acute ALL patients.

The specific aims of the studies were as follows:

1. To identify new markers and haplotypes in the *TPMT* gene explaining the variability in TPMT activity in randomly selected healthy individuals in Estonia (Ref. I).
2. To investigate the influence of SAM on TPMT activity in human subjects (Ref. II).
3. To search for new genetic markers, beyond *TPMT*, to explain the variability in TPMT activity in the general population and among ALL patients using a genome-wide approach (Ref. III).

MATERIALS AND METHODS

The population cohort studies in Ref I–III were approved by the Ethics Review Committee on Human Research of the University of Tartu, Estonia. The study of ALL and IKP (Institut für Klinische Pharmakologie) cohort in Ref III was approved by the ethics committees of the Charité, Humboldt University in Berlin, Germany and the University of Tübingen in Tübingen, Germany in accordance with the principles of the Declaration of Helsinki. Informed consent and filled questionnaires were obtained from all study participants.

3.1. Study subjects and sample preparation

First phase (Ref I)

Estonian population cohort

Venous blood samples were collected from 253 healthy blood donors (127 males, 126 females) at the Blood Centre of Tartu University Hospital in Estonia. DNA was extracted from whole blood. TPMT activity was measured in hemolysates prepared from RBCs.

Second phase (Ref II)

Estonian population cohort

Individuals were recruited from the Estonian Genome Center at the University of Tartu in Estonia. The cohort was composed of 1017 healthy individuals (511 males, 507 females) with a wide range of health statuses and demographic information. DNA was extracted from whole blood samples; hemolysates prepared from RBCs were subjected to TPMT and SAM measurements. A set of 19 biochemical parameters from plasma and ten hematological parameters from whole blood were measured at Tartu University Hospital.

Second phase (Ref III)

Estonian population cohort

A portion of the Ref II cohort was recruited (N = 844; 414 males, 430 females).

ALL study cohort

Children diagnosed with ALL (N = 245) who participated in the Berlin-Frankfurt-Münster trials were included. None of the patients had been transfused within 3 months prior to blood sampling. The samples were obtained before ALL maintenance therapy. Genomic DNA was extracted from whole blood; RBC lysates were prepared for TPMT assay.

IKP liver cohort

Histologically normal liver tissues (N = 124) and corresponding blood samples (N = 150) were collected from patients undergoing liver surgery at the Department of General, Visceral, and Transplantation Surgery, University Medical Center Charité in Berlin, Germany. DNA was extracted from whole blood; cytosol was prepared for TPMT assay.

3.2. Biochemical measurements

First phase (Ref I)

TPMT activity was measured in the RBC hemolysates obtained for all 253 subjects. The samples were submitted to HPLC with UV detection and 6-MP as the substrate at the Department of Pharmacology at the University of Tartu in Estonia.

Second phase (Ref II)

TPMT activity and SAM levels were measured in all 1017 RBC hemolysate samples by HPLC at the Department of Clinical Biochemistry, University of Ljubljana, Slovenia. For the TPMT assay, 6-MP was used as a substrate.

Second phase (Ref III)

TPMT activity was measured in the RBC hemolysate samples from all three cohorts by non-radioactive HPLC with 6-TG as a substrate at the Dr. Margaret Fischer-Bosch Institute of Clinical Pharmacology in Stuttgart.

3.3. Genotyping and imputation

3.3.1. *TPMT* genotyping

First phase (Ref I)

The coding region of *TPMT* was analyzed for 154 subjects, including all 45 individuals with intermediate TPMT activity, all 19 with high TPMT activity, and a sample of 90 individuals with normal enzyme activity. To avoid amplification of the TPMT processed pseudogene, intron-specific polymerase chain reaction (PCR) primers were designed and the PCR primers were used to sequence the coding region of the TPMT gene. The program ChromasPro 1.34 was used for sequence analysis (Technelysium Pty Ltd).

Second phase (Ref II and Ref III)

The Estonian population cohort was genotyped for TPMT*2, *3B, and *3C alleles by TaqMan Genotyping Assays (Applied Biosystems). The ALL and IKP-liver cohorts were genotyped for the same alleles by TaqMan Genotyping Assays (Applied Biosystems) or matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (Sequenom).

3.3.2. Whole-genome genotyping

Second phase (Ref III)

Genome-wide genotyping for the Estonian population, ALL, and IKP-liver cohorts was performed with HumanCNV370, Human Omni1-Quad, and HumanHap300 arrays (Illumina, Inc), respectively. After conducting quality control, imputation, and filtering, the following markers were included in the subsequent association analyses: 8,617,769 markers in 844 Estonian cohort samples; 8,224,478 markers in 245 ALL cohort; 7,481,872 markers in 123 IKP-liver cohort samples.

3.3.3. Next-generation sequencing

Second phase (Ref III)

In the IKP-liver cohort samples, *TPMT* was analyzed for the presence of genetic variations by targeted exome sequencing in the HiSeq2500 platform (Illumina, Inc) at the Center for Genomics and Transcriptomics in Tübingen, Germany.

3.4. TPMT mRNA and protein quantification

Second phase (Ref III)

TPMT mRNA was quantified with a TaqMan® Gene Expression Assay (Hs00909011_m1, Applied Biosystems). TPMT expression was normalized against ß-actin measured with a HUMAN ACTB Endogenous Control Assay (Applied Biosystems). The measurements were conducted on a Fast Real-time PCR System (7900HT, Applied Biosystems). TPMT protein expression was quantified by immunoblot analyses of liver cytosol with a specific rabbit anti-TPMT antibody (Mayo Clinic). TPMT protein levels (available for 122 samples) were quantified through immunoblotting.

RESULTS

4.1. Identification of known and new *TPMT* variants in healthy Estonians (Ref. I)

4.1.1. Distribution of TPMT activity and influence of gender

Mean (± standard deviation, SD) TPMT activity in 253 healthy Estonians (127 males, 126 females) was 90.60 ± 32.27 ng/ml/h (range, 21.5–185.5 ng/ml/h). Males had a higher mean enzyme activity level (97.07 ± 34.66 ng/ml/h) than females (84.18 ± 28.41 ng/ml/h; Mann-Whitney U-test, $p = 0.0022$). Values in the range of 60–140 ng/ml/h were considered normal (arbitrary cut-off values); values below and above this range were considered intermediate and high, respectively. No subjects had a complete enzyme deficiency. Shapiro-Wilk W-test indicated a non-normal distribution of the wild-type population as a whole ($W = 0.98$, $p < 0.001$) as well as of the normal TPMT activity group ($W = 0.94$, $p < 0.0001$). Based on the aforementioned cut-off criteria, 45 subjects were placed in the intermediate, 189 in the normal, and 19 in the high TPMT activity groups (Fig. 10).

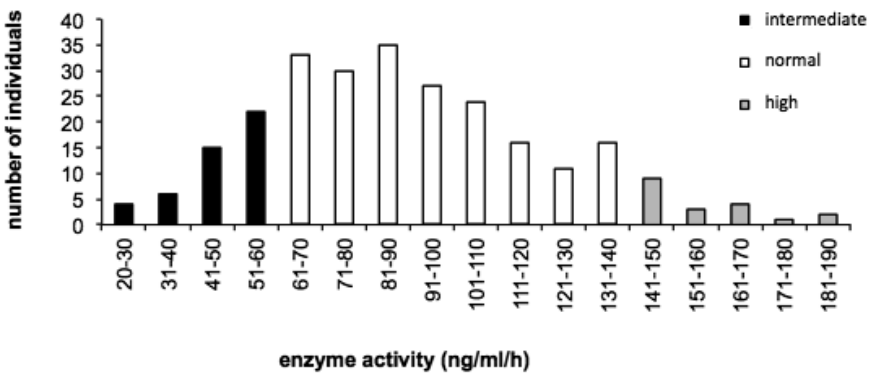


Figure 10. Distribution of TPMT activity among 253 healthy Estonians. Components of the distribution that formed the intermediate, normal, and high enzyme activity groups are indicated (graphic legend).

4.1.2. Identification of *TPMT* sequence variants

After sequencing the coding region of *TPMT* (exons 3–10) in 154 individuals (45 intermediate, 19 high, and 90 normal enzyme activity), 16 markers were identified, of which three were new (Table 2). Five previously known mutant alleles were detected: *TPMT*2*, **3A*, **3C*, **9* and **12*. Subjects who were carrying these mutant alleles include 15 *TPMT*1/*3A*, 2 *TPMT*1/*3C*, and 2 *TPMT*1/*2* heterozygotes, as well as 1 *TPMT*1/*9* and 1 *TPMT*1/*12* heterozygote.

Table 2. Single marker analysis of markers identified in the present study with their associated enzyme activity levels.

Marker	Location	N	rs number	AA	Mean TMPT activity level ± SD, ng/ml/h			*P*
					Homozygote major allele	Heterozygote	Homozygote minor allele	
–30T>A	5'-UTR	1	376768623	–	87.87 ± 36.17	56.47	–	0.3843
114T>A	Intron 3	17	3931660	–	92.96 ± 34.97	47.20 ± 11.35	–	**<0.0001**
1111A>T	Intron 3	119	12529220	–	86.97 ± 38.89	88.27 ± 33.53	87.95 ± 39.17	0.9915
10A>G	Intron 3	1	201529425	–	87.47 ± 36.17	117.4	–	0.4071
35T>C	Intron 4	105	4449636	–	84.76 ± 37.61	87.83 ± 33.53	92.31 ± 41.03	0.6749
238G>C	Exon 5	2	1800462	Ala80Pro	88.14 ± 36.10	51.6 ± 20.51	–	0.1527
356A>C	Exon 5	1	151149760	Lys119Thr	87.85 ± 36.18	59.67	–	0.4350
399C>T	Exon 5	1	17839843	Thr113Thr	87.18 ± 35.73	163	–	0.0346
58C>T	Intron 5	107	2518463	–	85.71 ± 38.12	87.19 ± 33.34	92.31 ± 41.03	0.7340
374C>T	Exon 6	1	200220210	Ser125Leu	87.91 ± 36.13	49.97	–	0.2928
94T>A	Intron 6	18	12201199	–	92.44 ± 35.25	51.61 ± 18.14	–	**<0.0001**
460G>A	Exon 7	15	1800460	Ala154Thr	92.01 ± 35.21	47.46 ± 11.5	–	**<0.0001**
719A>G	Exon 10	17	16880254	Tyr240Cys	92.71 ± 34.96	47 ± 11.11	–	**<0.0001**
474T>C	Exon 7	58	2842934	Ile158Ile	86.30 ± 36.5	88.69 ± 35.75	98.95 ± 36.95	0.6475
14G>T	Intron 8	140	2842949	–	73.77 ± 36.75	85.15 ± 35.88	91.82 ± 35.9	0.1742
145A>G	3'-UTR	1	–	–	88.01 ± 36	35.1	–	0.1417

N, number of individuals; AA, amino acid; P values of significant markers with multiple testing correction are indicated in bold.

The three new mutations were a silent –30T>A mutation in the 5'-untranslated region (UTR), a 10A>G mutation in intron 3, and a 145A>G mutation in the 3'-UTR. All three were present heterozygously.

4.1.3. Genotype-phenotype comparison and haplotype analysis

An association analysis revealed no significant differences in marker frequency between the normal and high TPMT activity groups. In the intermediate enzyme activity group, four markers [114T>A, 94T>A, 460G>A (**3B*), 719A>G (**3C*)] were found to be in strong linkage disequilibrium (LD), and the frequencies of these markers differed significantly from those in the control group (normal and high activity groups combined; $p < 0.001$, Table 2, Fig. 11).

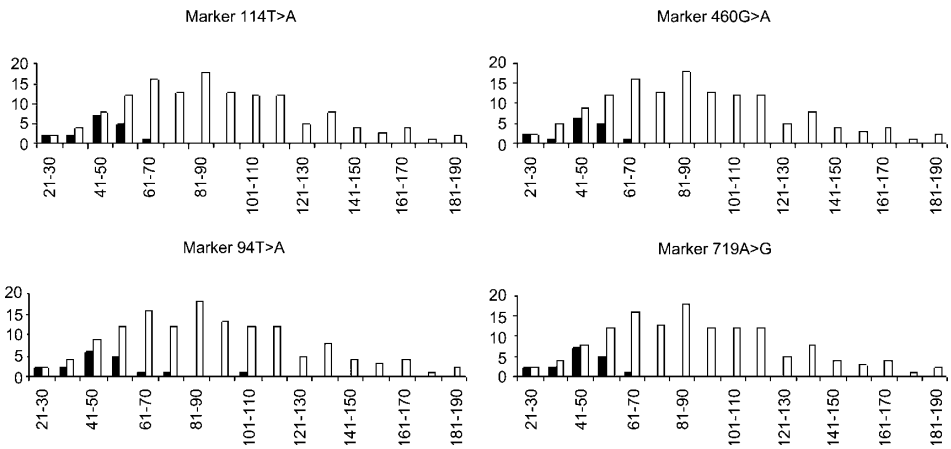


Figure 11. Comparison of enzyme activity between wild-type (□) and heterozygous (■) individuals regarding four significant markers. X-axis, enzyme activity (mg/ml/h); y-axis, number of individuals.

The haplotype analysis yielded two haploblocks. The haplotype frequencies for intermediate, normal, and high TPMT activity groups, as determined in Haploview, are presented in Table 3. The haplotype analysis further revealed that haplotype (H3) from the second haploblock was associated with reduced enzyme activity ($p < 0.001$). None of the haplotypes from the first haploblock were associated with TPMT activity.

Table 3. Haplotype frequencies in three TPMT activity groups.

Block 1	1111A>T	35T>C	58C>T	Intermediate	Normal	High
H1	T	T	C	0.575	0.500	0.523
H2	A	C	T	0.375	0.446	0.477
H3	A	T	C	0.050	0.043	–
H4	A	T	T	–	0.011	–

Block 2	474T>C	14G>T	Intermediate	Normal	High
H1	C	G	0.200	0.217	0.205
H2	T	T	0.575	0.766	0.795
H3	T	G	0.225	0.016	–

H1-H4, haplotypes 1–4

4.2. SAM modulates TPMT activity (Ref. II)

4.2.1. TPMT activity, SAM levels, and *TPMT* genotypes

In this study cohort of 1017 subjects, 828 (81.4%) exhibited normal (≥26.1 nmol/g Hb/h; 26.12–102.50 nmol/g Hb/h) and 189 (18.6%) exhibited low (<26.1 nmol/g Hb/h; 12.80–26.09 nmol/g Hb/h) TPMT activity. Meanwhile, 520 subjects (51.1%) had high SAM levels (≥16.6 nmol/g Hb; 16.61–50.90 nmol/g Hb) and 497 (48.9%) had low SAM levels (<16.6 nmol/g Hb; 1.60–16.60 nmol/g Hb).

Genotyping for *TPMT*2, *3A*, and **3C* indicated that of the 1017 subjects, 961 (94.5%) had the wild-type *TPMT* genotype (*TPMT*1/*1*), 50 (5.1%) were *TPMT*1/*3A* heterozygotes, and 6 (0.6%) were *TPMT*1/*3C* heterozygotes. No mutant homozygotes or *TPMT*2* alleles were found in the cohort. Of the 961 *TPMT* wild-type individuals, 818 (85.1%) had normal and 143 (14.9%) had low TPMT activity, respectively. Meanwhile, of the 56 *TPMT*-heterozygous subjects, 46 (82.1%) had low and 10 (17.9%) had normal TPMT activity.

4.2.2. In addition to *TPMT* genotype, SAM is a main predictor of the TPMT activity

The influence of *TPMT* genotype, SAM levels, and 41 other factors (objective, demographic, biochemical, and hematological values) on TPMT activity was investigated. Although several factors had an association *p*-value suggestive of influencing TPMT activity (<0.05) initially, after correction for multiple testing, only *TPMT* genotype and SAM levels remained statistically significant ($p \leq 1 \times 10^{-13}$).

4.2.3. Influence of SAM on TPMT activity is more pronounced in *TPMT*-heterozygous individuals

The correlation between SAM levels and TPMT activity was much stronger in *TPMT*-heterozygotes (r = 0.651, N = 56) than in wild-type individuals (r = 0.252, N = 961). The distribution of TPMT activity across the four *TPMT* genotype/SAM level groups showed the expected pattern, with the highest enzyme activity occurring in the *TPMT*-wild-type–high SAM group and the lowest activity being observed in *TPMT*-heterozygous individuals with low SAM levels (Fig. 12), indicating that healthy individuals can be divided into four TPMT-phenotype groups on the basis of *TPMT* genotype and SAM level. The influence of SAM on TPMT activity was significant within *TPMT* genotype groups, and was particularly pronounced in *TPMT*1/*3* subjects (Fig. 12).

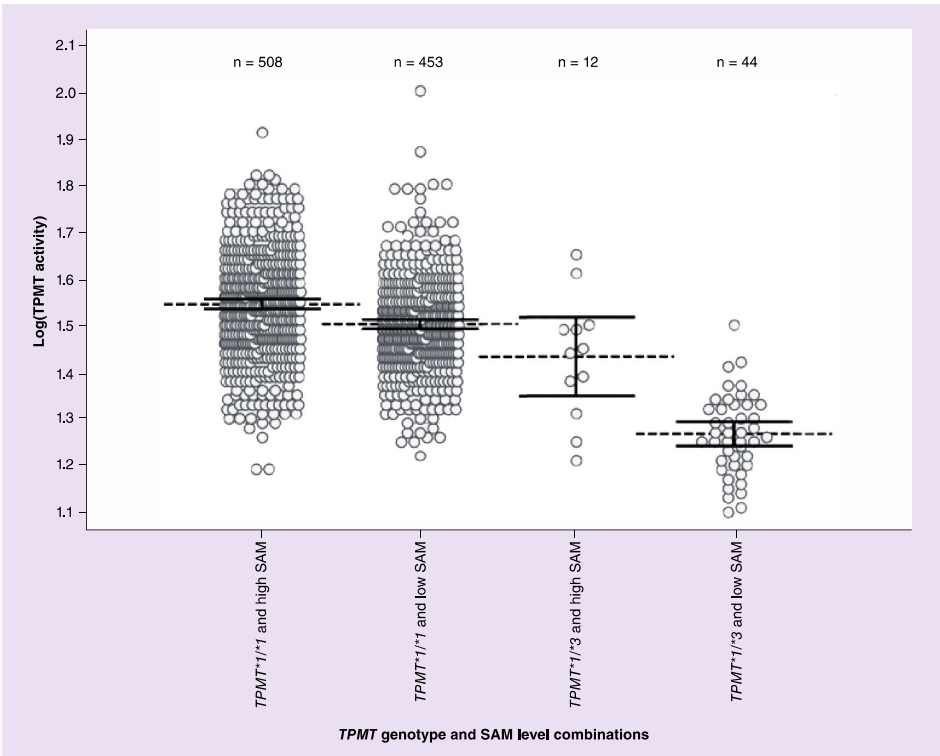


Figure 12. Distribution of TPMT activity according to *TPMT* genotype and SAM levels. TPMT activity differed significantly across the four *TPMT* genotype/SAM level combination groups ($p = 1 \times 10^{-13}$). Individuals with the *TPMT*-wild-type (*TPMT*1/*1*) genotype and high SAM levels had the highest TPMT activity, while heterozygous (*TPMT*1/*3*) individuals with low SAM levels had the lowest TPMT activity. SAM levels had a significant influence within the *TPMT* genotype groups. Error bars show 95% confidence intervals of the mean values. Dashed line show mean values for each *TPMT* genotype/SAM level group.

Next, we looked at the correlation between SAM and TPMT activity in four TPMT genotype-phenotype groups shown in Figure 12. The four presently observed TPMT genotype-phenotype groups were consistent with clinical observations, where the majority of patients belong to two genotype-phenotype concordant groups (wild-type genotype with normal activity and heterozygous genotype with intermediate activity) and a minority of individuals have discordant *TPMT* genotypes and phenotypes (wild-type genotype with low activity or heterozygous genotype with normal activity). This discordance is a major obstacle to more extensive implementation of pharmacogenetics in clinical practice. In our cohort, the two groups with concordant TPMT genotypes and phenotypes had very similar correlation coefficients between TPMT activity and SAM (wild-type with normal activity, r = 0.259; heterozygous with intermediate activity, r = 0.299), while the coefficients obtained for the two discordant groups differed substantially. In *TPMT*-wild-type with low TPMT activity, TPMT activity did not correlate with SAM levels (r = –0.059). By contrast, in *TPMT* heterozygotes with normal TPMT activity, we observed a very strong correlation between the TPMT activity and SAM levels (r = 0.879), indicating that the discrepancy between TPMT genotype and activity in individuals carrying a mutated *TPMT* allele might be due to differing SAM levels.

The discovery that unexpectedly high TPMT activity in some *TPMT*-heterozygous individuals might be due to higher than typical SAM levels is supported by the finding that the difference in the mean SAM levels between the genotype–phenotype-concordant and -discordant groups was much more pronounced in *TPMT*1/*3* heterozygotes than in *TPMT*-wild-type individuals. As illustrated in Figure 13, *TPMT*-wild-type individuals with low enzyme activity had a mean SAM concentration only 2 units lower than *TPMT*-wild-type individuals with normal enzyme activity, a difference that is unlikely to be clinically significant despite the p-value. On the other hand, among *TPMT*1/*3* subjects, those with normal TPMT activity had a mean SAM level 10.5 units higher than those with low enzyme activity, a difference that could be important in a clinical setting.

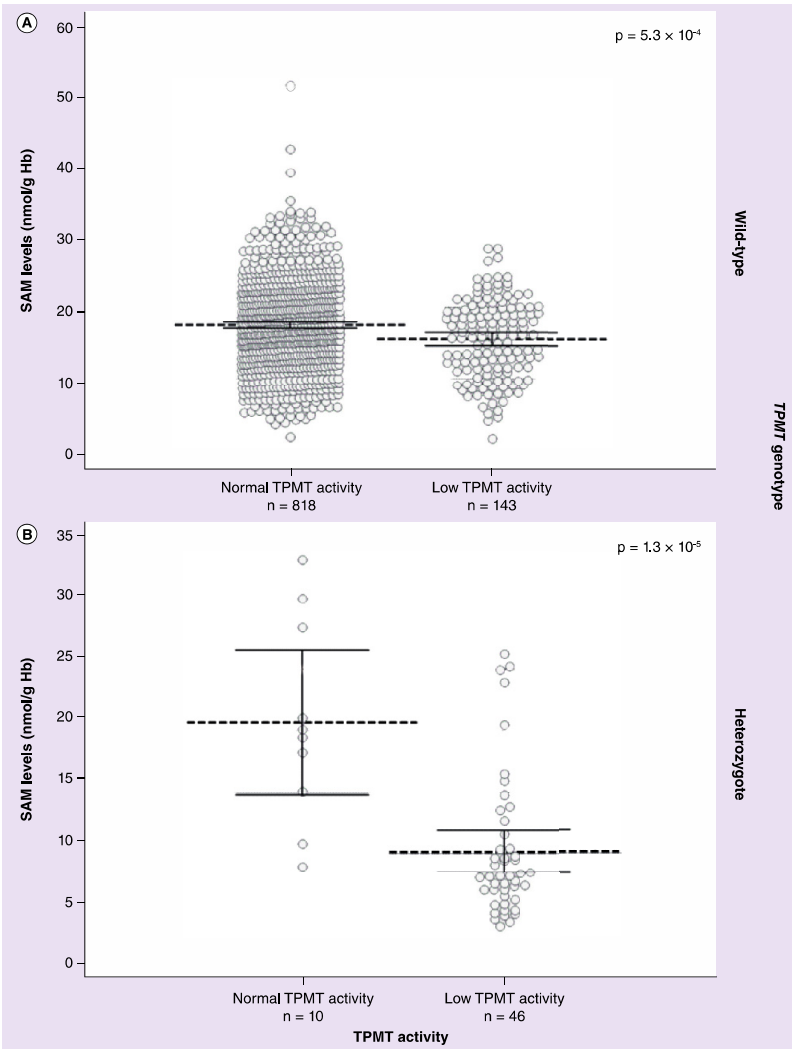


Figure 13. Comparisons of SAM levels between normal- and low-TPMT activity individuals within two *TPMT* genotype groups. In both wild-type and heterozygous individuals, SAM levels were higher in the group exhibiting normal enzyme activity (means, 17.5 nmol/g Hb and 20.7 nmol/g Hb, respectively) than in the groups with low TPMT activity levels (15.5 nmol/g Hb and 10.2 nmol/g Hb, respectively). However, the difference in SAM levels was greater in *TPMT* heterozygotes (i.e., 20.7 *vs.* 10.2 nmol/g Hb), indicating that the unexpectedly high TPMT activity in some heterozygous individuals might be due to higher SAM levels. Error bars show 95% confidence intervals of the mean values. Dashed lines show mean SAM levels for each genotype-phenotype group. Hb, hemoglobin.

4.3. A genome-wide meta-analysis revealed the *TPMT* locus as the main determinant of TPMT phenotype (Ref. III)

4.3.1. TPMT phenotype-genotype associations in three studied cohorts

First, we investigated the relationship between genome-wide genotypes and TPMT activity in a cohort of Estonians from the general population. TPMT activity measured in RBCs showed a bimodal distribution of TPMT activity without any cases of TPMT deficiency. The association analysis of *TPMT*3* alleles with TPMT activity showed significantly lower TPMT activity in heterozygotes (median 21, range 13–33 nmol/g Hb/h) than in wild-type homozygotes (median 40, range 18–64 nmol/g Hb/h; Fig. 14A).

Next, we investigated whether the same relationship between genotype and TPMT activity exists in pediatric ALL patients. The distribution of TPMT activity showed two patients with a profound *TPMT* deficiency (activity ≤2 nmol/g Hb/h): one with the *TPMT*3A/*11* genotype and one *TPMT*3A/*3A* homozygote. *TPMT* wild-type carriers had significantly higher TPMT activity levels (median 30, range 9–69 nmol/g Hb/h) than heterozygous carriers of defective *TPMT* alleles (median 21, range 9–33 nmol/g Hb/h; Fig. 14B).

After making these observations, we sought to identify genetic markers that influence TPMT activity in the liver, which is composed of the most important thiopurine metabolizing tissue in the human body. TPMT activity in liver cytosol showed a bimodal distribution and none of the samples were *TPMT*-deficient. Liver cytosol samples from carriers of the *TPMT*2* and *TPMT*3A* alleles showed reduced hepatic TPMT activity (median 1.2, range 1.1–1.9 nmol/h/mg) compared to those of wild-type *TPMT* homozygotes (median 2.7, range 1.5–4.1 nmol/h/mg; Fig. 14C).

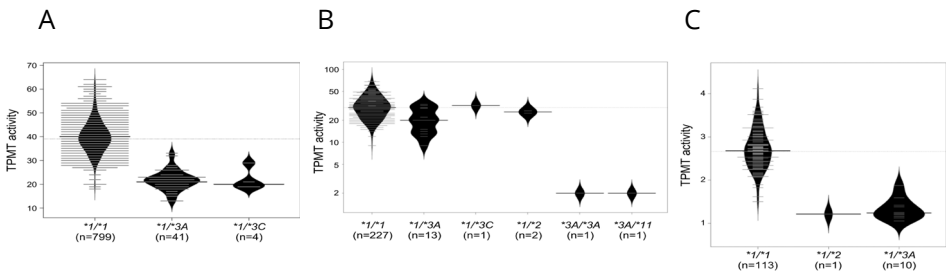


Figure 14. Association of TPMT activity with wild-type (*TPMT*1)* and common non-functional *TPMT* alleles (*TPMT*2, *3A, *3C*) in Estonian (A), pediatric ALL (B), and IKP liver (C) sample cohorts.

4.3.2. Genome-wide association hits in three studied cohorts

A total of 8,617,769 genotyped and imputed genetic markers in the Estonian population cohort (see section 3.3.2) were included in a GWAS for TPMT activity, using sex and age as covariates. As shown in a Manhattan plot (Fig. 15A) and regional association plot (Fig. 15B), the locus showing the most significant association with TPMT activity was in the *TPMT* gene region at 6p22.3. In total, 169 genetic variants (Table S1 in Ref. III) on chromosome 6 were significant at the genome-wide threshold ($p < 5 \times 10^{-8}$) with the lowest *p*-value being 2.73×10^{-76}.

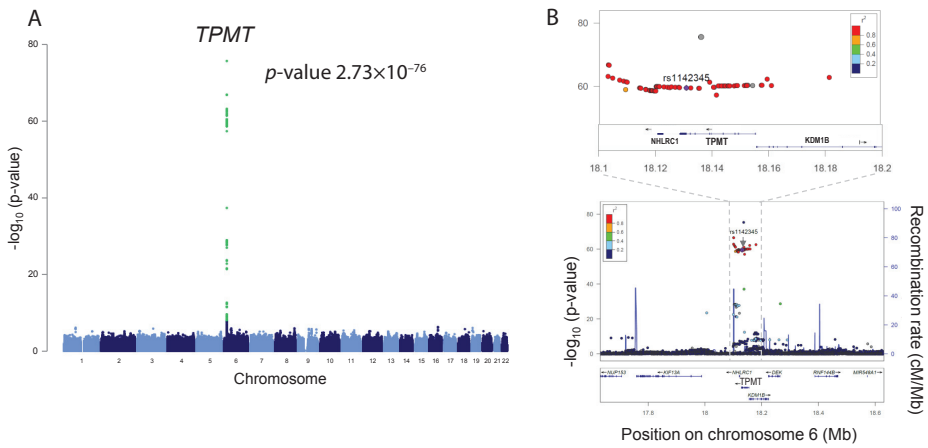


Figure 15. Results of GWAS for TPMT activity in the Estonian cohort, using age and sex as covariates. **A.** Manhattan plot showing the association *p*-values of genetic variants across chromosomes 1–22. Significantly associated genetic variants, $p < 5 \times 10^{-8}$, are marked in green. **B.** Regional association plot highlighting the genomic region containing *TPMT*. Recombination rates and LD estimates (r^2) of variants with the *TPMT* variant *719A>G* (rs1142345) are displayed.

A parallel GWAS of 8,224,478 genotyped and imputed markers in the ALL cohort was conducted. As shown in Figure 16, only genetic variants within the *TPMT* gene region were significantly associated with TPMT activity. A list of all SNPs that were genome-wide significant ($p < 5 \times 10^{-8}$) is provided in Table S2 in Ref. III. The common variant *719A>G* (rs1142345), which is present in the non-functional *TPMT*3A* and *TPMT*3C* alleles, displayed a notably low p-value.

Subsequently, we performed a GWAS of 7,481,872 genotyped and imputed markers in the IKP-liver cohort, corrected for age and sex, to determine whether the association results found in previous cohorts could be further confirmed. As shown in Figure 17, only genetic variants within the *TPMT* region were found to be significantly associated with TPMT activity ($p < 5 \times 10^{-8}$) (Table S3, Ref. III).

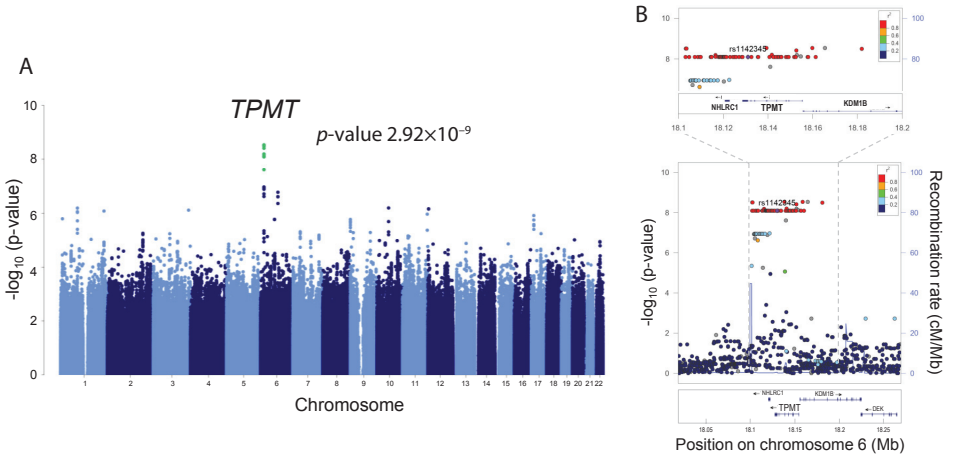


Figure 16. Results of GWAS for TPMT activity in the ALL cohort, using age and sex as covariates. **A.** Manhattan plot showing the association *p*-values of genetic variants across chromosomes 1–22. Significantly associated genetic variants, $p < 5 \times 10^{-8}$ are marked in green. **B.** Regional association plot highlighting the genomic region containing *TPMT*. Recombination rates and LD estimates (r^2) of variants with the *TPMT* variant *719A>G* (rs1142345) are displayed.

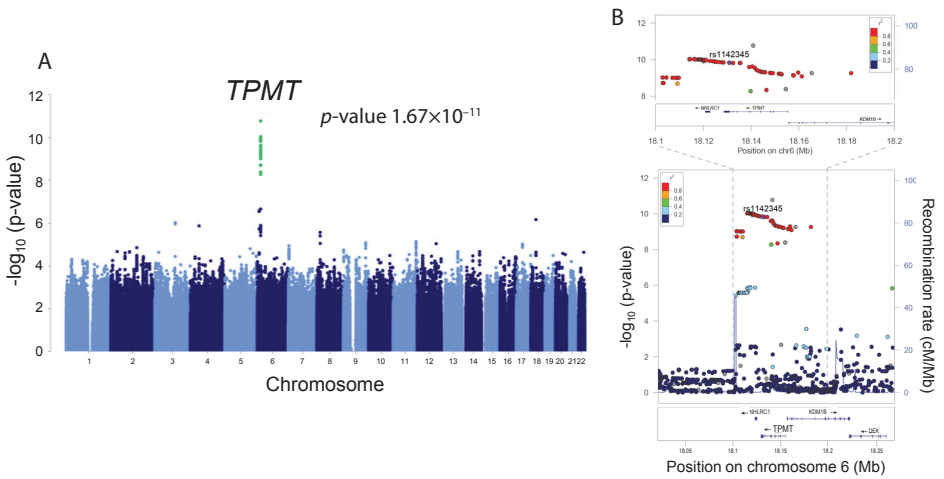


Figure 17. Results of GWAS for TPMT activity in IKP human liver samples, using age and sex as covariates. **A.** Manhattan plot showing the association *p*-values across chromosomes 1–22. Significantly associated genetic variants, $p < 5 \times 10^{-8}$ are marked in green. **B.** Regional association plot of these values focusing on the genomic region on chromosome 6 (hg19) in which *TPMT* is located. Recombination rate and LD estimates (r^2) of variants with the *TPMT* variant *719A>G* (rs1142345) are shown.

Further GWAS analyses focused on the most frequent non-functional *TPMT* alleles (*TPMT*3A* and **3C*) were performed in all three cohorts to search for genetic factors independent of *TPMT*. However, no further variants were found to be significantly associated ($p < 5 \times 10^{-8}$) with TPMT activity. Moreover, previously identified candidate SNPs were not found to be related to TPMT activity in the Estonian, ALL, or IKP-liver cohorts in our GWAS results (Table 4).

Table 4. Previously implicated SNPs were associated with TPMT activity in the present cohorts.

					Estonian cohort		ALL study		Liver cohort	
gene	rs number	chro-mo-some	non-effect allele	effect allele	effect size	P-value	effect size	P-value	effect size	P-value
PACSIN2§	rs2413739	22	c	T	0.06	0.23	–0.07	0.48	–0.02	0.91
XDH#	rs494852	2	C	T	0.07	0.36	–0.13	0.26	0.004	0.98
IMPDH1	rs4731448	7	A	G	0.02	0.73	–0.01	0.90	–0.03	0.83
SLC28A3#	rs17428030	9	A	G	–0.11	0.26	0.04	0.77	–0.30	0.26
ABCC4#	rs17268122	13	G	T	0.03	0.62	0.02	0.84	0.15	0.36
FAM8A6P#	rs1040637	6	A	G	0.02	0.72	–0.10	0.31	–0.40	0.002
HIVEP2, AIG1#	rs200148	6	G	A	0.05	0.27	–0.13	0.12	0.13	0.34
*NUDT15**	rs554405994	13	–	GGAGTC	–0.34	0.44	–	–	–	–
*NUDT15**	rs186364861	13	G	A	–	–	–	–	–	–
*NUDT15**	rs116855232	13	C	T	0.18	0.61	–0.70	0.20	–0.14	0.87
*NUDT15**	rs147390019	13	G	A	–	–	–0.55	0.45	–	–

§ (Stocco et al., 2012), # (Matimba et al., 2014), *(Moriyama et al., 2016)

Because systematic data on TPMT expression and *in vivo* function in the liver were not available, we conducted a correlational analysis between cytosolic TPMT activity and TPMT protein levels, determined by immunoblotting and obtained a highly significant correlation ($r_S = 0.58$, $p < 2.2 \times 10^{-16}$) between TPMT's protein expression level and its enzyme activity level. TPMT *mRNA* expression did not correlate with either TPMT activity or TPMT protein levels in the human liver, even after exclusion of *TPMT* variant cases. These results are in accordance with previously published data (Tai et al., 1997)

4.3.3. Meta-analysis of the studied cohorts

A joint meta-analysis of all three of the aforementioned datasets was performed to increase power and reliability of our GWAS analyses. The meta-GWAS revealed significantly associated genetic variants on chromosome 6; among them was a sentinel marker of the *TPMT* gene region (rs73726531, $p = 1.2 \times 10^{-72}$, effect size: –2.2; Fig. 18A). As shown in the associated regional association plot in Figure 18B, this sentinel marker was observed to be tightly linked with the *TPMT* variant *719A>G* (rs1142345). Altogether, 148 markers on chromosome 6 exceeded the genome-wide significance threshold ($p < 5 \times 10^{-8}$) in the meta-GWAS (Table S5 in Ref. III).

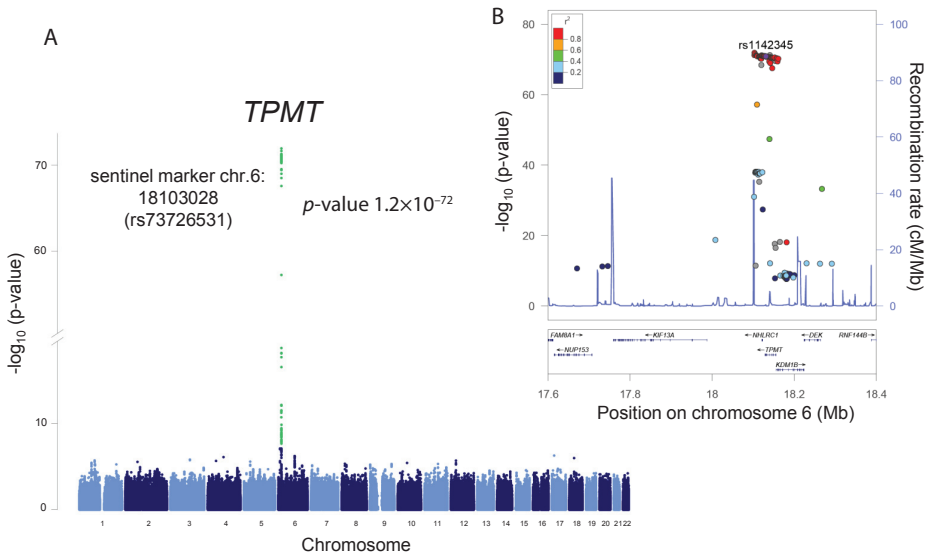


Figure 18. Results of meta-GWAS for TPMT activity of all three cohorts. **A.** Manhattan plot showing the association *p*-values, revealing significant results only within the *TPMT* locus. The sentinel marker was at chromosome 6:18103028 (rs73726531; minor allele frequency = 0.03, $p = 1.2 \times 10^{-72}$; effect size: –2.2), which is linked to *TPMT* variant *719A>G* (rs1142345). **B.** Regional association plot of these values focusing on genomic region hg19 in which *TPMT* is located.

5. DISCUSSION

Ref I was the first study focusing on the distribution of TPMT enzyme activity, and the identification and frequency of known and novel sequence variants in *TPMT* in the Estonian population. It is very well known that the frequency of mutations that influence drug responses in one population may not be a causative variant in another. The variety of *TPMT* allele patterns that have been observed thus far in different ethnic group populations is summarized in Table 5. Our finding of higher mean enzyme activity in males than in females is consistent with the findings of Schaeffeler et al. (2004), who observed good correlations of gender, as well smoking, with enzyme activity.

Table 5. TPMT allele frequencies in different populations.

Population	**Allele frequency (%)**						**Reference**
	***2**	***3A**	***3B**	***3C**	***6**	**Other alleles**	
British Caucasian (N = 199)	0.5	4.5	0	0.3	NA	NA	(Ameyaw et al., 1999)
Swedish (N = 800)	0.06	3.75	0.13	0.44	NA	NA	(Haglund et al., 2004)
American Caucasian (N = 282)	0.2	3.2	0	0.2	NA	NA	(Hon et al., 1999)
African-American (N = 248)	0.4	0.8	0	2.4	NA	NA	(Hon et al., 1999)
Ghanaian (N = 232)	0	0	0	6.75	0	3.45 (*8)	(Schaeffeler et al., 2008)
Chinese (N = 192)	0	0	0	2.3	NA	NA	(Collie-Duguid et al., 1999)
Japanese (N = 151)	0	0	0	0.3	NA	NA	(Kubota and Chiba, 2001)
Russian (N = 700)	0.14	2.6	0	0.36	NA	0	(Nasedkina et al., 2006)
Korean (N = 900)	0	0	0	1.44	0.17	0.07 (*16, *32), 0.11 (*38)	(Kim et al., 2015)
Estonian (N = 253)	0.4	2.75	0	0.2		0.3 (*9, *12)	(Tamm et al., 2008)

N, number of individuals; NA, not available.

The most frequent TPMT alleles in Caucasians are *TPMT*2*, **3A* and **3C* (Ameyaw et al., 1999; Haglund et al., 2004; Schaeffeler et al., 2004). Consistent with the results obtained in our study, the allele frequencies of *TPMT*3A* among the different populations have been reported to be in the range of 3.2–5.7%, and those of *TPMT*2* and *TPMT*3C* have been reported to be in the range of 0.2–0.8% *3C* (Ameyaw et al., 1999; Haglund et al., 2004; Schaeffeler et al., 2004). The rest of the *TPMT* alleles that have been identified to date are very rare (i.e., 1 in 300 individuals, Liu et al., 2015; Weinshilboum and Sladek, 1980). Our study cohort included a single carrier of *TPMT*9* and a single carrier of *TPMT*12*, both of whom were heterozygotes. The aforementioned variants result in reduced TPMT enzyme activity, as was confirmed by our findings of substantially lower TPMT activity levels for heterozygous individuals (48.8 ± 11.3 ng/ml/h) than for wild-types (93.3 ± 31 ng/ml/h, $p < 0.001$).

Three new mutations were identified, including an intronic SNP (10A>G, rs20152942), a SNP in the 3'UTR (–30T>A, rs376768623), and a SNP in the 5'UTR (145A>G). The effects of these mutations on enzyme activity are unknown. Given that the rs376768623 allele was paired with the known low-activity allele *TPMT*3A* and the carrier had intermediate (some preserved) activity, the rs376768623 allele may have no or only a negligible effect on enzyme activity. All three of these new mutations are extremely rare, with prior examples of only two of them (rs376768623, rs201529425) in very few individuals being included in the dbSNP database. The intronic mutations 114T>A and 94T>A were relatively common in our intermediate TPMT activity group. However, additional studies will be necessary to understand if these have any regulatory impact on enzyme activity.

Although genetic variants were found in association with intermediate TPMT activity, the present results cannot explain high (or ultra-high) TPMT activity. Similar to Dewit et al. (2010), we observed few individuals with ultra-high TPMT activity. Notably, the TPMT activity in one subject who was heterozygous for the extremely rare 399C>T variant was found to be almost double the median activity level of the wild-type individuals. This variant allele may have a gain-of-function effect, but no final conclusion can be made based on data from our single subject nor data in the dbSNP. To date, published analyses have not associated any variations in the *TPMT* open reading frame region with ultra-high enzyme activity. However, Roberts et al. (2008) reported a triple repeat in the promoter region of *TPMT* that was associated with very-high TPMT enzyme activity. We also contributed samples to that work, but the effect appeared to be quite specific for individuals of British origin. An alternative explanation for heightened TPMT activity may be gene duplication or enzyme induction. For example, ultrahigh CYP2D6 enzyme activity has been attributed to a functional gene duplication (Dalén et al., 1998). We have contributed samples to other studies looking for *TPMT* gene duplication, but none of the studies have yielded positive results. In addition, previous studies examining the effect of VNTRs in *TPMT* promoter region on enzyme activity have produced contradictory results (Arenas et al., 2004; Marinaki et al., 2003;

Zukic et al., 2010). Finally, it has been established that epigenetic patterns affect adsorption-distribution-metabolism-excretion gene expression (Kacevska et al., 2012), but no such studies in the literature have included data related to *TPMT* as of yet.

Our analysis of common markers shared by study participants, regardless of their enzyme activity, revealed that several markers formed two haploblocks (Table 3). The most interesting result obtained from this analysis was that although the markers 474T>C and 14G>T did not associate independently with intermediate enzyme activity, their haplotype (H3) may predict reduced TPMT activity because it was more frequently present in the intermediate TPMT activity group (Table 3). We used the D' confidence interval algorithm (Gabriel et al., 2002) for block definition in Haploview because that haplotype framework provides substantial statistical power in association studies of common genetic variation within regions.

One must consider the fact that TPMT represents only one part of a complicated network of biochemical pathways involved in drug metabolism. Accordingly, thiopurine drug toxicity has been associated with other genes described in this thesis, which contribute to the metabolism of thiopurine drugs and may influence TPMT activity directly or indirectly via other factors.

Even though the correlation between *TPMT* genotype and thiopurine therapy response is good in most cases, an increasing number of patients whose therapy responses cannot be predicted on the basis of *TPMT* genotype alone are being seen in clinical practice (Hindorf and Appell, 2012; van Egmond et al., 2012). Therefore, the identification of additional (genetic or non-genetic) biomarkers of thiopurine response profile is of great importance.

Studies performed in various experimental paradigms – including an *in vitro* protein model (Scheuermann et al., 2004), cell lines (Milek et al., 2009; Milek et al., 2012), and indirect studies of human subjects (Arenas et al., 2005; Dorababu et al., 2012; Karas-Kuzelicki et al., 2009) – have shown that SAM may modulate TPMT activity as a non-genetic cofactor. Arenas et al. (2005) suggested that some cases of patients with a homozygous *TPMT*-wild-type genotype exhibiting low TPMT activity might be explained by the presence of the *MTHFR* 677 TT genotype. More than a decade ago, they hypothesized that their result might be due to the fact that MTHFR is a key enzyme in SAM biosynthesis, but a direct correlation between SAM and TPMT activity had not been reported. Since that time, cell biology studies have demonstrated SAM-mediated stabilization of TPMT via a posttranslational mechanism that occurs in both tumor-derived transformed and primary cells (Milek et al., 2012).

In our study (Ref II) – which is to our knowledge the first to study the direct influence of SAM on TPMT activity in a large number of individuals – we showed that SAM is an important modulator of TPMT activity in healthy individuals not exposed to thiopurine drugs. More specifically, SAM had a greater influence on TPMT activity in *TPMT*1/*3* than *TPMT*-wild-type genotype individuals (Fig. 13). This finding is in concordance with the findings of Tai et al. (1997) indicating that stabilization of the TPMT protein by SAM is

especially pronounced in the presence of mutant TPMT. Our results do not lead to the conclusion that SAM levels can explain discrepancies in TPMT activity and thiopurine drug responses among wild-type individuals. However, our results do support the notion that SAM levels could be an important factor modulating TPMT activity in *TPMT* heterozygotes. Notwithstanding, these data should be interpreted with caution due to the small number of subjects in the normal activity–heterozygous group. Although this discordant group represents only approximately 1% of the population (according to both the present study and that conducted by Hindorf and Appell, 2012), in terms of clinical practice, this finding means that every fifth to tenth heterozygote may be at increased risk of ineffective therapy and relapse if classified only by genotype. Given the fairly weak correlations between SAM and TPMT activity in some TPMT genotype–phenotype groups, we believe that there are additional modulators of TPMT activity that have yet to be identified.

It should be kept in mind that even though good correlations were obtained with TPMT activity data obtained for RBCs and liver cytosol, these measurements are only an approximation of thiopurine-relevant metabolic processes that take place mostly in the liver and the various modulators/cofactors of drug metabolism may not be represented in RBCs. Duley and Florin (2005) pointed out that diagnostic values for TPMT activity and TGN levels are a RBC phenomenon poorly equated to the liver, which is the major site of drug methylation. Thus, some cases of thiopurine-induced leukopenia cannot be explained by RBC TPMT activity or TGN levels, and some individuals with low TPMT activity in RBCs can tolerate normal doses of the drug.

It is also important to note that the present study was conducted in individuals who were not undergoing thiopurine therapy. Therefore, prospective studies on thiopurine-treated ALL and IBD patients are necessary to evaluate the clinical usefulness of determining SAM levels as a predictive factor of thiopurine therapy response. Diagnostically and economically, it may be reasonable to measure SAM levels only in *TPMT*-mutated patients because such patients, when confirmed to have high SAM levels, should be able to tolerate standard doses of 6-MP, and thereby experience better therapeutic efficacy than they would with a reduced dose. Meanwhile, in *TPMT*-mutated patients with low SAM levels, SAM supplementation during 6-MP therapy could lower the incidence of adverse effects. Currently, there is no clinical evidence base for SAM supplementation during thiopurine therapy. Thus, further studies in patients receiving thiopurines are warranted in the near future.

The possibility that human microbiota may play an important role in drug response in terms of efficacy and ADRs has been gaining attention recently (Spanogiannopoulos et al., 2016). In the case of TPMT, which is highly evolutionarily conserved, bacterial TPMT reacts with AZA. Moreover, bacterial TPMT confers resistance to the bactericidal drug tellurite, highlighting how bacterial enzymes can act promiscuously on drugs used to treat humans. Such unplanned actions may inactivate a drug before it reaches the intended target tissue (Nayak and Turnbaugh, 2016; REMY, 1963).

As mentioned earlier in this discussion, concerns remain with regard to whether *TPMT* genetics determine exclusively the substantial inter-individual variability of TPMT activity measured in RBCs, or whether other genes may contribute substantially. Clinically, it is well-known that in addition to *TPMT* heterozygous individuals, patients with a homozygous wild-type *TPMT* genotype are at risk for thiopurine-related hematotoxicity. Although non-genetic factors (like SAM) may underlie thiopurine-related hematotoxicity, the mechanisms are not yet well understood as discussed extensively in literature overview of this thesis.

Our meta-analysis (Ref. III) results indicate that *TPMT* genetics have a fundamental impact on TPMT activity (Fig. 18) in humans and provide little support for the proposal that other genes may significantly contribute to the inter-individual variability of TPMT activity. In addition to investigating blood samples from healthy and diseased human cohorts, we also investigated, for the first time, human liver samples and the results revealed that only variants on chromosome 6 (*TPMT* region) were significantly associated with TPMT activity (Fig. 17). Noticeably, the genome-wide significant hits for TPMT activity were found to extend to other genes (e.g. *KDM1B*) near *TPMT*. As shown in the regional association plot of the meta-GWAS (Fig. 18B), these variants were linked with the presence of non-functional *TPMT*3* alleles. Further analyses conditioned on the *TPMT*3A* and **3C* alleles revealed no variants that were significantly associated with TPMT activity in all three cohorts.

Of note, we observed TPMT genotype-phenotype discordance in ALL patients, compared with the Estonian general population cohort. That discordance was attributed to the ALL disease process and anemia of patients resulting in lower TPMT activities in RBCs due to degraded TPMT enzyme.

We attempted to replicate effects associated with non-*TPMT* related candidate genes previously identified by a genome-wide study approach with HapMapCEU lymphoblastoid cell lines (LCLs) (Matimba et al., 2014; Stocco et al., 2012). Of note, LCL, including non-HapMap LCLs, have been promoted as useful model systems for cellular pharmacology, biochemistry, and enzymatic reaction studies (Zhang and Dolan, 2009). Matimba et al. (2014) proposed a minor association ($p > 5 \times 10^{-4}$) between SNPs within "thiopurine-related" genes (*XDH*, *IMPDH1*, *SLC28A3*, *ABCC4*) and "non-thiopurine pathway" SNPs (*FAM8A6P*, *AJG1*/*HIVEP2)* with thiopurine cytotoxicity in LCLs, with further validation in pediatric ALL patients. Additionally, using a genome-wide approach, Liu et al. (2016) sought to identify novel predictors of TPMT activity in LCLs and found 96 genes that ranked higher than *TPMT* itself. With the exception of *SLC22A16*, none of those genes were located on chromosome 6. However, our findings provide little support for any such effect being mediated through TPMT directly because there was only negligible correlation with respect to TPMT activity in our cohorts (Table 4). Noticeably, we did not find any variants of *SLC22A16* that were significantly associated with TPMT activity. Thus, use of LCLs in studies aimed at discovering novel pharma-

cogenetic loci may have serious limitations. Regardless, validation of results obtained in LCLs require validation in large-scale population/patient cohorts.

SNPs localizing to *PACSIN2*, a gene previously reported to influence TPMT activity and to be related to MP-related gastrointestinal toxicity (Stocco et al., 2012), were not associated with TPMT activity in our analyses (Table 4). Our negative finding in this regard is consistent with the work of Roberts et al. (2014b), who failed to confirm an association between *PACSIN2* genotype and thiopurine-related ADRs in IBD patients.

Yang et al. (2014) reported that a *NUDT15* variant was associated with thiopurine-induced leukopenia firstly in a retrospective cohort of Korean IBD patients, and that finding was confirmed in subsequent studies involving children with ALL and MP intolerance (Tanaka et al., 2015; Yang et al., 2015). Very recently, Moriyama et al. (2016) provided mechanistic evidence of how *NUDT15* variants may alter levels of active thiopurine metabolites, independent from *TPMT*, in a manner that increases thiopurine cytotoxicity. However, we did not observe any potential interaction of the *NUDT15* R139C variant (rs116855232) with TPMT activity in our three cohorts (Table 4). However, it should be noted that the frequency of this allele is extremely low in non-Asians, including Estonians.

Although the liver is the predominant site of thiopurine metabolism in humans, data demonstrating a close correlation between hepatic TPMT protein expression and cytosolic TPMT enzyme activity have been lacking. Here, we provide the first available systematic data on TPMT expression (mRNA, protein) and function (activity levels) in human liver samples. *TPMT* mRNA was not significantly correlated with either TPMT activity or TPMT protein levels, which supports previous findings demonstrating that the common *TPMT* polymorphisms 460G>A and 719A>G affect TPMT activity by way of post-translational modifications and increased protein degradation (for review see Moon and Loftus, 2016). Moreover, *TPMT* mRNA expression did not correlate with either TPMT activity or TPMT protein expression (in immunoblots) in subjects carrying the TPMT reference sequence (confirmed by next generation sequencing). These data are in contrast to prior results showing a significant correlation between normal/high TPMT enzyme activity in RBCs and *TPMT* mRNA levels extracted from whole blood samples taken from 29 individuals (Lindqvist et al., 2003). It remains questionable whether the relationship between white blood cell mRNA and RBC TPMT enzyme activity is relevant for drug metabolism.

The main limitations of our work are that this study was designed based on SNP array data and did not investigate gene duplications/deletions or genomic rearrangements, which might alter TPMT activity. Additionally, we did not investigate epigenetic regulation of TPMT expression (e.g. by non-coding RNAs such as microRNAs), which may be a plausible explanation, at least in part, for the lack of reliable correlation between *TPMT* mRNA expression and TPMT protein levels in the liver. Finally, the present study was not designed to identify genetic variants associated with thiopurine-related toxicity. Thus, we cannot

exclude the possibility that genes beyond *TPMT* are involved in determining thiopurine toxicity risk or treatment outcome.

The identification of novel rare variant alleles beyond *TPMT* that are associated with TPMT activity will require whole genome next-generation sequencing approaches. Our meta-GWAS of data from a combined cohort of 1212 subjects did not reveal any novel factors that were significantly associated with TPMT activity. Notably, we did not find confirmation of previously reported associations with other genes besides *TPMT*. Additional studies are needed to test whether such prior associations can be replicated. Moreover, in-depth research studies are needed to clarify the extent to which and the mechanisms through which the present findings may explain the inter-individual variability seen in TPMT activity.

6. CONCLUSIONS

This thesis provides an overview of different layers of -omics studies exploring inter-individual variability in TPMT activity.

The following conclusions can be drawn from this dissertation:

1. None of the newly detected markers in *TPMT* could be used to explain the discrepancies seen in TPMT genotype-phenotype correlations; the causes of ultra-high TPMT activity remain to be discovered.
2. We identified a new pharmacometabolomic marker and therapeutic agent. Of the 43 biomarkers tested, only *TPMT* genotype and RBC SAM levels were found to influence TPMT activity significantly. The influence of SAM levels on TPMT activity was particularly pronounced in *TPMT*-heterozygotes.
3. Our studies did not reveal any additional genetic markers outside of *TPMT* that were predictive of TPMT activity. Although our data confirm that *TPMT* genotype is a robust predictor of TPMT activity in most individuals, *TPMT* genotype alone is insufficient to predict TPMT activity reliably.

SUMMARY IN ESTONIAN

Uute tiopuriinmetüültransferaasi aktiivsust mõjutavate biomarkerite otsingul

Alates inimese genoomi (peaaegu) lõpliku nukleotiidse järjestuse avaldamisest 16 aastat tagasi, uute ja suure läbilaskevõimega järjestus- ehk sekveneerimistehnoloogiate arengu ning sellega kaasnevate suurte andmemahtudega, mis sisaldavad informatsiooni individuaalsete genoomide varieeruvuse kohta, on personaalmeditsiin saanud peamiseks suunaks inimesegenoomikas. Selle kõige olulisem eesmärk on uute ja usaldusväärsete komplekstunnuseid mõjutavate leidude juurutamine kliinilisse praktikasse kasutades inimeste genoomiprofiile haigusriskide ja ravimivastuse hindamiseks. Farmakogeneetika/-genoomika on selle protsessi üks olulisi harusid, mille abil on võimalik muuta raviotsuste tegemine täpsemaks määrates igale patsiendile sobivaima ravimi või ravimidoosi, arvestades tema individuaalseid geneetilisi eripärasid.

Üheks oluliseks võtmeteguriks personaliseeritud kasvajate ja immuunhaiguste ravis on ensüüm tiopuriinmetüültransferaas (TPMT), mis asub raku tsütoplasmas. Kuna TPMT rakusisest substraati ei ole veel tuvastatud, käsitletakse seda kui faas II ravimimetaboliseerijat, mis vahendab metüülrühma ülekannet S-adenosüül-metioniinilt aromaatsetele ja heterotsüklilistele väävliühenditele. Nende väävliühendite hulka kuuluvad ka tiopuriinravimid: 6-merkaptopuriin, asatiopriin, 6-tioguaniin, mis metülatsiooni tulemusel inaktiveeruvad. Tiopuriinravimeid kasutatakse tänapäeval peamiselt lapseea leukeemia, autoimmuunhaiguste ravis ning siirdamisjärgse äratõukereaktsiooni vältimiseks. Suures osas sõltub TPMT aktiivsusest eelnimetatud ravimite poolt põhjustatud kõrvaltoimete teke. TPMT madala aktiivsuse tõttu tekib indiviididel tiopuriinide standarddooside kasutamisel nn ravimimürgistus. Mitmed uuringud on näidanud, et TPMT aktiivsus on pöördvõrdelises seoses tsütotoksiliste metaboliitide (tioguaniinnukleotiidide) moodustumisega ja kõrvaltoimete esinemisega st, mida madalam on ensüümi aktiivsus, seda kõrgem on tioguaniinnukleotiidide tase organismis ja vastupidi.

Populatsiooniuuringutest on selgunud, et TPMT aktiivsus on kirjeldatav trimodaalse jaotusmudeli järgi. Valge rassi hulgas läbiviidud uuringud on näidanud, et keskmiselt omab ~0,3% indiviididest väga madalat ensüümi aktiivsust, ligikaudu 11% on ensüümi aktiivsus osaliselt vähenenud ja 89% on ensüümi aktiivsus normaalne. Seega teoreetiliselt on umbes 11% patsientidest ohustatud kõrvaltoimete tekkest eelnimetatud ravimite standarddooside kasutamisel. Samas on erinevates populatsiooniuuringutes välja joonistunud grupp indiviide, kes omavad tavapärasest kõrgemat ensüümi aktiivsust, kuid siiani ei ole leitud selle geneetilisi põhjuseid. Mutatsioonid/SNP-d TPMT geenis võivad mõjutada, peamiselt vähendades, ensüümi aktiivsust ning seega kutsuda esile kõrvaltoimeid, eelkõige vereloome pärssumist, ravimite standarddooside kasutamisel. Samas tavapärasest kõrgema ensüümi aktiivsusega indiviidide ravimine

standarddoosidega ei anna soovitud ravitulemust, kuna ravim metaboliseeritakse organismis väga kiiresti.

Hoolimata mitmetest TPMT ensüümi aktiivsust vähendavate *TPMT* geenivariantide avastamisest, on kliinilises praktikas suureks probleemiks TPMT genotüügi-fenotüübi erinevused, eriti nn keskmise ensüümi aktiivsusega indiviidide seas. See tähendab, et genotüübi ehk *TPMT* geenivariantide abil ei ole võimalik ensüümi aktiivsust õigesti hinnata. On näidatud, et normaalse (*wild-type*) *TPMT* genotüübiga indiviidide seas esineb madalama ensüümiaktiivsusega patsiente ja vastupidi, mis tähendab, et tekivad kõrvaltoimed ravimite standarddooside kasutamisel või puudub loodetud raviefekt. See fakt annab alust uute geneetiliste ja mitte-geneetiliste biomarkerite otsimiseks, et muuta geneetilise info kasutamise kliinilises praktikas täpsemaks ja usaldusväärsemaks.

Käesoleva töö kirjanduse ülevaates antakse põhjalik ülevaade farmakogenoomikast ja selle erinevatest olulistest komponentidest ning samuti aspektidest, mis mõjutavad selle kliinilist juurutamist. Lisaks on iseloomustatud TPMT ja tiopuriinravimite metabolismiradu, TPMT aktiivsust mõjutavaid teadaolevaid geneetilisi variante ning ka uusi avastatud potentsiaalseid biomarkereid.

Töö eksperimentaalosa on jagatud kahte etappi. Esimene hõlmab esmakordselt eestlaste kohordis (n=253) läbiviidud TPMT genotüübi-fenotüübi uuringut. Selle raames sekveneerisime TPMT geeni ning tuvastasime teadaolevaid ja uusi variatsioone. Leidsime, et eestlastel esineb ka kõige rohkem *TPMT*3A* alleeli heterosügootsust, mis on kooskõlas teiste valge rassi uuringutega. Lisaks avastasime kolm uut geenimuutust, kuid nende mõju ensüümi aktiivsusele ei ole teada. Samas ei tuvastanud me ühtegi geenivarianti, mida võiks seostada väga kõrge TPMT ensüümi aktiivsusega. Haplotüüpide analüüs näitas ühe haplotüübi üleesindatust keskmise ensüümi aktiivsusega indiviidide hulgas, kuid antud leid vajab edasisi uuringuid.

Töö teine etapp keskendub mitte-geneetiliste ja uute geneetiliste biomarkerite otsingutele. Kõigepealt uurisime suuremas eestlaste kohordis (n=1017) mittegeneetilise faktori S-adenosüül-metioniini (SAM) mõju TPMT aktiivsusele. Kuna varasematest töödest on teada, et SAM on TPMTle metüülrühma doonoriks ning *in vitro* katsed on näidanud selle varieeruva taseme mõju ensüümi aktiivsusele. Meie eesmärk oli näidata tervete inimeste kohordis, et SAMi varieeruv tase on seotud TPMT ensüümi aktiivsuse muutustega. Uuringu tulemused kinnitasid, et lisaks TPMT genotüübile on teine oluline ensüümiaktiivuse mõjutaja SAM ja seda just mutantse alleeli suhtes heterosügootsete, kuid TPMT normaalse aktiivsusega indiviidide seas. Samas, enne uue näitaja kliinilisse kasutusse rakendamist, tuleb saadud tulemusi kinnitada suuremas uuringus, kuna käesolevas töös kuulus sellisse gruppi (heterosügootne genotüüp-normaalne ensüümi aktiivsus) vaid 10 indiviidi ning samuti ei ole selge tuvastatud seos tiopuriine manustavatel patsientidel.

Seejärel viisime läbi suure assotsiatsiooniuuringu (n=1212) leidmaks uusi geneetilisi biomarkereid lisaks *TPMT* geenile, mis võiksid seletada suurt TPMT ensüümi aktiivsuse varieeruvust. Uuringusse kaasati tervete eestlaste (n=844)

ning saksa päritolu ägeda lapseea leukeemia patsientide kohort (n=245) ja maksaproovid (n=123), mis on peamine ravimimetabolismi kude. Assotsiatsioonianalüüsi ja meta-analüüsi tulemused näitasid, et ainult TPMT geeni variandid olid seotud TPMT ensüümiaktiivsuse varieeruvusega. Samas ei kinnitanud me oma töös varem avaldatud uuringute leide.

Kokkuvõttes näitavad tulemused, et me tuvastasime uue mitte-geneetilise biomarkeri, mille edasised kinnitavad uuringud on väga olulised, selle integreerimiseks kliinilisse kasutusse. Samas me ei leidnud uusi ja ei kinnitanud teaduskirjanduses varem avaldatud geneetiliste markerite seost TPMT ensüümi aktiivsusega. Kuna tulemused on ebaselged, siis kindlasti ei ole võimalik kliinilises igapäevapraktikas kasutada ainult TPMT genotüüpi ensüümiaktiivsuse ja ravimivastuse ennustamiseks. Hoolimata juhtnööridest geneetiliste markerite kasutamise ja tulemuste tõlgendamise kohta arstide igapäevatöös, tuleb väga täpselt jälgida patsiendi ravikulgu kasutades siiamaani kaustuses olevaid meetodeid ja raviskeeme.

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