Pharmacogenetic studies of thiopurine methyltransferase genotype-phenotype concordance and effect of methotrexate on thiopurine metabolism

Anna Zimdahl Kahlin

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During the course of the research underlying this thesis, Anna Zimdahl Kahlin was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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Abstract

The thiopurines (6-mercaptopurine [6-MP], 6-thioguanine and azathioprine) are cytotoxic drugs used in the treatment of acute lymphoblastic leukemia (ALL), inflammatory bowel diseases, certain autoimmune diseases and after transplantation. The metabolism of thiopurines is complex with several enzymes involved in the conversion into active drug metabolites. One of the enzymes, thiopurine methyltransferase (TPMT), is one of the best examples of implemented pharmacogenetics so far. Due to lowered TPMT enzyme activity caused by genetic polymorphism, carriers of heterozygous or homozygous defective TPMT alleles need dose reduction to avoid cytotoxic adverse reactions like myelosuppression or hepatotoxicity if treated with thiopurines.

To determine TPMT status before the start of treatment, genotyping (for the three most occurring TPMT alleles) and/or phenotyping (TPMT enzyme activity measurements) are used in the clinical setting. In the focus of this thesis, concordance of these methods was investigated in a large cohort of unique samples (n=12,663) collected in the routine analysis service of TPMT status determinations in Linköping. By sequencing all exons in samples where the results of the two methods differed, rare or novel TPMT alleles were discovered. Four TPMT alleles (TPMT*41, *42, *43, *44), not previously described, were characterized in terms of clinical *in vivo* data as well as protein structure and stability data obtained from recombinant human TPMT (rTPMT) produced by *E. Coli* and biophysical methods.

The clinical cohort was also used in the search for other factors (except genetic factors) that influence TPMT enzyme activity, and both age and gender turned out to affect TPMT enzyme activity level. In addition, TPMT enzyme activity in the early treatment of ALL was investigated and shown to be significantly lower at time of ALL diagnosis.

In the treatment protocol of ALL, the combined treatment using 6-MP and methotrexate (MTX) has increased the positive outcomes since the start in the 1950s. Despite this, the synergistic effect of these drugs is not yet fully understood. To evaluate the effect of MTX on thiopurine metabolism specifically, TPMT enzyme activity, TPMT gene expression, and thiopurine metabolite levels were determined before and after MTX infusions *in vivo* and after cotreatment in lymphoblasts *in vitro*. In the presence of MTX, TPMT enzyme activity and metabolite levels decreased, both *in vivo* and *in vitro*, although dose- and time-dependent. In addition, MTX bound to rTPMT and caused inhibition of rTPMT enzyme activity.

The results found in the scope of this thesis may be used for further individualization of thiopurine treatment.

Populärvetenskaplig sammanfattning

I kroppen finns ärftliga faktorer (gener) som till största delen är identiska hos alla människor, men små skillnader finns som gör att vi exempelvis får olika ögonfärg eller hårfärg. I den här avhandlingen studerades en gen som ger upphov till enzymet tiopurinmetyltransferas (TPMT), en molekyl som omvandlar läkemedel i kroppen till aktiva substanser (metaboliter) som har medicinsk effekt. Ungefär 10% av befolkningen har en annan variant av TPMT enzymet. Beroende på vilken variant av genen man har sker omvandlingen till aktiva substanser olika effektivt, man benämner detta som enzymets aktivitet. Man kan därför behöva anpassa dosen av läkemedlet så att man får en lagom mängd aktiva substanser med medicinsk effekt. Läkemedlet som TPMT omvandlar är en sorts cellgift (tiopuriner, 6-MP) som används för att behandla blodcancer (leukemi) hos barn, inflammatoriska tarmsjukdomar (ex. Crohns sjukdom), autoimmuna sjukdomar (ex. reumatism) samt efter organtransplantation.

I avhandlingen studerades hur resultatet av två olika metoder för att bestämma TPMT enzymets funktion (genotypning av de tre vanligaste varianterna jämfört med fenotypning) överensstämde i en stor grupp individer (12 663 individer). Genom utvidgad analys av de prover där resultaten från de två metoderna inte överensstämde kunde vi identifiera 15 individer med ovanliga eller tidigare okända TPMT varianter. Dessutom studerades om andra faktorer, utöver genetisk variation, påverkade TPMT enzymets aktivitet. Studierna visade att individens ålder och kön har betydelse för TPMT aktiviteten och dessutom hade leukemipatienter en lägre enzymaktivitet vid diagnos än senare i behandlingen.

Idag finns ett väl fungerande protokoll för behandling av barnleukemi. Hörnstenen i behandlingen är kombinationen av 6-MP och en annan sorts cellgift (metotrexat, MTX). Underligt nog så har man inte full förståelse för hur dessa läkemedel interagerar och varför kombinationen är så fördelaktig. Genom att mäta TPMT aktivitet samt nivåer av 6-MP metaboliter i patienter innan och efter att de fått MTX samt i leukemiceller odlade på laboratoriet och som behandlats med 6-MP/MTX kombinationen visades att MTX påverkade omvandlingen av 6-MP genom att sänka TPMT enzymets aktivitet samt orsaka lägre nivåer av metaboliter, men också att resultaten var beroende av behandlingstid och dos av MTX.

Genom att producera mänskligt TPMT enzym med hjälp av bakterier, studerades bindningen mellan MTX och TPMT. Genom att mutera TPMT enzymet och tillverka de varianter vi hittat hos patienter kunde orsaken till lägre enzymaktivitet i TPMT varianterna studeras.

Resultaten i denna avhandling kan bidra till en ytterligare individanpassad tiopurinbehandling

List of papers

Paper I

Comprehensive study of thiopurine methyltransferase genotype, phenotype, and genotype-phenotype discrepancies in Sweden

Anna Zimdahl Kahlin, Sara Helander, Karin Skoglund, Peter Söderkvist, Lars-Göran Mårtensson, Malin Lindqvist Appell.

Biochemical Pharmacology. **2019**; 164: 263-272

doi: 10.1016/j.bcp.2019.04.020

Paper II

One amino acid makes a difference – Characterization of a new TPMT allele and the influence of SAM on TPMT stability

Iu Yan Ping Heidi*, Sara Helander*, **Anna Zimdahl Kahlin**, Cheung Chun Wah, Chi Chung Shek, Leung Moon Ho, Björn Wallner, Lars-Göran Mårtensson, Malin Lindqvist Appell. (*Co-first author).

Scientific Reports. **2017**; 7: 46428, doi: 10.1038/srep46428

Paper III

Methotrexate binds to recombinant thiopurine S-methyltransferase and inhibits enzyme activity after high-dose infusions in childhood leukaemia

Patricia Wennerstrand, Lars-Göran Mårtensson, Stefan Söderhäll, **Anna Zimdahl** and Malin Lindqvist Appell

European Journal of Clinical Pharmacology. **2013**; 69(9): 1641–1649

doi: 10.1007/s00228-013-1521-9

Paper IV

Higher TPMT gene expression and lower thiopurine metabolite levels combining 6-mercaptopurine and methotrexate in pre-B and T-ALL

Zimdahl Kahlin Anna, Helander Sara, Vikingsson Svante, Mårtensson Lars-Göran, Appell Malin L.

Submitted. **2020**

List of papers outside of the thesis

The role of TPMT, ITPA, and NUDT15 variants during mercaptopurine treatment of Swedish pediatric acute lymphoblastic leukemia patients

M. Wahlund, A. Nilsson, **A. Zimdahl Kahlin**, K. Broliden, I. Hed Myrberg, M. Lindqvist Appell*, A. Berggren* (*Contributed equally)

The Journal of Pediatrics. **2020**; 216: 150-157

doi: 10.1016/j.jpeds.2019.09.024

Robust and convenient analysis of protein thermal and chemical stability

M. Niklasson, C. Andresen, S. Helander, M.G.L. Roth, **A. Zimdahl Kahlin**, M. Lindqvist-Appell, L.-G. Mårtensson and P. Lundström

Protein Science. **2015**; 24: 2055-2062, doi: 10.1002/pro.2809

P658 Effects of allopurinol on thiopurine metabolism and gene expression levels in HepG2 cells

Haglund, S., **Zimdahl, A.**, Vikingsson, S., Almer, S., Soderman, J.

Journal of Crohn's and Colitis. **2014**; 8 (1): S345

doi: 10.1016/S1873-9946(14)60777-7

Abbreviations

6-MP	6-mercaptopurine, drug
6-TG	6-thioguanine, drug
6-TUA	6-thio uric acid, inactive metabolite
ADR	adverse drug reaction
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
ALL	acute lymphoblastic leukemia, disease
ATIC	aminoimidazolecarboxamide ribonucleotide formyltransferase, EC 2.1.2.3
(d)AMP/ ADP/ ATP	(deoxy) adenosine mono/di/tri phosphate
AZA	azathioprine, drug
c.234G>T	substitution from guanine into tyrosine in cDNA position 234
CD	circular dichroism, method
CD	Crohn's disease, IBD
CI	confidence interval
CV	control vehicle, treatment with only solvent
DHFR	dihydrofolate reductase, EC 1.5.1.3
DME	drug metabolizing enzyme
FPGS	folylpolyglutamyl transferase, EC 6.3.2.17
GMPS	guanine monophosphate synthetase, EC 6.3.5.2
(d)GMP/ GDP/GTP	(deoxy) guanine mono/di/tri phosphate
HGPRT	hypoxanthine-guanine phosphoribosyl transferase, EC 2.4.2.8
HPLC	high performance liquid chromatography, method
Hx	hypoxanthine
IBD	inflammatory bowel disease
IMPDH	inosine monophosphate dehydrogenase, EC 1.1.1.205
IQR	interquartile range
K _d	dissociation constant
LC-MS/MS	liquid chromatography-tandem mass spectrometry, method
meMP	methyl mercaptopurine, inactive metabolite
meTIMP	methyl TIMP, active metabolite
MRD	minimal residual disease
MTX (LD/HD-)	methotrexate, 4-amino-10-methyl-pteroylglutamic acid, drug, (low/high dose-)
MTXPG	polyglutamated MTX, active metabolite with 2-7 glutamyl residues
NOPHO	Nordic Society of Paediatric Haematology and Oncology

p.Y240C	substitution of tyrosine into cysteine in amino acid position 240
PCR	polymerase chain reaction
PDNS	purine de novo synthesis
PPAT	phosphoribosyl pyrophosphate aminotransferase, EC 2.4.2.14
PRPP	phosphoribosyl pyrophosphate, cofactor for HGPRT
RA	rheumatoid arthritis, disease
RBC	red blood cells, erythrocytes
RT-PCR	RTqPCR, real-time quantitative PCR
rTPMT	recombinant TPMT, human TPMT protein produced with E. Coli
SAM	S-adenosyl-L-methionine, methyl donor and cofactor for TPMT
SAH	S-Adenosyl-L-homocysteine, coproduct of TPMT
SLE	systemic lupus erythematosus, disease
SNP	single nucleotide polymorphism
TGN	thioguanine nucleotides, active metabolites
TIMP	thioinosine monophosphate, inactive metabolite
T _m	thermal melting point
TPMT	thiopurine S-methyltransferase, EC 2.1.1.67
TYMS	thymidylate synthase, dTMP synthase, EC 2.1.1.45
TX	thioxantine
TXMP	thioxantine monophosphate, inactive metabolite
UC	ulcerative colitis, IBD
UPLC	ultra performance liquid chromatography, method
VNTR	variable number tandem repeat
WBC	white blood cells
XO	xanthine oxidase, EC 1.17.3.2

1. Introduction

1.1 Very short introduction to DNA, mRNA and proteins

The human body consists of cells in which a lot of reactions are happening, very quickly, all the time. Almost every cell has a nucleus containing our genetic material, our genome. The genome consists of DNA, or more precisely 3.12 billion nucleotides [1]. Our DNA includes information about everything from eye and hair colour to biological gender, inherited traits of diseases and ability to metabolize drugs.

DNA act like a template for all proteins and RNA molecules produced in the body. Transcription factors decide what part of DNA will be read and produced and at what levels. DNA consist of two strands of DNA bases (A, T, C or G) which will pair and form a double helix of two complementary strands. This structure was solved by Jim Watson and Francis Crick in 1953 [2].

When time for production, one DNA strand is transcribed and a complimentary copy (RNA) is made, **Figure 1**. All DNA does not contain information needed for the end product, therefore some parts of the RNA (introns) will be removed (by splicing) and the protein coding parts (exons) will form mRNA. This sequence codes for amino acids, the body's building blocks. Three DNA bases (a codon) codes for one amino acid, start or stop. There are 20 amino acids and 64 possible codons available, which make more than one codon code for a single amino acid and is defined in the genetic code. This process is named translation and is performed in the cytoplasm where mRNA binds to ribosomes and amino acid specific tRNA transports the amino acids and attaches them to a chain according to the mRNA sequence.

The chain of amino acids (polypeptide chain) will link to each other by peptide bonds. When finished, and possibly posttranslationally modified, it will fold into secondary (α -helices, β -sheets and random coil) and tertiary (3D) structures, e.g., based on the containing amino acids and their characteristics and form a protein. Proteins that will be located somewhere other than the cytoplasm could be sorted to the right location through the endoplasmic reticulum (ER) using a signal peptide.

Depending on the protein's characteristics, it can form complexes with other proteins, it can act as a membrane protein, transporter,

transcription factor, protein processor or enzyme that is converting chemical compounds into another form. The part of DNA that is coding for one protein is defined as a gene [3], and the human genome is estimated to contain about 20-25,000 protein-coding genes [1, 4]. All DNA are available in two copies (alleles) of which each is inherited from one parent.

Figure 1. Overview of the process of gene expression. (The folded protein was made in PyMOL, 2BZG).

1.2 Pharmacogenetics

1.2.1 Drug metabolism

The body's liver metabolism system is originally evolved for processing natural substrates and to protect against toxification. Nowadays, it is also important for drug metabolism. Once a drug is administrated, four steps can (depending on how it is administrated) affect how fast the drug's effects appear, the intensity of the effect, and how long the effect will continue. Those four steps are absorption (from site of administration into plasma), distribution (entering intestinal or intracellular fluids), metabolism (enzymatic transformation in liver or other tissues) and elimination (out of the body) [5].

The liver contains many drug metabolizing enzymes (DMEs) responsible for several metabolic reactions, which are divided into two major categories, phase I and phase II reactions. Phase I reactions include oxidation, hydrolysis and reduction and will often make the molecules more hydrophilic. Phase II reactions are methylation, acetylation and conjugation (with sulfate, amino acids or glucuronic acid). Phase II enzymes transform the molecule into an even more water-soluble form that will facilitate elimination through the bile or kidney [6].

Patients given the same drug in the same dose will respond differently, which depends on factors such as weight (fat composition), age, kidney or liver condition, interaction with other drugs or food or on the interindividual genetic variation [1]. The last is studied in the research field of pharmacogenetics.

1.2.2 Pharmacogenetics and concept definitions therein

Already 510 BC, Pythagoras observed that some individuals got haemolytic anemia by eating broad beans, while others did not [7]. In 1909, Garrod [8] publicised observations of individual response to chemicals in humans. In the beginning of 1930s, Fox [9] showed that some individuals were able to sense a bitter taste in phenylthiocarbamide, while others could not. These results were soon followed by the publication of the first pharmacogenetic study where Snyder [10] reported that this was a genetic phenomenon by investigating the ability of tasting bitter within families. In the 1950s observations regarding interindividual variation, unexpected adverse drug reactions, drug metabolism and inheritance patterns for the drugs isoniazid (tuberculosis), primaquine (malaria) and succinylcholine

(muscle relaxant during surgeries) were made and in 1959 Vogel [6, 7, 11, 12] was using the term pharmacogenetics for the first time.

Pharmacogenetics is the study of a single gene's influence on drug response or drug response having a hereditary basis, often reflecting differences in DMEs causing adverse reactions or insufficient treatment. The whole-genome application of pharmacogenetics is defined as **pharmacogenomics**. Both these concepts are sometimes overlapping and the abbreviation PGx refers to both [13-15].

Genetic polymorphism is “the occurrence, together in the same population, of more than one allele or genetic marker at the same locus with at least a frequent allele or marker occurring more frequently than can be accounted for by mutation alone” (Daly, 2010 [11]). Genetic polymorphisms are normally occurring in a population with a frequency of at least 1%, otherwise it is defined as an isolated mutation or sequence variant. In pharmacogenetic research, mainly the polymorphisms with significant functionally effects, such as altered expression or lower or absence of activity of the resulting enzyme, are studied.

The knowledge of **pharmacokinetics** includes how polymorphisms affect drug disposal and drug metabolism [11], or more easily explained “what the body does to the drug” [5, 14]. **Pharmacodynamics** is factors that affect drug targets, of which about 70% are receptors, 5% enzymes and 30% channels [1, 11], but it could also be explained as “what the drug does to the body” [5, 14].

The most common genetic polymorphism is **single nucleotide polymorphism (SNP)**, which consequently is a substitution of a single nucleotide in the gene. One estimates that 10-15 million SNPs are occurring today [1] or 1 of every 600 bases [16, 17]. However, this small change may have dramatic consequences for the protein.

Genotype is the composition of nucleotides in a defined gene at a specific position (locus) in the genome. The most occurring genotype in a population, and preferably one not causing disease, is defined as the **wildtype**. However, for some polymorphism, the most common genotype varies between ethnical populations, which complicates the usage of the concept wildtype [17]. Two identical alleles of a genotype are referred to as **homozygous**, whereas a **heterozygote** individual is carrying one deviating allele. **Haplotype** is several genetic variants inherited together on the same allele. The **phenotype** is a gene's

observable expression and depends on, e.g., if the genetic trait in the alleles are dominant or recessive.

1.2.3 Brief overview of the pharmacogenetic field today

The aims of pharmacogenetics today are to aid the selection of the right drug and/or right dose to each patient to reach the best effect with or without decreased adverse reactions (ADRs). There are two types of ADRs and the most common type depends on the drug concentration and is often associated with genetic polymorphism in DMEs [11]. Of the DMEs in phase I, the group cytochrome P450 enzymes (the CYP-enzymes) are the most important and catalyze the oxidation reactions of up to 80% of all prescribed drugs [11, 18]. The three most important are CYP3A4, CYP2D6 and CYP2C9, metabolizing, e.g., antidepressant desmethylimipramin, analgesic codein, breast-cancer treatment tamoxifen, immunosuppressing cyclosporin, chemotherapy drug vincristine and the anticoagulant warfarine. Several of the CYP-enzymes have genetic variants causing a low or absence of enzyme activity or have extra high activity due to several copies of the enzyme. All of them have a few genotypes that are more common, and several rare genotypes, with differences of presence in ethnical populations [1, 6, 11].

In phase II, the main DMEs are UDP-glucuronyltransferases (UGT), N-acetyltransferases (NAT) and methyltransferases. The first implemented and most pharmacologically important DME was thiopurine methyltransferase (TPMT) [6, 11], which will be extensively described in this thesis.

Other targets within the pharmacogenetic research are drug transporters, mainly membrane transporters of drugs and metabolites in or out of important cells (e.g. ATP-binding-cassette [ABC]-transporters and solute carrier organic anion transporters); drug target receptors, e.g., the G-protein-coupled receptors like dopamine, serotonin and adrenergic receptors important for a drug's response when treating heart rate, hypertension, heart failure and asthma; and enzyme targets like the gene VKORC1 encoding Vitamin K epoxide reductase, which is a target for anticoagulants like warfarin [7, 11].

In later years, genome-wide association studies (GWAS) have highly increased the identification of genotypes associated with drug response and disease or ADR susceptibility by combining analyses of polymorphisms in the entire human genome in cases and controls. This method is very unbiased and could find unexpected candidate genes as well as confirm existing knowledge [11].

Implementation into clinical pharmacogenetic routine testing is not very expanded. Most used and recommended today is testing of TPMT, NUDT15 [19] (both used in thiopurine treatment, described in **chapter 1.3**) and UGT1A1 (treatment with irinotecan, used against, e.g., metastatic colon cancer). Other drugs where pharmacogenetic testing have been proposed or recommended are warfarin (up to 40% of the variation in drug response between individuals are reported to come from the VKORC1 and CYP2C9 genotypes) and tamoxifen [6] (the proportion of CYP2D6 poor metabolizers is up to 12% in some populations whereas ultra-rapid phenotype frequencies are up to 29% of other populations) [13].

1.3 Thiopurines and thiopurine methyltransferase

1.3.1 The fascinating history

In the 1950s acute leukemia in children was treated deficiently with only methotrexate and steroids available and the expected life span after diagnosis was 3-4 months with only 30% surviving one year. Gertrude Elion and colleagues synthesized antipurines following Hitchings theory that it might be possible to inhibit the growth of rapidly multiplied cells by nucleic acid antagonists since nucleic acids are required for all cells to divide, and consequentially, a nucleic acid antagonist should have the most effect on rapidly dividing cells. Notable was that, in these years, the knowledge about nucleic acids and anabolic pathways was highly limited. 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), **Figure 2**, were found to be effective against tumors and leukemia in mice and in the following clinical trials, treatment with 6-MP in children with acute leukemia led to an increased life span of 12 months, and even complete remissions occurred. In 1953, about two years after its synthesis, 6-MP was approved for treatment of acute leukemia by the US Food and Drug Administration (FDA) [20].

Further studies provide that 6-TG was more active and more toxic than 6-MP. Azathioprine (AZA) was synthesized in an attempt to protect the sulfur from oxidation and hydrolysis and act as a prodrug for 6-MP, with the same activity, but less toxicity than 6-MP. In 1958, Robert Schwarz was the first to describe the immunosuppressant effect of 6-MP [21] and in 1960 Roy Calne increased the survival of dogs with kidney transplantation from an unrelative donor from 10 days to 44 days by daily 6-MP doses [22]. In 1962, the first successful unrelated kidney transplants in humans, using i.a. AZA as immunosuppressant,

Figure 2. The thiopurines 6-thioguanine (6-TG) and 6-mercaptopurine (6-MP) were synthesized by substitution of the oxygen to sulphur in the endogenous purines guanine and hypoxanthine, respectively. Azathioprine (AZA) was made as a prodrug for 6-MP to protect the sulphur and thus prevented it from degradation and be less toxic.

was performed [20, 23]. In 1988, Gertrude Elion and George H. Hitchings were awarded the Nobel Prize in Physiology or Medicine for their discoveries of several drugs based on antimetabolites to the nucleic acid metabolism.

In 1980, Weinshilboum and Sladek [24] described the large variability and trimodal distribution of TPMT enzyme activity in nearly 300 individuals. They also showed that the enzyme activity was inherited within families. By collaboration with Lennard and Lilleyman, the phenotyping of TPMT was included in ongoing ALL studies and in 1983 Lennard developed a method for measuring the levels of the thiopurine metabolite thioguanine nucleotides (TGN) [25] and the correlation between TPMT phenotype, TGN levels and the ADR myelosuppression was found [26-28]. Since the early 1990s the phenotyping has been available for clinical testing [15]. The TPMT gene was first described in 1993 by Honchel and colleagues [29]. In the year 2000, clinical testing of the TPMT phenotype was available in Sweden and in 2006, TPMT genotyping was also performed. In 2003, the US FDA highlighted TPMT as a valid biomarker for pharmacogenetics [15] and, today, TPMT polymorphism is one of the best examples of clinically important pharmacogenetics.

1.3.2 TPMT gene and polymorphism

The TPMT gene is a polymorphic gene of a total of 34 kb located on chromosome 6p22.3, including 10 exons and 9 introns. Normally, eight of the exons (exon 3-10) encode for the phase II metabolizing enzyme TPMT [30]. Exon 2 is subject to altered splicing [29-31]. TPMT cDNA consists of 738 nucleotides. The TPMT promoter is located -166 to -36 bp upstream from the transcription start [32] and contains a variable number tandem repeat (VNTR) region with varying numbers of repeats [33]. TPMT has a pseudogene of 96% identity to the cDNA, which is mapped to chromosome 18 [31].

Only two years after the isolation of the TPMT gene, the first SNP (c.238G>C) causing low enzyme activity was reported [31]. And the two following year c.719A>G was described, both alone and in combination with c.460G>A [30, 34]. The genetic variations are autosomal codominant inherited [35].

The nomenclature of TPMT alleles is handled by the TPMT nomenclature committee [36]. The wildtype allele are defined as TPMT*1 and the three most common SNPs in the TPMT gene are TPMT*2 (c.238G>C, p.A80P, rs1800462), TPMT*3A (c.460G>A, p.A154T, rs1800460) + c.719A>G, p.Y240C, rs1142345) and TPMT*3C (c.719A>G, p.Y240C, rs1142345). These three SNPs are, together, responsible for 95% of the intermediate and low enzyme activity cases [37]. In contrary to TPMT*3A and *3C, the genotype TPMT*3B (c.460G>A, p.A154T, rs1800460) is a very rare genotype [30], **Table 8** (p. 63).

Furthermore, the presence of defect alleles as well as the frequency of the specific alleles of TPMT varies among populations. In Caucasians, about 10% of the population are carrying a non-functional TPMT allele while the proportion in Asian populations are 2-4% [38-40]. TPMT*3A is the most commonly occurring defect allele in Caucasians (with a frequency of 3.2-5.7%). The frequencies of TPMT*3C and TPMT*2 alleles, in Caucasian populations, are 0.2-0.8% and 0.2-0.5%, respectively [37, 41]. TPMT*3C is the most commonly occurring defect allele in South-East and East Asians (frequency 2%) and Africans [30, 38-40, 42]. In these populations, the TPMT*3A allele is close to non-existing [43]. TPMT*3A is the most common allele in South-West Asians [43]. In Afro-Americans are TPMT*3C accounting for 50% of all defective alleles [44].

1.3.3 TPMT enzyme and effects of genetic variation

TPMT is a cytosolic enzyme of 245 amino acids and a mass of 28 kDa [45, 46], which is expressed globally in the human tissues [47] and catalyses the S-methylation of sulfhydryl groups in thiopurine drugs using S-adenosyl-L-methionine (SAM) as a cofactor and methyl donor [48].

In 1963, Remy and colleagues identified the TPMT protein for the first time in mammalian tissue [45]. The protein structure of TPMT was solved in 2007 by Wu and colleagues [49] using x-ray crystallography. The TPMT protein structure consists of one single domain and is folded in a typical Class-I methyltransferase fold by nine β -sheets with three α -helices on each side and two additional α -helices, **Figure 3**. TPMT has an internal solvent channel all through the structure, connected to both the binding pocket of the cofactor and the probably oblate shaped active site of substrate.

Figure 3. Structure of thiopurine S-methyltransferase. A) Cartoon visualization B) Surface and the coproduct SAH (red) in the active site pocket. The figures are illustrated in PyMOL, coordinates obtained from RCSB Protein Data Bank, accession code 2BZG.

The awareness of TPMT and its clinical importance was early known, and it has an essential role in the metabolism and, thereby, treatment with thiopurines because of its significant intra-individual variations caused by genetic polymorphism. A heterozygous defect allele gives rise to an intermediate phenotype and two defect alleles cause very low or absent TPMT enzyme activity [35, 50, 51].

In Caucasian populations, the TPMT enzyme activity frequency distribution shows a trimodality with about 10% of the individuals carrying one non-functional allele and 1/300 (0.3%) have two non-functional alleles [24, 37]. This is the common pattern of an autosomal codominant inherited gene. However, in Asian populations, the TPMT enzyme activity distribution is described as bimodal [38].

Strangely enough, the natural substrate of TPMT is still unknown [47]. A study of TPMT knock-out mouse models declares that TPMT deficient mice were not noticeably different in comparison to heterozygous or wildtype mice (length of life, fertility, histology analyses of several organs, rate of purine de novo synthesis [PDNS] or toxicity response to another chemotherapeutic drug) when not treated with thiopurines [52]. Two hypothesis concerning the natural functions of TPMT have been reported. First, recombinant TPMT methylates the reactive selenocystein in the presence of SAM [53] and bacterial TPMT has been suggested to be involved in a selenium methylation process in freshwater [54], both indicating a detoxification mechanism by TPMT of selenol-containing compounds. Recently, it was claimed that TPMT catalyzes the molybdenum cofactor Moco [47].

1.3.4 Metabolism and clinical action of thiopurines

After oral administration, 6-MP has a rapid absorption with 6-MP serum peak after 20-120 minutes [47, 55]. The thiopurine drugs act as prodrugs and are dependent on their metabolic conversion into active metabolites, which cause the cytotoxic and immunosuppressive effects of the drugs. The cellular uptake of the thiopurine drugs are probably through nucleoside transporters (SLC23A, SLC28A and SLC29A) [56-59]. The metabolism of thiopurines are extensive and complex due to several enzymes involved. Additionally, thiopurines have a relatively narrow therapeutic window.

Since 6-MP is an analogue to the purine hypoxanthine (Hx), the metabolism is similar to the endogenous purine salvage pathway (**Figure 4 and the last page**). 6-MP is converted through three competitive metabolic processes, of which two are inactivating. The major reaction is through oxidation by XO, mainly performed in the liver and intestines, and is responsible for the low bioavailability (about 20%) of oral 6-MP. Administration by intravenous injections increase the bioavailability due to eliminated first-pass metabolism. The inactive metabolite 6-TUA is the main 6-MP metabolite detected in urine [47, 55, 60-63]. The second inactivating reaction of 6-MP is the methylation

performed by TPMT into meMP. The variation herein is most influencing the metabolite levels by making more 6-MP available for the third process, which is conversion into active metabolites, a multistep reaction started by the HGPRT conversion into TIMP.

TIMP is also acting as a substrate for TPMT and is methylated into meTIMP, which is an active metabolite. TIMP can, in addition, be processed into TXMP by IMPDH and further into TGN by GMPS [64]. TGN is the collection of the active metabolites thioguanine nucleotides, i.e., thioguanine (deoxy) mono-, di- and triphosphates which are produced by several kinases and reductases [65].

AZA is a prodrug to 6-MP and, *in vivo*, both converted enzymatic by GST and non-enzymatic by thiol reaction into 6-MP [66, 67]. 6-TG is a guanine analogue, which also acts as substrate for HGPRT and is converted directly into TGMP. In addition, 6-TG could also be inactivated by TPMT methylation, also making TPMT variations important during 6-TG treatment. In contrast to AZA and 6-MP, 6-TG is not producing any meTIMP metabolites.

Figure 4.
Simplified
overview of
the thiopurine
pathway.

For more
details and
abbreviations,
see the last
page of the
thesis.

Hx can be incorporated in DNA/RNA as both adenine and guanine (deoxy)ribonucleotides, while thiopurines can only be incorporated in the form of thioguanine (deoxy)ribonucleotides [20, 68]. The exact mechanism of action is still discussed. However, most researchers agree in that the drugs exert their effect through 1) incorporation of t(d)GTPs in DNA and RNA which leads to apoptosis of the cell; and 2) inhibition of the PDNS enzyme PPAT by meTIMP, which causes lack of endogenous ATP and GTP and thereby inhibit proliferation [20, 69-74]. Since these

mechanisms disturb the DNA replication and biosynthesis of new nucleotides, rapidly dividing cells like activated T-cell lymphocytes in inflammatory bowel diseases (IBD) or lymphoblasts in ALL, are the most affected [75]. In addition, the lack of endogenous nucleotides increases the proportion of incorporated TGN [72, 76].

The DNA mismatch repair (MMR) system has been reported to recognize the incorporated tdGTP and trigger apoptosis [77]. However, MMR-deficient cells could also be sensitive against thiopurine treatment, indicating several mechanisms of apoptosis. Other enzymes involved in DNA repair and replication have been shown to have altered enzyme activity and, in addition, a DNA repairing protein complex has been proposed to have higher affinity in the presence of tdGTP-incorporated DNA [74]. The immunosuppressive effect has further been explained by apoptosis induced in CD4+ T lymphocytes by tGTP binding to Ras-related C3 botulinom toxin substrate 1 (Rac1), important for the activation of T-cells. However, only in combination with stimulation from CD-28 [78].

The concentration of E-TGN correlates negatively with the RBC TPMT enzyme activity, meaning that lower TPMT enzyme activity causes higher amounts of TGN. Carriers of defective TPMT alleles, especially individuals with homozygous total defect TPMT, are in high risk of severe ADR like myelosuppression [27, 51, 79-81]. High levels of meTIMP cause higher risk of hepatotoxicity [82] and myelotoxicity [83].

1.3.5 Methotrexate in ALL treatment

The folate analogue methotrexate (MTX, 4-amino-10-methyl-pteroylglutamic acid) is used in the treatment of rheumatoid arthritis (RA), ovarian cancer, osteosarcoma, psoriasis, IBD, after transplantations and in the ALL treatment [61]. Before the discovery of 6-MP, MTX was the drug used in ALL treatment. In the 1950s the combination of 6-MP and MTX was introduced in ALL treatment [84] and it was soon clarified that the combinational treatment using 6-MP and MTX had even better antileukemic effect than the use of either drug alone. The combination of low dose (LD)-MTX and 6-MP have been the cornerstone of ALL maintenance treatment ever since [84-86].

The main target for MTX is the inhibition of DHFR, which reduces the availability of reduced folate cofactor. Acting as a substrate for FGPS, MTX is polyglutamated (MTXPG) by up to seven glutamic acids. MTXPG accumulates in the cell and are strongly inhibiting the DHFR, TYMS and AICAR enzymes in the PDNS. The MTXPG can be converted back to

MTX by FPGH [61, 87, 88]. Also the active metabolite 7-OH-MTX and the inactive metabolite DAMPA are produced from MTX [61].

LD-MTX is normally administrated orally once every week during maintenance therapy. The bioavailability is 50-95 % and the maximum plasma concentration is reached after 1.5-2.5 h. MTX enters the cell by active transport through the reduced folate carrier SLC19A1 and is pumped out of the cell by ABC transporters. The elimination is mainly by unmetabolized MTX in the urine after 4-6 hours [61].

In 1960s high dose (HD)-MTX was introduced in ALL treatment to decrease the risk of relapses, especially CNS relapses. HD-MTX (0.5-12.0 g/m² or higher) are administered intravenous during 24 hours followed by Leucovorin rescue [61, 84, 89]. The aim of HD-MTX treatment is to increase the uptake of MTX, also by passive uptake, to reach higher intracellular concentrations of MTX and thereby MTXPG [61]. During repeated HD-MTX infusions, the proportion of MTXPG increase with more glutamate residues [85] and high levels of long chained MTXPG in lymphoblasts are related to faster elimination of ALL-cells [90]. The mechanisms of action are discussed in **chapter 6.4**. For a more detailed figure of the complexity of the metabolism and interactions of 6-mercaptopurine and methotrexate, see the **pathway on the last page** of the thesis where also all abbreviations are explained.

1.3.6 Usage of thiopurines today

Today, almost 70 years after its introduction, the thiopurines are still widely used. 6-MP is a cornerstone during the ALL treatment, 6-TG is used in acute myeloblastic leukemia in elderly and AZA during IBD treatment [15]. Thiopurines are also used to treat RA, SLE, dermatological conditions, chronic active hepatitis and as immunosuppressants after transplantations [20, 91]. See also **Table 1** (p. 30) for diagnosis distribution in the patient material of the thesis.

The implementation of clinical determination of TPMT status before start of treatment is increasing and in the NOPHO-ALL2000 and 2008 treatment protocols, TPMT determination is included. The differences between conventional and individualized thiopurine treatment according to TPMT, **Figure 5**, are lower doses for individuals carrying TPMT heterozygous and homozygous defective alleles.

Thiopurines in ALL treatment

Acute lymphatic leukemia (ALL) is the most common cancer in children, with approximately 300 individuals diagnosed annually in the Nordic

Figure 5. The thiopurine dose reduction of TPMT heterozygous and homozygous defectives (b) erases the differences in TGN exposure and hematopoietic toxicities. Figure adapted from [59] (originally from [92]) with permission of Springer Nature.

countries. In healthy bone marrow, stem cells are developed into red blood cells (RBC), thrombocytes or white blood cells (WBC). WBC consist of monocytes, granulocytes and lymphocytes. In ALL the lymphocytes are transformed into malignant immature cells, lymphoblasts, which divide unrestrained. ALL can be divided into three subgroups; T- and B-lineage, which are developed from T- or pre-B-lymphocytes, respectively, and the unusual Burkitt leukemia which is developed from mature B-lymphoblasts. Of those diagnosed with ALL, approximately 85% are B-lineage ALL and 15% T-lineage ALL [59, 93]. During ALL, the large amounts of lymphoblasts outcompete healthy blood cells which cause symptoms of increased susceptibility to infections, anemia, pain in the bones and increased bleeding tendency. Duration of the symptoms is usually days to weeks before diagnosis which is primarily based on blood count, occurrence of lymphoblasts and coagulation disturbances, and finally determined by bone marrow examination and extensive blood sampling.

ALL in children (from 1 year old) are treated according to a common treatment protocol for the Nordic countries, NOPHO (Nordic Society of Paediatric Haematology and Oncology). The NOPHO-ALL 2008 protocol [93] constitutes a detailed scheme of drug treatments and examinations with exact days for each event and plans for further treatment according to each analysis response and stratification (three

levels of risk are described, standard, intermediate and high risk) of patients according to risk factors. Individuals having risk factors (e.g., high leucocyte count, T-lineage ALL, some translocations and haplodiploidy <45 chromosomes) are treated with more intensive protocols (intermediate or high risk).

Overall, the treatment protocol consists of three main phases: induction, consolidation and maintenance. During the first induction phase, the aim is to deplete all cancer cells in the body and consists of intensive treatment with chemotherapy (i.a. intrathecal MTX) and cortisone. By day 29, most of the patients have no ALL cells in their peripheral blood or bone marrow. The result of the induction phase is followed up by examination of the bone marrow by minimal residual disease (MRD), which predict relapses well and further stratification is possible.

The consolidation phase aims to prevent return of cancer cells. Here are the patients treated with daily oral 6-MP and courses of HD-MTX with intrathecal MTX followed by leucovorin rescue in combination with other chemotherapeutic agents.

The last and longest phase is the maintenance therapy which started at week 17-61 depending on risk stratification, and the total treatment period of ALL is 2-2.5 years long. During the maintenance therapy, chemotherapeutic agents are administrated in pulses and several HD-MTX infusions are included as well as continuously oral daily 6-MP+weekly MTX treatment.

Individuals with higher risk stratification have up to two extra intensification phases, and hematopoietic stem cell transplantation could also be necessary.

In 1992, NOPHO established their first common ALL treatment protocol and this was updated in 2000 and 2008. In the 2000 update, TPMT determination and dose reduction were included (wildtype: 75 mg/m²/day, heterozygous: 50 mg/m²/day, defective: 5 mg/m²/day [19]) and from 2008 all treatment with irradiation was omitted and replaced by intrathecal chemotherapy (CNS ALL). In the 2008 protocol, the target WBC was decreased from 3.5 to 3.0 x 10⁹/L. The updated protocols also included better risk factor stratification. Overall, the survival outcomes were improved in the new more intensive protocols and, hence, the adverse reactions have increased [94]. The 2008 ALL protocol is also used to treat adults of 18-45 years, to try to improve the outcome in this patient group.

Nowadays, the overall survival of ALL is over 90%. There are still incidences of relapses and the improvement of the treatment protocols are always running to further adjust doses, use the optimal combination of drugs at the exactly right time during the treatment and usage of new parameters to estimate risk and needed dose adjustments, all to improve the treatment protocol further [85, 93]. The biggest challenge has been described as finding the right balance between too high and too low cytotoxic effect during the treatment. From 2020, there will be a new protocol, ALL-Together, in place for children and adults running in many European countries including the Nordic countries.

Thiopurines in inflammatory bowel diseases

The two main types of inflammatory bowel diseases (IBD) include Crohn's disease (CD) and ulcerative colitis (UC). These are both chronic relapsing inflammatory causing ulceration in the gut. CD affects the small bowel and the colon and can develop fistulae and bowel blockage, the inflammation is deep in the bowel wall and is discontinuous. UC affects the colon and rectum and are continuously extending inflammation of the mucosa. Symptoms of both diseases are primarily of gastrointestinal origin with diarrhea and rectal bleeding in addition to weight loss and fever [95].

IBD is more common in northern Europe, the UK and North America, and the prevalence is higher in white and Afro-American populations [96].

Both CD and UC are treated with 5-aminosalicylic acid (5-ASA) drugs as first choice and steroids are used during disease relapse. Thiopurines are used in steroid-dependent disease as an immunomodulating and steroid-sparing agent and for maintenance of remission of the diseases. Azathioprine is the first choice thiopurine, mostly due to clinical experience of this drug. Target doses of AZA are most commonly >2 mg/kg BW and 6-MP 1-1.5 mg/kg BW [97, 98].

In opposite to ALL treatment, therapeutic drug monitoring (TDM), i.e., determination of the thiopurine metabolite levels TGN and meTIMP are used to monitor the efficacy of the IBD thiopurine treatment [75, 99], mostly when patients do not respond to treatment and to study adherence of the treatment [97]. Target metabolite levels are TGN 250-400 pmol/8 x 10⁸ RBC and meTIMP <11,000 pmol/8 x 10⁸ RBC [98, 100].

In IBD, an interesting phenomenon of thiopurine metabolism is known but so far unexplained. Of IBD patients, 15-20% display a skewed metabolism profile of metabolites. This implies high meTIMP/TGN metabolic ratio or more specific conversion of higher levels of meTIMP and untherapeutic low levels of TGNs in RBC. These individuals are normally treated with 25-33% of normal dose AZA in combination with allopurinol (normally used to treat gout and originally developed by Elion to increase the effect of thiopurines) and the combinational treatment restores the balance of metabolites [20, 101, 102]. Surprisingly, skewed metabolism is not a pronounced issue in the ALL treatment [55], even though single case studies have been reported.

2. Aims of the thesis

2.1 Overall aims

The general aim of the thesis was to investigate the interindividual variation in the TPMT gene and enzyme activity as well as drug-drug-interactions in the metabolism for a further individualised, more effective treatment causing fewer adverse reactions.

2.2 Specific aims

- To describe a large amount of available material of TPMT geno- and phenotyping results, analyzing the concordance in between and to evaluate the importance of combining the two strategies.
- To characterize the TPMT single nucleotide polymorphisms not previously described and to characterize the cause of low enzyme activity.
- To study factors other than allelic variation that influence TPMT enzyme activity.
- To investigate the effect of MTX on TPMT enzyme and thiopurine metabolite levels *in vivo* and *in vitro*.

3. Materials

In this chapter, the patient cohorts and the cell lines used in Papers I-IV are described. Some results, in the form of descriptive statistics of the cohorts, are included here.

3.1 Patient cohorts

3.1.1 Paper I –Database from clinical laboratory

The cohort described in [Paper I](#) includes information from 15,975 individuals with requested TPMT/thiopurine status from the Clinical Pharmacology division, Department of Medicine and Health Sciences, Linköping University between year 2000-2015. Information available and used was age, gender, time for sampling, results from TPMT enzyme activity, **Figure 6**, diagnosis given in the letter of referral, **Table 1**, and genotyping measurements. From the year 2004, a pyrosequencing method for determination of the three most common SNPs in the TPMT gene (*2, *3A, *3C) was available and included in the TPMT analyses.

Figure 6. The TPMT enzyme activity in 15,968 unique individuals, referred from Swedish clinics for TPMT status determination, ranged from 0 to 40.8 U/ml pRBC and showed a trimodal distribution (88% were within the normal range of TPMT enzyme activity (≥ 9.0 U/ml pRBC), 11.6% had intermediate activity and 0.4% had low activity (< 2.5 U/ml pRBC)). Figure adapted from [Paper I](#).

Table 1. Referring clinics of TPMT samples

Referring clinic/diagnosis	Number of individuals	Frequency (%)
Gastroenterological clinics	11,341	71%
Other from gastroenterological clinics ^A	7,328	46%
Confirmed Crohn's disease	1,849	12%
Confirmed ulcerative colitis	1,671	11%
Hepatitis/other liver diagnosis	4,93	3.1%
Dermatology or Rheumatology clinics	2,241	14%
RA/rheumatology diagnosis	1,523	9.5%
SLE/psoriasis/eczema/allergy diagnosis	499	3.1%
Skin clinic/dermatology diagnosis	219	1.4%
Hematology clinics	954	6.0%
Other from hematology clinics ^A	747	4.7%
Confirmed ALL	207	1.3%
Other clinics	315	2.0%
Other kidney diagnosis ^A	196	1.2%
Transplantation (kidney, liver etc.)	119	0.7%
Vasculitis	52	0.3%
Unknown^B	1072	6.7%
Total	15,975	100%

^A Diagnosis not given in the referring letter or undiagnosed patient. ^B Not possible to categorize, e.g., sent via clinical chemical laboratories. ALL, Acute Lymphoblastic Leukemia; RA, Rheumatoid Arthritis; SLE, Systemic Lupus Erythematosus.

Of the individuals with at least one TPMT phenotype result and complete data (n= 15,968), 50.8% were female and 49.2% were male.

TPMT genotype (testing for *2A, *3A and *3C) was available from 12,663 unique individuals and 90.4% were classified as wildtype (TPMT*1/*1), 9.2% heterozygous, and 0.4% were homozygous defective.

The cohort material was used in Paper I to evaluate the concordance between the phenotyping and the genotyping methods and for analysis of factors influencing the TPMT enzyme activity, **Table 2.**

After the introduction of genotyping to the clinical TPMT analyses, the phenomenon of discrepancies (when genotyping and phenotyping results do not correlate) was found. A routine for extended sequencing of discrepant samples was established and some rare or novel TPMT SNPs were found, **Table 3.** This was a continuous work and the characterization of the additional three last found TPMT SNPs are included and described as results in this thesis.

Table 2. Summary of the distribution of TPMT genotype, age and gender in the data material, n=12,654.

Wildtype			
Age	n	Gender	n
0-18 years	2,534	Female	1,107
		Male	1,427
19-40 years	3,598	Female	1,820
		Male	1,778
41-60 years	2,831	Female	1,531
		Male	1,300
61+ years	2,462	Female	1,355
		Male	1,107
Heterozygotes			
Age	n	Gender	n
0-18 years	256	Female	118
		Male	138
19-40 years	353	Female	170
		Male	183
41-60 years	294	Female	154
		Male	140
61+ years	266	Female	141
		Male	125
Homozygotes			
Age	n	Gender	n
0-18 years	8	Female	5
		Male	3
19-40 years	19	Female	11
		Male	8
41-60 years	22	Female	14
		Male	8
61+ years	11	Female	3
		Male	8

Table 3. Overview of rare or novel TPMT SNP found in the TPMT cohort.
Note that this table does not include results found in the scope of this thesis.

Gender, year of birth	Diagnosis	TPMT genotype	TPMT EA		Relatives			Ref
			First	Last	Relation	Genotype	EA	
M, 1946	CD	*1/*9	6.9	8.5				
F, 1963	UC	*1/*9	6.2	7.8				
M, 1980	IBD	*1/*9	8.7	9.2				
F, 1986	UC	*1/*9	8.5	8.3				
M, 1999	-	*1/*14	7.2					
F, 1982	IBD	*3A/*14	0.2	2.3	father	*1/*14	6.3	[103]
					mother	*1/*3A	5.0	
M, 1975	IBD	*3A/*15	0.2	0.4	father	*1/*3A	6.0	[103]
					mother	*1/*15	6.9	
F, 1974	UC	*1/*23	6.7	7.2				
F, 1993	IBD	*1/*23	8.4	8.8				
M, 1944	KC	*23/*3C	0					
F, 1975	IBD	*3A/*23	0.6	0.7	mother	*3A/*23	0.8	[104]
					sister	*3A/*23	0.5	
M, 1940	IBD	*1/*31 (prev. named *1/*28)	8.1	5.8	wife	*1/*1	14.7	[105]
					daughter	*1/*1	14.7	
					son	*1/*31	8.8	

EA, TPMT enzyme activity [U/ml pRBC]; M, male; F, female; CD, Crohn's disease; UC, ulcerative colitis; IBD, other inflammatory bowel disease; KC, kidney clinic

3.1.2 Paper III – ALL cohort

Fifty-three children, treated according to the NOPHO-ALL2000 protocol, were included for a study of the ALL treatment's effect on TPMT and metabolite levels. The children were classified into risk groups with varying treatment intensity (standard (SI), intermediate (II), intensive (I), very intensive (VI) and extra intensive (EI)) depending on pre-therapeutic factors (WBC count, age, central nervous system ALL, T- or pre-B cell ALL, chromosome abnormalities, testis-ALL) and continuous response to the treatment. From day 50 all children received, daily, 6-MP of 25 mg/m² (SI, II) and 60 mg/m² (I, VI, EI) respectively. During maintenance treatment, the daily target dose of 6-MP was 75 mg/m² (adjusted to 50 or 5-10 mg/m² for TPMT heterozygous and homozygous, respectively) and oral MTX was given in a single weekly

target dose of 20 mg/m² except during weeks of HD-MTX treatment. According to the treatment protocol, doses of both 6-MP and MTX were adjusted to WBC count. At 42 hours after the start of HD-MTX infusions, calcium folinate rescue was given. RBC transfusions were given during the treatment when needed. For patient characteristics, see [Paper III](#), Table 1. The childrens' TPMT data were added to the clinical laboratory database and are therefore also included in the cohort of [Paper I](#).

3.1.3. Paper II - TPMT discrepancy from China

In [Paper II](#), a patient was treated at a rheumatology clinic in Hongkong, China and, as an immunosuppressive agent, 25 mg of AZA was given daily. A blood sample for TPMT genotyping was collected and a deviating TPMT genotype was found. In a collaboration with the Chinese research group, our efforts were to confirm the presence of the SNP and to characterize it. Therefore, this individual was not part of any other study cohort in this thesis and ethical approval (**chapter 4.16**) was given in China.

3.2 Cell lines

Cell lines used in the thesis ([Papers III and IV](#)) were MOLT-4 and NALM-6 (American Tissue Culture Collection, ATCC, Rockville, MD). MOLT-4 is a suspension culture of human T-lymphoblasts and was initially obtained from a 19-year-old male in relapse of ALL. NALM-6 is a human B-cell precursor leukemia cell type established from another 19-year-old male also diagnosed with ALL and who had relapsed. Both cell lines were cultured in RPMI1640-medium supplemented with 10% fetal calf serum (Gibco) at 37°C in an atmosphere of 5% CO₂ and subcultivated twice every week.

4. Methods

In this chapter are the methods used in Papers I-IV described in a general and principle manner. In addition, I have included method considerations, motivations for choice of methods and results of method developments performed during my PhD. For an accurate description of the execution of the respective methods, the reader is referred to the method parts of Papers I-IV.

Table 4. Overview of materials and methods in the publications.

Material and Methods	Paper I	Paper II	Paper III	Paper IV
<i>In vivo</i> samples from patients	X	X	X	
<i>In vitro</i> cell lines			X	X
Recombinant enzymes	X	X	X	
Datasimulations		X		
DNA/RNA isolation	X	X	X	X
Sanger sequencing	X	X		
Pyrosequencing	X	X	X	
RT-PCR				X
Enzyme activity RBC	X	X	X	
Enzyme activity WBC - HPLC			X	X
Metabolite monitoring, LC-MS/MS				X
Circular dichroism	X	X		
Binding study - fluorescence			X	
Binding study - isothermal calorimetry		X		
Enzyme activity - absorbance			X	
Enzyme activity - fluorescence		X		

4.1 Sampling

To determine TPMT enzyme activity *in vivo* (Paper III), blood samples were collected at point of diagnosis and treatment day 15 and 29 (induction treatment), day 106 (consolidation), approximately day 400 (maintenance) and one occasion 3-24 months after ending treatment. To avoid discomfort beyond the treatment, times of sampling were chosen to be at occasions when the children left blood samples for the treatment monitoring. Sample to determine the TPMT genotype was collected at day 0. To evaluate the effect of HD-MTX on TPMT enzyme activity and metabolite levels (TGN and meTIMP), two separate samples were obtained at the time of HD-MTX infusions during maintenance therapy. One sample was collected before the start of infusion and the second approximately 66 hours after start of HD-MTX infusion.

4.2 Isolation and amplification of nucleotide acids

Genomic DNA (gDNA) was extracted in a clinical routine laboratory using whole blood and the Maxwell DNA purification kit, together with a Maxwell 16 robot (Promega).

For pyrosequencing, target sequences of exon 5, 7, and 10 were amplified using PCR with one primer in each pair biotinylated, **chapter 4.6**. For Sanger sequencing, the sequence of the full exon 2-10, respectively, was amplified separately. For both pyrosequencing and Sanger sequencing, the primers were designed to avoid amplification of the TPMT pseudogene, by primer binding to sequences where TPMT and the pseudogene deviates and in the introns, respectively.

RNA was usually isolated using the PAXgene Blood RNA kit (PreAnalytiX, Qiagen). The PAX gene tube used for blood sampling stabilizes RNA and prevents degradation by RNases and further gene expression.

The RNeasy Plus Universal kit (Qiagen) was used for RNA isolation from the biobank sample of EDTA stored whole blood. This method was used when patients declined further sampling and hence the RNA isolation had to be done on stored EDTA blood. RNA is not stable in EDTA tubes for long term storage [106]. However, since the RNA was only used for cDNA sequencing, possible stability changes in RNA levels was not a problem. As a control, another sample of a wildtype individual stored at the same condition and for the same time was used. After discussions

with Qiagen, two additional steps before the manufacture's original protocol was added.

The cDNA which corresponds to TPMT mRNA exons 3–8 or exons 5–10 was amplified using PCR and primers hybridizing over the exon-boundaries to exclude contaminating gDNA and the TPMT pseudogene [107].

The amplified cDNA product was analyzed using agarose gel electrophoresis and the sample containing the novel allele was compared to the control (TPMT*1/*1) which results in one distinct band on the agarose gel. **Altered splicing** causes several bands of deviating sizes and each DNA product was extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen).

For **RNA isolation of lymphoblastic cell lines**, the RNeasy Mini Kit protocol (Qiagen) was used followed by conversion of RNA into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

4.3 Spectroscopic methods

Spectroscopic methods are methods based on the principal of light's (electromagnetic radiation's) interaction with matter or molecules. One common spectroscopic method is to measure the **absorption** of a molecule in a solution using a spectrophotometer. Another more sensitive and commonly used method called **fluorescence** is based on the phenomena that molecules (often aromatic residues) that are excited by light, emit a photon when returning to a ground state.

In proteins, the indole group of tryptophan residues is the main reason for fluorescence, absorbing near 280 nm. The emission of indole is highly sensitive to changes in the local environment, solvent polarity and thereby protein structure. This can be used to study the protein structure's native and unfolding state and ligand binding. A quencher is a molecule that decreases the emission intensity [108]. Fluorescence was used in sequencing methods, RT-PCR and for determining recombinant enzyme activity.

The principle of **circular dichroism (CD)** is that optical active molecules (i.e., asymmetric molecules, having a chiral center), absorb right and left-handed circularly polarized light differently. The difference of absorption could be measured and is expressed as ellipticity (θ , deg

cm²/dmol). Proteins are optical active molecules due to the chiral structure in the polypeptide backbone, the 3D-structure and aromatic side chains (Trp, Phe, Tyr)ⁱ and the CD signal can therefore be used for studies of protein secondary and tertiary structure, folding changes, ligand binding and stability.

Far UV, 180-240 nm, is the interval where the peptide bond absorbs light and is used for studies of the protein's secondary structure (α -helix, β -sheet and random coil) where all three have their own characteristic CD spectra (α -helix, maximum at 195 nm, minimum at 208-210 and 222 nm; β -sheet, maximum at 198 nm and minimum at 215-216 nm; random coil, minimum at 198-200 nm).

By monitoring the CD signal at 222 nm (α -helix) or 216 nm (β -sheet), the protein unfolding process can be studied during increasing temperature, time or concentration of denaturants. The melting point (thermal melting point, T_m), at 50% of the native state, are used as a measure of the protein stability, **Figure 7** [6-9].

Figure 7. General figure of the data obtained from a thermal denaturation analysis using CD for determination of the melting point (T_m), indicating the thermal protein stability .

In this thesis, circular dichroism was used for determining changes in the overall secondary structure of modified rTPMT protein and in the presence of SAM (Paper I and II) by running CD spectra in the Far-UV interval. The stability of the different TPMT protein variants were studied by monitoring the CD signal at 222 nm (α -helices) during increased temperatures (Paper I and II), to study the stability of the

ⁱ Amino acids: Tryptophan, Trp, W; Phenylalanine, Phe, F; Tyrosine, Tyr, Y.

protein structure. In similar arrangements, the stability over time with a constant temperature of 37°C was investigated in [Paper II](#).

4.4 Chromatography methods

Chromatography methods were used for separation of substances before usage or detection in several methods of this thesis.

The principle of chromatography is a stationary phase of (coated) particles packed into a column or as a film covering the inside of a column to which the analyte will be exposed using a mobile phase to flow through the stationary phase and the interaction(s) between analytes and the stationary phase will cause separation of the analytes. Additionally, a system for sample handling, injecting, fraction collecting, and/or detection is used, which can be automatic or manual.

Gel filtration is one type of chromatographic method where the analytes are added to a mobile phase that will flow thru a column filled with a stationary phase of beads with pores by gravity. The analytes are separated by size and shape. In affinity binding chromatography the analytes are binding to the stationary phase due to high affinity. By changing the content of mobile phase into a solute with a higher affinity to the stationary phase than the analytes, the analytes will be eluted.

By using isocratic elution, the same mobile phase is used during the whole separation, which depends on the analytes interaction(s) with the stationary phase. A gradient elution changes conditions (as pH, salt concentration or polarity) during separation and affects the analytes' behavior and interaction with the stationary phase to increase the separation of analytes.

The most widely used liquid chromatography form is the reversed-phase liquid chromatography, which has a non-polar stationary phase and a polar mobile phase, normally consisting of water or aqueous buffers or mixtures.

Smaller particle sizes of the stationary phase cause higher resistance for the mobile phase to flow through. In high-performance liquid chromatography (HPLC), **Figure 8**, the column contains a stationary phase of particles, normally in size of 3-5 μm in diameter and can manage a pressure of up to 50 MPa. Because of its cheap and wide area of use for high resolution separation, HPLC is the most popular chromatography method today. Recently, the technique in this area has advanced and the

ultra-performance liquid chromatography (UPLC) has been developed. In UPLC, the stationary phase consists of particles less than 2 μm in diameter and the column can handle pressure of up to 150 MPa, which makes the system faster than HPLC.

The method of detecting the eluted analytes is possible to vary due to suitable application. However, the response of chromatography is always described as a function of retention time or elution volume and is visualized in a chromatogram through peaks of eluted analytes. In a successful chromatography method, the peaks are symmetric, well separated and defined, and each peak describes one of the desired analytes. The amount of an analyte can be described either by the height of the peak or the area under the peak. The retention time of each analyte depends on the chemical properties of the stationary and mobile phase, as well as physical dimension of the column, temperature and the flow rate of mobile phase. To identify which peak is associated by which analyte, the retention time of the unknown peak is compared to a known reference standard. By use of, e.g., a mass spectrometer as a detector, the analytes' structures could be described [109].

Figure 8. Overview of a HPLC system.

4.5 Mass spectrometry

The mass or the amount of a molecule can be determined by a mass spectrometer. The first step is to separate the molecule, which could be

performed either by gas chromatography (GC) or liquid chromatography (LC), making the analytes enter the mass spectrometer at different retention times. The second step is to convert the molecule into a gas-phase ion using, e.g., electron ionization (EI) or electro-spray ionization (ESI). During the ionisation process, electrons are beamed into the molecule to split it and two types of ions are then able to be created; the loss or addition of a proton ($M-H^-$ or $M-H^+$) or creation of a radical cation by loss of an electron ($M^{\cdot+}$).

In ESI, the LC eluent is pumped into a capillary with an applied current cone voltage and charged liquid aerosols are created as the analytes exit the capillary. The droplets evaporate and gas ions are flowing through the extractor cone and further to the mass spectrometer due to vacuum and electrostatic attraction, **Figure 9**.

Figure 9. The principle of the LC-MS/MS method used in Paper IV. The samples containing the analyte (M , mass) were injected into an ultra-performance liquid chromatography (UPLC) column for separation (RT, retention time) and the separated metabolites were detected using positive $[M+H]^+$ electro-spray ionization (ESI) in positive mode where only analyte ions with one extra H^+ are allowed to enter the extractor cone and further to the MS/MS analyzer. MS/MS implies two quadrupole filters/mass analyzers (Q_1 and Q_3) with a collision cell (Q_2) of high pressure of argon gas fragmenting (depending on the collision energy) the analyte precursor ion (M^+) into several product ions (P^+) of which only one is allowed to reach the detector. Detection (time interval is regulated by the dwell time) results in chromatograms from which the amount of a specific analyte can be determined.

Third step, is to determine the weight or, more precisely, the weight to charge ratio (m/z) of the ions, which is performed using a mass analyzer. The most used type of analyzer is the quadrupole which consists of four parallel rods working as electromagnets. By quickly and precisely changing the polarity of these rods, only ions with specific m/z ratios can pass through while the others either collide with the rods or are left between the rods. The quadrupole acts as a mass filter where only ions with a specific mass range can pass.

By using tandem/triple quadrupoles (MS/MS), the first quadrupole only (Q1) let precursor ions with a certain m/z ratio pass through. The ions are then colliding with an other molecule (often argon gas) in the Q2, which nowadays is a specialized collision cell rather than a quad forming different product ions. The product ions then enter Q3, which is another quadrupole mass filter set to only let a specific m/z ratio pass through to the detector. This allows for a very specific detection of a molecule and is referred to as MRM (multiple reaction monitoring).

By rapidly changing the settings of the quadrupoles to allow different m/z ratios, a mass spectra can be generated. This is a plot of the relative signal of the ion versus their mass-to-charge ratios (m/z) and can be generated for both precursor and product ions [109, 110].

4.6 Sequencing methods

Sanger sequencing was used in [Papers I and II](#) to investigate all coding TPMT exons in the search for rare or novel TPMT genotypic variants.

Sanger sequencing was invented in 1977 [111] and is also referred to as terminator sequencing because of its course of action where labelling of the DNA are performed as an amplification process with both nucleotides (deoxyribonucleotide triphosphates [dNTPs]) and dideoxynucleotides (ddNTPs) available for incorporation in the new DNA strands, **Figure 10**. The incorporation of a dNTP will be followed by a further growing strand while incorporation of ddNTP will terminate the sequence and cause DNA products of all lengths. In addition, the four ddNTPs are labelled with four separate fluorophores. By sorting the DNA fragments based on length, using capillary electrophoresis, and detecting the terminating ddNTP-fluorophore, the DNA sequence can be determined. Up to 800 bases can be read using Sanger sequencing [112].

Figure 10. Overview of the Sanger sequencing method. A labeling reaction where the sequence of interest is amplified using normal dNTPs and labeled ddNTPs, which terminate the sequence, are followed by length sorting using capillary electrophoresis and detection of the ddNTPs.

After the discovery of that DNA polymerization could be measured by detecting light following pyrophosphate (PPi) release, the **pyrosequencing method** was invented in 1988 [113, 114]. During the PCR amplification of the template aimed for sequencing, one of the primers must be biotinylated in the 5'-end. The DNA is denatured and single stranded DNA could be captured on streptavidin beads attached to a preparation tool.

The addition of the four dNTPs (dCTP, dGTP, dTTP, dATPaS [deoxyadenosine alphathiotriphosphate]) is performed separately and sequentially in a predetermined order. If the released nucleotide is complementary for the single stranded template, it is incorporated in the growing strand by DNA polymerase, **Figure 11A**. During the incorporation, PPi will be released and is converted by ATP sulfurylase into ATP. ATP is used in the conversion of luciferin into oxyluciferin, which generates a detectable light using luciferase (from a firefly, not recognizing dATPaS, making it possible to separate the signal from added base A and incorporation PPi-ATP release). The light signal will be visible in the pyrogram, **Figure 11B**, with the height in proportion to the ATP concentration and, consequently, to the number of incorporated nucleotides. Nucleotides not incorporated into the DNA strand and ATP are degraded by apyrase before the release of the next dNTP [112, 115].ⁱⁱ

ⁱⁱ Pyrosequencing Technology and Platform Overview, www.qiagen.com

Figure 11. A) The principle of pyrosequencing. Figure adapted from [116] with permission from Springer Nature. B) Raw data (top) and theoretical (bottom) pyrogram investigating the sequence [T/G]ATAACTTTT with a heterozygous result in the position of interest (marked in grey/yellow). The empty C is used as restoring negative control. Adapted from Paper II, supplemental information.

This method is performed in a 96-well plate format and is a high throughput method, making it well suitable for clinical laboratories. With pyrosequencing, it is possible to read up to a 400 base long sequence [112]. In this thesis, pyrosequencing was used in Papers I and III as a method to determine the genotype for the three most common genetic variants of TPMT in the clinical routine. In Paper II, pyrosequencing was used for confirmation of the new SNP found with Sanger sequencing.

4.7 Real-Time Quantitative PCR

Real-time quantitative PCR (qPCR or RT-PCR, not to be confused with reverse-transcriptase-PCR) was used to determine the levels of expressed TPMT mRNA in lymphoblastic cell lines in Paper IV.

RT-PCR is a method based on PCR with the qualification of quantitate the number of mRNA (converted into cDNA) copies and, thereby, measuring the expression level of a specific gene. In RT-PCR, as well as normal PCR, thermal cycles are used for denaturation into single cDNA strands, annealing of primers and extension from the primer into double

stranded cDNA. However, in the annealing phase of RT-PCR, both primers and a probe are hybridizing with the cDNA strand, **Figure 12**.

Figure 12. Principle of real time quantitative PCR. Double stranded cDNA is separated. Gene specific primers and a probe containing reporter dye (R) and quencher dye (Q) hybridize to the single stranded cDNA. When Taq-DNA-polymerase (Taq) builds a complementary strand, it cleaves of the reporter dye and its fluorescence emission (R_F) is measurable. The procedure is repeated, and for each new copy of the gene, the intensity of the fluorescence emission in-crease.

The probe, according to Taqman methodology, contains two fluorescent dyes, one reporter dye in the 5'-end, and a quencher dye in the 3'-end [117]. In Paper IV, a FAM (6-carboxyfluorescein) reporter was used in combination with a non-fluorescent quencher (NFQ) in an minor groove binder (MGB) probe which is shorter but has a higher specificity.ⁱⁱⁱ The probes were specific to the TPMT gene and the huCYC gene, which has previously been described as an excellent housekeeping gene in the TPMT gene expression method used in this paper [118].

ⁱⁱⁱ Product overview of #4316034 (MGB probe), #4331182 (TPMT) and #4351372 (human cyclophilin huCYC/PPIE), www.thermofisher.com, 2019-11-05

When the probe is intact and the reporter and quencher are therefore close, the quencher absorbs the fluorescence emission of the reporter dye. With the primer as a starting point, the Taq-polymerase (*Thermus aquaticus*, thermal stable DNA polymerase often used in PCR methods) is building a complementary strand in the extension phase of the PCR. However, when reaching the probe, the Taq-polymerase uses its 5' → 3'-exonuclease activity to cleave off the reporter dye of the probe [119]. The released reporter dye results in a fluorescence emission with a 518 nm peak, where intensity could be monitored in real time. Since this occurs in the production of every copy in the PCR reaction, the result could be used to calculate back to the original concentration (number of gene copies) at the start. The levels of gene expression could be determined by comparing the levels to a serial dilution of cDNA as a standard curve. Often a housekeeping gene is used for normalization.

In Paper IV, the results are expressed in TPMT/huCYC ratio which is normalized to CV treated sample set as 100%. Since small volumes are used, even small pipetting errors could cause large variations in the results. Therefore, to increase accuracy, pipetting robots were used for transferring cDNA and mastermix into a 384 well plate.

4.8 Cell incubations with drugs

In Paper III, which was the first performed study in which cell incubations with drugs were used, MOLT-4 cells were treated with 90 µM MTX. Since both MTX and 6-MP are difficult to solve and a solvent which was non-toxic for the cells were required, different solvents and concentrations were tested. In the end, 50 mM sterile filtered NaOH was used for solving MTX and 51.4 µl solvent and drug were added to 20 ml cell culture, resulting in 0.3% CV (final concentration 0.15 mM NaOH). The effect of CV was studied by including untreated cells to the CV and drug treated in Paper III. No significant differences in TPMT enzyme activity (**Figure 29**, p. 74) or signs of unhealthy cells were observed. Therefore, in Paper IV, the same solvent 50 mM NaOH was used to solve the drugs. In this paper, lower concentrations of MTX were used and 6-MP was also added to the cells (MOLT-4 and NALM-6). The addition of drug in/or 50 mM NaOH used in Paper IV was 30 µl to 50 ml cells (0.06% CV, 30 µM NaOH). All cell culturing was performed without the normally added penicillin-streptavidin (PEST) due to previous studies in the research group indicating that antibiotics may affect the TPMT enzyme activity (results not shown).

4.9 Sorting out living cells after drug incubation

After cell incubation with MTX and/or 6-MP, cells were counted and the same amount of cells were transferred to a new tube and binding buffer containing MACS MicroBeads (which are magnetic beads that attach to apoptotic or dead cells by recognizing an antigen in the plasma membrane of the cells) were added. After incubation, the mix of cells and beads were transferred to MiniMACS columns, which were placed in MiniMACS separators that apply a magnetic field to the column. Labeled cells were stuck in the column due to the magnetic field and were not directly bonded to the column matrix. Living cells, which were not labeled, were able to pass through the matrix in the column and be collected for further investigation.^{iv}

4.10 Production of recombinant TPMT enzyme

The construction of the TPMT defective genes (p.Y240C, p.Y240S, p.Y166C)^v was performed using site directed mutagenesis [3, 109], **Figure 13**, on a plasmid containing the sequence of TPMT wildtype cDNA, either by our research group at Linköping University or by GenScript (Piscataway, New Jersey, USA). The modified vector was transformed into *Escherichia coli* (*E. Coli*) cells and growth in 37°C LB medium supplemented with chloramphenicol and kanamycin for specificity through the *E. Coli* and vector. The expression of the recombinant TPMT (rTPMT) protein was induced using IPTG (isopropyl-beta-D-thiogalactopyranoside) due to usage of the lac operon [109]. The vector also included the sequence coding for a His-tag (6xHis)^v and a thrombin protease cleavage site, located next to the TPMT gene. This makes it possible to purify the rTPMT using the His-tag's affinity to Ni-NTA matrices and remove the His-tag using biotinylated thrombin which could be captured using streptavidin beads [109].

Figure 13 → *The method of site directed mutagenesis of recombinant TPMT. Primers designed with the desired mutation are used for amplification of the plasmid. Restriction enzyme DpnI degraded the vectors not containing the mutation by recognizing methylations from the E. Coli. The translation and purification procedures to obtain a purified TPMT are described more extensively in the text.*

^{iv} Product information for MACS Dead Cell removal kit (130-090-101) and MS Columns (130-042-201), Miltenyi Biotec, 2019-11-04

^v Amino acids: Tyrosine, Tyr, Y; Cysteine, Cys, C; Serine, Ser, S; Histidine, His, H.

4.11 TPMT enzyme activity measurements

The methods used for determination of the enzyme activity of TPMT are based on the first TPMT enzyme activity method, which was developed in 1978 by Weinshilboum *et al.* [120] (modified in 1992 by Klemetsdal *et al.* [121]) for measurement in RBC. The assay uses TPMT's function of converting 6-MP into meMP with the cofactor SAM acting as methyl group donor and forms SAH, **Figure 14**. By adding the substrate 6-MP and radiolabeled co-factor [^{14}C]-SAM, the TPMT enzyme forms labeled [^{14}C]-meMP which can be detected by a scintillation counter. The amount of the product meMP is related to the activity of TPMT.

Figure 14. The principle of the TPMT enzyme activity methods. By adding the substrate 6-MP and cofactor SAM ([*] labeled methyl group), the activity could be determined by measuring the amount of meMP, formed by the TPMT enzyme.

4.11.1 TPMT enzyme activity in RBC, WBC and cell lines

A method for **TPMT enzyme activity measurement in RBC**, based on the original method, was established as Sweden's first clinical laboratory TPMT phenotyping test in year 2000 at Department of Medical and Health Sciences, Linköping University, as described by Petterson *et al.* [3]. The method counts the product of radiolabeled [^{14}C]-meMP in counts per minutes (cpm) after precisely 60 minutes of incubation.

The within-run variation (intra-assay coefficient of variation) in the method was 3.3% (n=8, mean of samples 13.2 U/ml pRBC, range 12.5-13.7 U/ml pRBC) and the in-between (inter-assay) variation was 4.7% (n=19, mean of samples 13.5 U/ml pRBC, range 12.7-14.9). This method was used for TPMT phenotyping of all clinical patient samples in Papers I, II and III.

The clinical laboratory method has further been modified for measurement of **TPMT enzyme activity in WBC** as described in

Lindqvist Appell *et al.* 2010 [105] and were used in this thesis to measure **TPMT enzyme activity in cultured lymphoblastic cell lines**. Deviations compared to the clinical method was two hours of incubation and the product was separated using HPLC. Both [^{14}C]-SAM and [^{14}C]-meMP were detected by a UV detector (at 290 nm) and the amount of eluated [^{14}C]-meMP was determined by a radioactive detector.

4.11.2 Spectrophotometric assays for rTPMT activity

A spectrophotometric method for measurement of recombinant TPMT enzyme activity was described as an assay for study TPMT substrates by Krynetski *et al.* [122] and used as an activity assay by Scheuermann *et al.* [123]. The principle of this method is to continuously determine the **absorbance of 6-MP** at 320 nm after addition to a sample containing rTPMT protein. The TPMT enzyme activity was determined by monitoring the decreased signal due to conversion of 6-MP into meMP. Kinetics of the reaction was generated by varying the 6-MP substrate concentration to determine a suitable concentration of substrate. $V_{\max}$ was calculated to 73.8 nM/min/mg and K_M to 0.63 mM, **Figure 15**. The spectrophotometric method for TPMT enzyme activity was used in Paper I to determine the rTPMT enzyme activity in the presence of MTX.

Figure 15. Kinetics of the rTPMT catalyzation of 6-MP into meMP measured by a spectrophotometric assay and varying 6-MP substrate concentrations.
a) Saturation curve according to the Michaelis-Menten model.
b) Lineweaver-Burk plot.

Another method for measuring rTPMT enzyme activity was used in Paper II for studying the long-term stability in terms of enzyme activity after 0, 0.5 and 8 hours of incubation at 37°C. The SAMfluoro: **SAM Methyltransferase Assay** (G-Bioscience) is a continuous enzyme

assay where the activity of the methyltransferase of interest is determined by using the produced co-product SAH for further cascade formation of a measurable fluorescent product (Resorufin), whose amount could be detected by monitoring the fluorescence at excitation and emission wavelengths of 530-540 nm and 585-595 nm, respectively.

4.12 Metabolite level quantification

4.12.1 Thiopurine metabolite levels in RBC and cell lines

The method for measuring metabolite levels of TGN and meTIMP in patient RBC, [Paper III](#) was originally developed by Lennard and Singleton in 1992 [124]. The method was modified in 2002 by Pettersson *et al.* [125] and was implemented in the routine analysis laboratory at the division of Clinical Pharmacology, Linköping University. In this method RBC are lysed and the metabolites are converted to their corresponding nucleobases by acid hydrolysis. After extraction, the products are separated and detected using HPLC.

4.12.2 Monitoring metabolite levels in cell lines

The method for measuring the thiopurine metabolites TGN, meTIMP, TXMP and TIMP in lymphoblastic cell lines ([Paper IV](#)) was based on a method described in 1998 by Dervieux *et al.* [126]. However, as lymphoblastic cells are more difficult to homogenize than RBC a sonication step was introduced to lyse four million cells in water. Also, the intermediate thiopurine metabolites TXMP and TIMP were added to the method for a more comprehensive view of the metabolism. The LC-MS/MS method, used in [Paper IV](#), was first developed and validated as an HPLC method. However, when equipment was available, the method was optimized and transferred to LC-MS/MS to increase selectivity. For specific method parameters of the LC-MS/MS method and the HPLC method, the reader is referred to **Table 5** and **Table 6**, respectively.

Table 5. Method parameters of the metabolite method for LC-MS/MS

UPLC system:	Acquity UPLC System including column-, sample- and binary solvent manager (Waters)
Injection volume:	15 µl
Column:	Acquity UPLC BEH C18 column (100*2.1 mm, 1.7 µm) with a BEH VanGuard C18 guard column (5*2.1, 1.7µm) maintained at 55°C
Mobile phase:	A: 2 mM AmAc and 0.1% FA in water B: 2 mM AmAc and 0.1% FA in methanol Delivered at 0.45 mL/min, starting with 1.5 min isocratically at 2% B followed by a linear gradient to 50% B at 6.5 min and 1 min re-equilibration.
Runtime per sample:	7.5 min
Detector:	Xevo TQD LC-MS/MS system (Waters), positive ESI mode using MRM
<u>MS parameters</u>	
Source temp.:	150°C
Desolvation temp.:	500°C
Desolvation gas flow:	1000 L/h
Cone gas flow:	-
Collision gas, flow:	Argon, 0.15 ml/min

Analytes	TGN	TIMP	TXMP	meTIMP
detection range	27 - 5,300 pmol/mL*	40 - 8,000 pmol/mL*	67 - 13,000 pmol/mL*	270 - 53,000 pmol/mL*
Retention time (min)	1.02	1.15	1.43	2.44
Cone voltage (V)	36	36	30	20
Precursor ion (m/z)	168.0	153.0	169.0	158.0
Collision energy (eV)	20	20	18	10
Product ion (m/z)	150.9	119.0	151.9	110.0
Dwell time (ms)	61	61	61	128

*concentration in 60 µl supernatant, see protocol in [Paper IV](#). AmAc, ammonium acetate; FA, formic acid, ESI, electro-spray ionization; MRM, multi reaction monitoring

Table 6. Method parameters of the metabolite method for HPLC

HPLC system:	2695 Separations Module pump (Waters)			
Injection volume:	50 µl, sample temperature 5 °C			
Column:	Phenomenex Synergi MaxRP column 150x2 mm (4 µm, cooled to 10 °C)			
Mobile phase:	0.02 M phosphoric acid, 1.3 mM DTE, 0.75% acetonitrile and 0.25% methanol (v/v) Isocratically delivered at 0.45 mL/min			
Runtime per sample:	13 min			
Detector:	Dual λ Absorbance Detector 2487 (Waters)			
Analytes detection range	TGN 27 - 5,300 pmol/mL*	TIMP 40 - 8,000 pmol/mL*	TXMP 67 - 13,000 pmol/mL*	meTIMP 270 - 53,000 pmol/mL*
Retention time (min)	2.7	4.1	7.4	6.4
Wavelength (nm)	340	325	340	290

*concentration in 60 µl supernatant, see protocol in [Paper IV](#). DTE, dithioerythritol

Validation was performed using MOLT-4 cells and spiked concentrations. When the variation within batch was investigated (n=6), the accuracy was 95-109% (except for the limit of detection [LOD, standard concentration 1] where the accuracy was 77-114%). Precision within batch was ≤9.0%. Determining the variation between batches (n=3), the accuracy was 93-108% (except LOD which was 95-117%). Precision was ≤9.5% (except for LOD which was ≤18.5%). The extracted samples were stable in the autosampler (5°C) for 72 hours (n=3). No carry-over effect was detected.

4.12.3 Fraction of free base using LC-MS/MS

One single measurement of MOLT-4 cells (treated with 2.2 µM 6-MP) was analyzed without the hydrolysis step (boiling the sample in a mixture of PCA/DTE) as normally performed in the method, to give us a clue of the distribution of free nucleobase and nucleotides/ribosides in the samples, **Table 7**. The hydrolyzation into nucleobases occurred during the boiling.

Table 7. Single measurement of boiled and unboiled metabolite samples from MOLT-4 cells incubated with 6-MP for 16 and 26 hours, respectively, to investigate the proportions of free base and nucleotides.

	6-MP 16 hours [pmol/ml]			6-MP 26 hours [pmol/ml]		
	boiled	not boiled	%	boiled	not boiled	%
TIMP	237	19	8	6385	51	1
TXMP	770	170	22	3863	886	23
TGN	484	16	3	3323	108	3
meTIMP	202	-	-	1364	-	-

The results showed that TIMP and TGN were, to a large extent, consisting of nucleotides or ribosides (92-99%). TGN was not expected to be found in free base form. However, no free TIMP base means that almost no 6-MP was detected, probably due to easy diffusion out of the cell and thereby lost during sample preparation and washing. TXMP also includes 22-23% of free TX base which is unexpected since it should only be produced using the drug 6-TG. This could indicate that 6-MP could act as substrate for IMPDH. However most likely this is because TXMP was unstable during the method conditions and was, to some extent, already degraded before the boiling. Since both meTIMP and meMP are degraded into AMTCI during the boiling and detected as such, rather than the free base, this measurement was not possible to perform.

4.13 Interaction studies of rTPMT and MTX

The TPMT protein has five tryptophan residues, **Figure 16**. By monitoring the fluorescence signal from TPMT's tryptophans during addition of MTX in increasing concentrations, the binding of MTX could be characterized, Paper III. The specific excitation wavelength of tryptophan residues (295 nm) was used and emission spectra, with an increasing maximum intensity at 340 nm, were recorded during increased addition of MTX. The similar emission maximums during increased drug concentrations display a flexible region around the tryptophans.

Figure 16. The structure of human TPMT with five tryptophans highlighted in orange and the coproduct SAH in red. The figure is illustrated in PyMOL, coordinates obtained from RCSB Protein Data Bank, accession code 2BZG

4.14 Interaction between rTPMT and SAM

In this thesis, isothermal calorimetry (ITC) was used in [Paper II](#) to characterize the binding of cofactor SAM to modified rTPMT proteins. ITC consists of two cells, a sample cell and a reference cell. The thermodynamics of a binding event is detected by measuring the heat emission of the binding process, when enzyme or ligand is added to the sample cell. The energy consumption to keep the sample cell temperature consistent in comparison to the reference cell (containing a corresponding buffer), is detected as a function of time. From this, the binding can be characterized regarding, for instance, the dissociation constant, K_d [109], as done in [Paper II](#) for rTPMT variants and the cofactor SAM.

4.15 Statistics

In all papers, $P \leq 0.05$ was considered as significant, and statistical significance is plotted as $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***). All statistical analyses were performed using IBM, SPSS Statistics 19.0-23.0 software (SPSS Inc, IBM, Armonk, NY, USA), descriptive statistics were generated by Microsoft Excel 2013 (Microsoft, Redmond, US) and the graphs were created using the software GraphPad Prism 5.01 (SanDiego, CA).

Paper I - Descriptive statistics and graphs were used to present the database material. To avoid negative confidence interval in conditional proportions and concordance rates, the Clopper-Pearson method was used to calculate 95% confidence intervals (CI).

The general linear model used for analysis of factors influencing the TPMT enzyme activity can be described as a sort of common analysis of variance with two fixed factors (genotype and gender) and one continuous factor (age). The significant factors and interactions between factors were used for a model, from which a regression line (sort of trendline adjusted for the significant factors) was obtained. In **Figure 23**, p. 66, was also the data grouped and plotted descriptively.

For difference between T_m of rTPMT*1 and rTPMT*44, an unpaired t-test was used.

Paper II - Thermal denaturation and long-time stability data were analyzed using one-way ANOVA and post-hoc Dunnett's test, to compare the means of T_m and CD-signal, respectively in the presence of no SAM, 10x SAM and 50x SAM for each rTPMT protein variant separately.

For rTPMT enzyme activity, a two-way ANOVA was used comparing means with the factors time and TPMT variant.

Paper III - The patient sample analysis was considered not normally distributed; therefore, non-parametric statistics (median, interquartile range [IQR] and Wilcoxon's test for comparing group means) were used. Cell lines treated with CV or MTX were compared using t-tests.

Paper IV - The data of mRNA and TPMT enzyme activity were normalized, within each separate incubation set, to the CV as 100%. It was only interesting to examine if any of the MTX or MTX+6-MP treated samples were deviating from the CV. Therefore, t-test with test value 100 was performed and since it was not obvious how to define the number of comparisons, all significant p-values were reported.

However, it was also interesting to study if there was any difference between the MTX treated sample and the combined MTX+6-MP treated sample containing the same MTX concentration. For these comparisons, pairwise independent t-tests were performed.

4.16 Ethical approval

Paper I - When patients leave a blood sample for medical diagnosis or treatment use, a box on the letter of referral is available to cross if the patient declined their sample to be collected in a biobank. An approval from the local Ethics committee in Linköping (EPN dnr. 01-016) and updated approval (EPN dnr. 2014 194-31) allow the study to use the biobank samples for research and request further samples from patients through the patient's treating physician. The approval also include permission to ask the patient for permission of requesting blood samples from relatives. All individuals leaving further blood samples got written information about the study and gave their written content to be included.

Paper II - Since the patient, carrying the novel TPMT*41 allele, was treated in a hospital in Hongkong, China, the study was approved by Cluster Research Ethics Committee (KC/KE) of the Hongkong Hospital Authority at Queen Elizabeth Hospital, Hong Kong SAR in China (nr KC/KE-14-0077/ER-1). Before collecting blood samples for the study, informed consent was obtained from the patient.

Paper III - The local Ethics Committee at Linköping University approved the study (ref. no. 01-297) and all children and/or their parents received written patient information about the study and gave their written consent to be included in the study and the usage of their blood samples for research.

Paper IV - This paper included only cultured cell lines, why no ethical approval was needed.

5. Results

In this chapter, the main results of Papers I-IV are described and some additional results that did not fit into the papers are included. More details of the results can be found in Papers I-IV.

5.1 Concordance between TPMT geno- and phenotyping

(Paper I)

Individuals defined as TPMT wildtype by genotyping when testing for TPMT*2, *3A and *3C ranged between 2.0-40.8 U/ml pRBC in their first^{vi} TPMT enzyme activity measurement. Individuals classified as a carrier of a heterozygote TPMT defective allele had an enzyme activity of 0-14.0 U/ml pRBC and individuals with homozygous defective TPMT genotype ranged between 0-5.5 U/ml pRBC. The concordance of the TPMT enzyme activity measurement and genotyping (testing for TPMT*2, *3A, *3C) was 94.5% (95% CI; 94.1-94.9), **Figure 17**.

Concordance genotyping / phenotyping: 94.5 %

n=12,663

		TPMT enzyme activity (U/mL pRBC) n, conditional proportions % (95% CI)		
		High	Intermediate	Low
TPMT genotype	Wildtype	10,864 99.1 (98.9-99.2)	578 35.4 (33.0-37.7)	1 1.6 (0.0-8.8)
	Heterozygous defective	103 0.9 (0.8-1.1)	1,053 64.4 (62.0-66.7)	9 14.8 (7.0-26.2)
	Homozygous defective	0	4 0.2 (0.1-0.6)	51 83.6 (71.9-91.8)
		↓	↓	↓
Total:		10,967 (100%)	1,635 (100%)	61 (100%)

Figure 17. Comparison of genotyping (testing for TPMT*2, *3A, *3C) and the first measurement of TPMT enzyme activity in patient samples referred for TPMT status determination. Results in concordance with both methods are visible in the white boxes. Figure adapted from Paper I.

^{vi} Many individuals (9.6 %) have results from repeated TPMT enzyme activity measurements registered in the data base. The first TPMT enzyme activity is referring to the first determined TPMT enzyme activity value registered in the database.

5.2 Discrepancies between genotyping and phenotyping

(Paper I)

Compared to the routine TPMT genotype result, 4.6% (n=588) had a lower TPMT enzyme activity than expected and 0.8% (n=107) had higher than expected, **Figure 17**. Of the 11,443 individuals with genotype TPMT*1/*1, 579 individuals were classified as intermediate or low by the enzyme activity measurement.

The first action, when genotyping and phenotyping did not correlate, was to request an additional blood sample from the patient for renewed activity measurement. However, the second sample was not always obtained, **Figure 18**.

^{A, B} Two extra other TPMT alleles were found in these groups, even if samples in these groups were not usually sequenced.

Figure 18. Overview of the repeated TPMT enzyme activity measurements of TPMT*1 discrepancies.

Of 11,443 samples 579 (5.1%) phenotype measurements were lower than expected in comparison to the genotyping results. An additional requested sample was received in 310 cases and of these 228 reanalyzed samples (73.5%) were in the range of wildtype TPMT enzyme activity.

5.3 TPMT enzyme activity during ALL treatment

(Paper I and III)

The TPMT enzyme activity in the beginning of ALL treatment was studied by the sampling of 53 children at day 0, 15, 29, 106 and approximately day 400 of the ALL treatment and one occasion 3-24 month after ending treatment, **Figure 19**.

Figure 19. TPMT enzyme activity during ALL treatment in 47 TPMT*1 individuals. Figure from *Paper III*. (ns= non-significant differences between time points)

The TPMT enzyme activity was significantly lower at the point of diagnosis in comparison to treatment day 106. No differences in TPMT enzyme activity was detected after day 106.

When analyzing the concordance of genotyping (*2, *3A, *3C) and phenotyping method in individuals with hematology diagnoses (n=798, **Figure 20**), the concordance was only 70.7% (95% CI; 67.4-73.8). Most remarkable, but not unexpected, was that 78.7% of the individuals with intermediate enzyme activity were genotyped as wildtype.

Hematology diagnoses - Concordance genotyping / phenotyping: 70.7 %

n=798

		TPMT enzyme activity (U/mL pRBC) n, conditional proportions % (95% CI)		
		High	Intermediate	Low
TPMT genotype	Wildtype	502 98.6 (97.2-99.4)	225 78.7 (73.5-83.3)	1 33.3 (0.8-90.6)
	Heterozygous defective	7 1.4 (0.6-2.8)	61 21.3 (16.7-26.5)	1 33.3 (0.8-90.6)
	Homozygous defective	0	0	1 33.3 (0.8-90.6)
Total:		509 (100%)	286 (100%)	3 (100%)

Figure 20. Comparison of genotyping (testing for TPMT*2, *3A, *3C) and the first measurement of TPMT enzyme activity in samples from individuals with hematology diagnoses which were referred for TPMT status determination. Results in concordance with both methods are visible in the white boxes.

5.4 Discovery of rare or novel genetic variants of TPMT

(Paper I)

If the discrepancy between genotyping and phenotyping remained after the analysis of the second sample, the TPMT exon 2-10 were sequenced using Sanger sequencing. In 15 individuals (0.1% of all individuals [n=12,663] and 2.6% of the individuals with unexplained low TPMT enzyme activity [n=588]) a rare or novel TPMT allele was found, explaining the decreased activity, **Table 8**.

Four individuals carried the rare allele TPMT*9, two TPMT*14 carriers were found, and 4 individuals carried the TPMT*23 allele. One of each of the alleles TPMT*15, TPMT*31, TPMT*42, TPMT*43 and TPMT*44 were also discovered in the cohort. Characterization of the previously undescribed TPMT alleles (TPMT*42-44) are present chapter 5.7. Histogram of the TPMT enzyme activities in correlation to the extended TPMT genotyping results are visualized in **Figure 21**.

Table 8. Final table of all genotypes after the extended TPMT genotyping of discrepant samples. Table adapted from *Paper I*.

	TPMT genotype	n (individuals)	Frequency (%)
Wildtype	*1/*1	11,433	90.3
Individuals carrying one variant allele	*1/*2	17	0.13
	*1/*3A	1,030	8.13
	*1/*3B	3	0.02
	*1/*3C	110	0.87
	*1/*9	4	0.03
	*1/*14	1	0.01
	*1/*23	2	0.02
	*1/*31	1	0.01
	*1/*43	1	0.01
	*1/*44	1	0.01
Total	Heterozygotes	1,170	9.24
Individuals carrying two variant alleles	*3A/*3A	40	0.32
	*3A/*3C	11	0.09
	*3A/*3B	1	0.01
	*3C/*3C	1	0.01
	*2/*3A	2	0.02
	*3A/*14	1	0.01
	*3A/*15	1	0.01
	*3C/*23	1	0.01
	*3A/*23	1	0.01
	*3A/*42	1	0.01
Total	Homozygotes	60	0.47
Total	All individuals	12,663	100

Figure 21. TPMT enzyme activity of 12,663 individuals with genotyping results available, visualized as overlapping subgroups in the histogram.

5.5 Unexplained discrepancies

(Paper I)

A rare TPMT allele was found in far from every discrepant sample and many discrepancies so far remained as unexplained mysteries, **Figure 22**. In **chapter 5.7.4** one example of unexplained discrepancy was found.

Many of the discrepancies had TPMT enzyme activity values close to the limit set between the intermediate and normal activity intervals. However, a considerable proportion is in the middle of the intervals, and both too high (**Figure 22**, blue and green bars) and too low (**Figure 22**, orange and red bars) enzyme activities occurred, compared to the TPMT genotype.

Figure 22. Unexplained discrepancies in the database, $n=451$. The TPMT enzyme activity results are visualized and extended genotyping is included in the figure. The range of low, intermediate and high TPMT enzyme activity are marked in grey. If more than one TPMT enzyme activity result was available, the result of the most recent measurement was used in the figure.

5.6 Factors affecting the TPMT enzyme activity

(Paper I)

In the database, data concerning age (at time for sampling) and gender were available. These factors were used in an analysis of factors influencing the TPMT enzyme activity, **Figure 23**. Expected finding was the established knowledge of TPMT genotype as an influencing factor, **Figure 21**. However, age and gender were also found to affect the TPMT enzyme activity.

Men have higher TPMT enzyme activity than women in all three classes of genotypes. The difference between gender appeared to be less in younger individuals. Older individuals had higher TPMT enzyme activity than younger, and this phenomenon was most pronounced in wildtype individuals and heterozygous males, **Figure 23**.

Figure 23. Factors that influence the TPMT enzyme activity, $n=12,654$. A) Results from the general linear model. Adapted from *Paper I*. B) Regression lines derived from parameter estimates, between TPMT enzyme activity and age for males and females in TPMT genotypes, based on the general linear model. C-E) TPMT enzyme activities in males (M, blue) and females (F, red) divided according to genotype and age. Data plotted as mean and 95% CI.

5.7 Characterized TPMT alleles and their consequences

5.7.1 TPMT*41 – decrease in SAM affinity

(Paper III)

A novel allele named TPMT*41 was found in a woman treated at a rheumatology clinic with AZA. The patient had intermediate TPMT enzyme activity, 7.7 U/ml pRBC, and a heterozygous substitution of c.719A>C was found, **Figure 24**. Since the substitution had the same cDNA position as the common TPMT*3C, an interesting study was performed showing the effect of one amino acid on the rTPMT protein.

Figure 24. Sequencing histogram from TPMT exon 10 showing TPMT*1, TPMT*1/*3c and TPMT*1/*41. TPMT*3C and *41 cause amino acid shifts of p.Y240C and p.Y240S, respectively. Figure adapted from [Paper II](#).

All three variants of TPMT (TPMT*1/*1 [wt], TPMT*1/*3C [p.Y240C] and TPMT*41 [p.Y240S])^{vii} were produced as rTPMT and characterized using CD, fluorescent TPMT enzyme activity and ITC.

The thermal stability of rTPMT p.Y240S was lower than both rTPMT p.Y240C and rTPMTwt, **Figure 25**. The rTPMTwt protein was the most stable with 5.6 and 10.5°C higher thermal melting point (T_m), without cofactor SAM, compared to p.Y240C and p.Y240S, respectively. This pattern was also seen in the enzyme activity and long-time stability measurements (Table 1 and Figure 4 in [Paper II](#)).

When adding the cofactor SAM, the thermal stability, evaluated using T_m , was increased in all protein variants in the presence of the highest concentration of SAM, while only the T_m of rTPMTwt increased at 10-

^{vii} Amino acids: Tyrosine, Tyr, Y; Cysteine, Cys, C; Serine, Ser, S.

fold excess. Also, the long-time stability was increased for wt in both 10-fold and 50-fold excess of SAM, and for p.Y240C using the highest SAM concentration.

B)

rTPMT variant	Thermal melting points, T_m		
	-SAM T_m (°C)	+SAM _{10-fold} ΔT_m	+SAM _{50-fold} ΔT_m
wt	47.2 ± 0.4	+2.2*	+5.3***
p.Y240C	41.6 ± 0.4	+0.8	+4.7***
p.Y240S	36.7 ± 0.3	+1.5	+4.6***

T_m was obtained using CD spectroscopy monitored at 222 nm

Significant level; $p \leq 0.05$ (*) $p \leq 0.001$ (***)

Figure 25. A) Thermal stability of rTPMTwt (black), rTPMT p.Y240C (blue) and rTPMT p.Y240S (green) in 10-fold (dashed line), 50-fold (solid line) molar excess of SAM or absence (dotted line) of SAM. B) Thermal denaturation temperatures for TPMTwt, TPMT p.Y240C and TPMT p.Y240S. Figures adapted from [Paper II](#).

When studying the three rTPMT variant's binding affinity for SAM using ITC (Figure 5, [Paper II](#)), it was confirmed that rTPMT wt had the highest affinity for SAM and, due to signal levels, it appeared that p.Y240S had the lowest SAM affinity, which probably caused the low enzyme activity seen *in vivo*. K_d for SAM to rTPMTwt was determined to 2.7 μ M, the proportion of noise in comparison to signal was unfortunately too high in the other measurement to obtain a reliable K_d for the binding to SAM.

To further understand the mechanism behind the decreased stabilization and interaction with SAM, molecular dynamics simulations were performed and showed that the substitution of p.Tyr240Cys/Ser weakened, in wildtype protein, a strong hydrogen network including His182, Val184, His201, Ile204, Ile214, Cys216 and Tyr240^{viii}. In addition, the substitution caused lost interaction between Cys/Ser240 and Val184, which were located close to the binding site of 6-MP (Figure 6, [Paper II](#)).

5.7.2 TPMT*42 – nonsense amino acid sequence

(Paper I)

The TPMT*42 allele was found in a man diagnosed with CD. Three months after starting AZA treatment, he suddenly suffered from serious leucopenia and needed blood transfusions and intravenous antibiotics. Repeated measurements of TPMT enzyme activity showed results of 0.5 and 0.6 U/ml pRBC, respectively but the genotype was TPMT*1/*3A. Due to the discrepant result, new blood samples were requested from the patient and his family. By extended sequencing of exon 2-10, an insertion of an extra A in cDNA position 95_96 was found, **Figure 26**, causing an amino acid shift and consequential a nonsense amino acid sequence and a premature stop codon, resulting in a truncated TPMT enzyme. The allele, named TPMT*42, was inherited in the family and both the patient's mother and daughter were heterozygous carrier of TPMT*42.

5.7.3 TPMT*43 – loss of exon V

(Paper I)

A man diagnosed with SLE, was carrier of the novel TPMT*43 allele. TPMT status determination resulted in enzyme activity of 7.5 and 8.5 U/ml pRBC and the genotype TPMT*1/*1. In the extended sequencing, two SNP were found in exon 5, **Figure 27**. Since the patient declined

^{viii} Amino acids: Histidine, His, H; Valine, Val, V; Isoleucine, Ile, I; Cysteine, Cys, C; Tyrosine, Tyr Y.

TPMTwt

MDGTRTSLDIEEYSDTEVQKNQVLTLLEEWQDKWVNGKTAHFHQEQGHQLLKKHLDTLFKGKS...

TPMT p.K32KfsX58

MDGTRTSLDIEEYSDTEVQKNQVLTLLEEWQDKVGERQDCFSSGTRTSAIKEAFRYFPStop

Figure 26. Results concerning the TPMT*42 allele. The insertion of an A in cDNA position 95_96 caused a shift in the amino acid sequence and a premature stop codon. The variant was inherited from the patient's mother and further to his daughter. Figure adapted from *Paper I*.

Figure 27. Results concerning the TPMT*43 allele. The substitution of c.234G>T and c.262G>A caused altered splicing in allele 2 (A2), where exon V was missing. Figure adapted from *Paper I*.

further sampling, no other samples were obtained. A method for extracting RNA from the biobank sample was instead developed and used. Gel electrophoresis of the resulting cDNA showed several bands and new primers, amplifying 299 bp from exon III to exon VI was designed. The cDNA product using the new primers showed three separate bands on the gel, **Figure 27**, of which the two largest were possible to get sequences from. The largest band (~300 bp) resulted in the TPMT*1 cDNA sequence and the second largest band (~175 bp) showed the TPMT cDNA sequence without exon V. Theoretical amplification size without exon V was 166 bp.

These results concluded that the patient was a heterozygous carrier of a new allele containing two SNPs which caused deletion of exon V and lower TPMT enzyme activity. The allele was named TPMT*43.

5.7.4 TPMT*44 – decreased protein stability

(Paper I)

The allele TPMT*44 was found in a woman diagnosed with ALL. Her TPMT enzyme activities were 2.0 and 2.5 U/ml pRBC in repeated measurements and the genotype was determined to TPMT*1/*1. Extended sequencing found a heterozygous SNP in position c.497A>G which caused the amino acid substitution of TPMT p.Y166C, **Figure 28**. Additional blood samples from the patient and her family were requested and obtained. Gel electrophoresis showed one single band of expected size, indicating no altered splicing. The TPMT*44 allele was inherited to the patient from her mother and one of her half-sisters was also a carrier of the novel allele.

The result of the patient's first TPMT enzyme activity measurement was lower than expected also for a heterozygous carrier, but was most likely caused by the ALL disease, **chapter 5.3**. Interestingly, one of the half-sisters (nr 3, **Figure 28**) was subject for unsolved discrepancy, **chapter 5.5**. Both gDNA sequencing and cDNA sequencing resulted in the sequence of TPMT*1 and there were no signs of altered splicing. Despite this, the TPMT enzyme activity was 8.7 U/ml pRBC, which is in the intermediate range.

Measurements of TPMT enzyme activity in WBC were performed in samples from the family (results not shown in the figure). The half-sister with TPMT*1 and normal RBC TPMT enzyme activity (**Figure 28**, individual nr 5) had a WBC-TPMT enzyme activity of 1.76 nM meMP/μg total protein and hour and was set to 100%. Compared to nr 5, the

Figure 28. Results of TPMT*44 allele. The substitution of c.497A>G caused the amino acid substitution p.Y166C. The allele was inherited from mother to daughters. In comparison of rTPMT wt, rTPMT p.Y166C has decreased thermal stability. Figure adapted from Paper I.

WBC-TPMT enzyme activities were 54, 59 and 57% for individual nr 1, 2 and 4, respectively. The discrepant individual (nr 3) had 75% TPMT enzyme activity and the TPMT enzyme activity was thereby decreased also in WBC. The TPMT*44 allele caused loss of thermal stabilization, studied by measuring thermal denaturation on rTPMTwt ($49.4 \pm 0.3^{\circ}\text{C}$) and rTPMT p.Y166C ($36.6 \pm 0.4^{\circ}\text{C}$), **Figure 28**.

5.8 Effect of methotrexate on TPMT enzyme function

(Paper III and IV)

MTX is used in combination with 6-MP during the maintenance therapy of ALL and is administrated weekly by oral LD-MTX and HD-MTX infusion pulses. Plasma concentrations of LD-MTX are $0.02\text{--}0.2 \mu\text{M}$ MTX [127] and up to $1 \mu\text{M}$ [128] and in our study, individuals treated with HD-MTX had plasma levels of $84 \mu\text{M}$ (range $57\text{--}102 \mu\text{M}$, 23 hours after start of MTX infusion). Consequently, two different concentrations were used to study the MTX effect on the TPMT enzyme, $90 \mu\text{M}$ to simulate the HD-MTX dose and $0.01\text{--}0.5 \mu\text{M}$ to simulate the LD-MTX dose.

In vivo, comparing the TPMT enzyme activity in 21 children before and 66 hours after HD-MTX infusion, the TPMT enzyme activity had decreased by -9.2% (IQR: -13.0 to $+0.65$).

TPMT enzyme activity in MOLT-4 cells, incubated with HD-MTX ($90 \mu\text{M}$), was increased after 24 hours of incubation and then remarkably decreased with only 26 and 1.8% of the TPMT enzyme activity remaining after 48 and 72 hours, respectively, **Figure 29**.

Figure 29. TPMT enzyme activity in MOLT-4 cells after incubation at 24, 48 or 72 hours with $90 \mu\text{M}$ methotrexate (MTX). Results are shown as a mean percentage of resp. control vehicle (CV) $\pm$ SEM ($n=6$). No significant difference between untreated and CV (NaOH) were detected. Figure from Paper III.

When incubating cell lines with LD-MTX doses, the TPMT enzyme activity in B-lymphoblastic NALM-6 cells was higher than in T-lymphoblastic MOLT-4 cells (27.7 and 9.2 cpm/ μ g total protein during two hours of measurement, respectively). In this study, dead cells were removed after incubation. However, LD-MTX (0.01-0.5 μ M) did not change the TPMT enzyme activity in MOLT-4 or NALM-6 cells, either alone or in combination with 6-MP after 16 or 26 hours of incubation ([Paper IV](#), Figure 2).

The same procedures regarding cell incubations, and following handling of the cells, were used to investigate TPMT gene expression levels, **Figure 30**. The TPMT gene expression was significantly increased using the higher doses of MTX and the longest incubation time in both MOLT-4 and NALM-6 cells.

Figure 30. Gene expression of TPMT in MOLT-4 and NALM-6 cells after incubation at 16 and 26 hours with 0.01-0.5 μ M MTX (red bars), or the combination of both 6-MP and MTX (green bars). (* significant difference between MTX vs 6-MP+MTX). Figure adapted from [Paper IV](#).

For further evaluation of MTX effect on TPMT, MTX binding to rTPMTwt, **Figure 31**, and rTPMT enzyme activity in the presence of MTX were studied. MTX was proved to bind to rTPMT. By monitoring the fluorescence signal from tryptophans during titration of MTX to the rTPMT, a K_d of 24 μM was obtained. In presence of 1, 50, 100 and 200 μM MTX, the remaining rTPMT enzyme activity was 103, 85, 35 and 16% compared to the corresponding CV (DMSO, n=1 of each sample).

Figure 31. Fluorescence signal from tryptophans during titration of MTX to rTPMTwt. Figure from Paper III.

Table 9. Thiopurine metabolite levels (TGN and meTIMP) before and after HD-MTX infusions in ALL treated children. Table adapted from Paper III.

Metabolite	Levels pre-infusion (IQR)	~66 hours % change, IQR	Change versus pre-infusion values
TGN	111.5 ^β (79-271)	-18.1% (-26 to -14.6)	p=0.006, n=15
meTIMP	1,609 ^β (427-3,900)	0% (-20.5 to + 36)	p= 0.917, n=15

^βUnit: $\text{pmol}/8 \times 10^8 \text{ RBC}$. Metabolite levels are normalised to 6-MP dose (mg/day).

5.9 Methotrexates effect on the thiopurine metabolism

(Papers III and IV)

The interaction mechanism explaining the synergistic effect of 6-MP and MTX is not fully understood. The scope of Papers III and IV was to investigate MTX effect on the thiopurine metabolism levels. In both studies TGN and meTIMP metabolite levels were measured. However, in the last study, when measuring the metabolite levels in cell lines, two intermediate metabolites (TIMP and TXMP) were added to further study the MTX effect on the thiopurine metabolism.

The levels of the thiopurine metabolites TGN and meTIMP *in vivo* in RBC, were measured both before and approximately 66 hours after the start of HD-MTX in 15 children during the maintenance therapy, **Table 9**. The TGN level was decreased by 18% and the meTIMP was unchanged.

Figure 32. Cell count after 16 and 26 hours of incubation with MTX (red, A, B, $n=3$) or the combination of MTX and 6-MP (green, C, D, $n=6$). In three measurements of NALM-6 cell count (MTX+6-MP) were start cell concentrations 250,000 cells/ml and the results were normalized to 300,000 cells/ml.

Incubations with LD-MTX concentrations were performed in MOLT-4 (B-lymphoblasts) and NALM-6 (T-lymphoblasts) cells. The cell count after incubation at 16 and 26 h, **Figure 32**, showed the highest cell growth (with similar pattern) in cells treated with CV, 6-MP, 0.01 μM MTX and the combination of 6-MP + 0.01 μM MTX. Increased MTX concentration decreased the cell growth, both alone and in combination with 6-MP. The cell growth seems to be most affected by addition of MTX.

Figure 33. Metabolite levels after 16 and 26 hours of incubation with 6-MP or the combination of 6-MP+MTX (0.01-0.5 μM), $n=3$. ($n=2$). Figure adapted from [Paper IV](#).

Dead cells were removed after incubation. The same number of cells were used in all metabolite samples and the results were normalized by total protein concentration. Metabolites detected (in form of its nucleobases) were TIMP, meTIMP, TXMP and TGN, **Figure 33**. Since 6-MP is needed to form the metabolites, no untreated sample was studied.

In MOLT-4 and NALM-6 cells, **Figure 33**, the metabolite concentrations were 10-fold higher after 26 hours in comparison to 16 hours of incubation. NALM-6 cells produced higher levels of metabolites than MOLT-4.

The metabolite levels appeared to be unaffected by the lowest MTX concentration (0.01 μM) after both 16 and 26 hours of incubation. In both cell lines, TGN metabolite levels decreased in the presence of 0.05 μM MTX or higher concentration. The levels of TIMP and TXMP were affected in a similar pattern as TGN in both MOLT-4 and NALM-6 cells. However, when describing the graphs visually, the metabolite levels in MOLT-4 cells decreased in a more stepwise pattern during increasing MTX concentrations than the levels in NALM-6 cells, which had a dramatic decrease in metabolite levels in the presence of 0.05 μM or higher. The levels of meTIMP appeared to be less affected by MTX than other metabolites.

6. Discussion

For detailed discussions regarding the individual results, the reader is referred to the respective paper.

The research of pharmacogenetics is extensive. With increasing knowledge of the connection between genes, enzymes and metabolic functions and co-interactions it is also possible, in varying extent, to predict the resulting outcome of drug treatment and individualize the treatment before the start of treatment. To use pharmacogenetics successfully, some information regarding the gene is of great value.

- Extent and occurrence of variation in the current gene
- Clinical importance of the variation for the individual
- Possibility to detect the variance in a clinical routine method
- Mechanism of disfunction in the gene/enzyme
- The drug's way in the body and location of drug action
- Drug interactions in the current metabolism and treatment

TPMT was the first and most successful implementation of individualized treatment according to pharmacogenetics [15, 98]. However, this thesis highlights several challenges during thiopurine treatment, which in several ways reflects the general challenges of improved individualized drug treatment today.

6.1 TPMT genotype, phenotype, and clinical methods

The extent and occurrence of genetic variations in TPMT are well-studied and established. The three most common SNPs of TPMT (TPMT*2 [c.238G>C, p.A80P], TPMT*3A [c.460G>A, p.A154T + c.719A>G, p.Y240C] and TPMT*3C [c.719A>G, p.Y240C]) have been known since the mid-1990s [30, 31, 34, 37]. Additionally, many rare TPMT sequence variants, with the same clinical importance for the individual carrier, have been described during the later years. A nomenclature committee [36] was established to keep track of existing polymorphisms of TPMT and today 50 variants of TPMT are described, including the wildtype TPMT*1. Many additional genetic TPMT variants, not named by the nomenclature committee, are available in genetic databases like dbSNP. However, these variants are usually reported from large whole genome studies without specific interest in TPMT and therefore information regarding phenotype and clinical importance is missing. In addition,

many studies published in the TPMT field includes rather small populations and the search for rare or novel variants is far from always included, in both studies and clinics. Paper I in this thesis investigates the largest material of TPMT genotyping-phenotyping correlation so far, including 12,663 individuals. The distribution of individuals carrying TPMT wildtype, heterozygous and homozygous defective alleles are in accordance with previous studies [24, 41] with 90.3% TPMT wildtype (*1/*1), 9.2% heterozygous (*1/*variant) and 0.5% homozygous deficient (*variant/*variant).

The concordance analysis of genotyping vs phenotyping for TPMT status determination was 94.5%. Genotyping is the simplest and most cost-effective method of TPMT status determination, with a high detection rate and because of this, many clinical laboratories are only determining the genotype of the three most common SNPs. However, for the individuals that carry a rare allele [129], the consequences could be serious if their dysfunctional allele is not reported to the clinic. Additionally, the subsequent costs for the provided care could be high. In Paper I, 15 individuals carrying a rare or novel TPMT allele were found by using combined genotyping and phenotyping, and a routine of sequencing all coding TPMT exons if discrepancy between these was identified.

The distribution of TPMT alleles is not the same in different populations (**chapter 1.3.2**) and the relevance of studies of TPMT variants in different populations is decreasing with the increasing population transfer in the world. Therefore, it is questionable if testing only for the most common variants of the largest population of a country is correct. As an example, TPMT*8 has been found in high frequency in two separate African populations [130, 131] and in one African-American population [44] and TPMT*6 was found in two separate Asian populations [42, 132]. Neither of these were detected in our material. Additionally, the fourth most common TPMT allele is TPMT*9, which has been found in other studies [41]. Surprisingly, when it comes to frequency, on a shared fourth place in our material is TPMT*23, which has been found in 4 individuals (to our knowledge not related to each other) but has never been reported elsewhere.

The population in our study was not randomly selected since all were subjects for thiopurine treatment. However, since the natural function of TPMT, strangely enough, is still unknown, only individuals on thiopurine treatment are suffering from defective TPMT enzyme [19, 47] and consequently this group is the most relevant to study. Although,

disease or treatment could influence the results of TPMT enzyme activity measurement (**chapter 5.3**) and these are, together with high labor intensity and costs, the largest disadvantages of phenotyping. In Paper III, the significantly low TPMT enzyme activity in the beginning of the ALL treatment was described, **Figure 19** (p. 61), and it is also seen in the large database material, **Figure 20** (p. 62). This today is established knowledge, explained by lower TPMT enzyme activity in older RBC [133], and the NOPHO ALL treatment is therefore adjusted according to the TPMT genotype.

However, the major advantages of TPMT enzyme activity measurement is that it reflects the actual TPMT function in the body at time of sampling, which should be the factor that is most important for dosing. In addition, the functional consequence of all defective TPMT variants is detected and the method could separate the phenotype of genetic haplotype variants (like TPMT*1/*3A and TPMT*3B/*3C). The clinical TPMT enzyme activity measurements in this thesis were performed in RBC [125]. Other methods are measuring the TPMT enzyme activity in whole blood [134], which for better or worse also includes drugs which could be inhibiting the TPMT enzyme activity. Proposed inhibitors for TPMT are, for example, benzoic acid derivatives, salicylic acid, diuretics, NSAIDs and the antibiotic trimethoprim-sulfamethoxazole [135-143]. However, by performing both genotyping and phenotyping and interpreting the combined result, many of the separate method disadvantages could be eliminated and all defective alleles would be found.

Even if many clinicians are aware of the importance of TPMT status determination, thiopurine treatments that start without knowledge of TPMT status still occur. Out of 122 gastroenterologists in Sweden in 2011 [97], 74% did normally adjust thiopurine dosage according to TPMT status. However, 69% did not wait for the result before starting the treatment. In other studies, TPMT status usage in thiopurine treatment has been reported to be between 35-60% [97]. Because of this, unnecessary serious ADRs occur, as exemplified by a case report from the Netherlands in 2019 [144] where a patient got AZA and later on 6-TG without previous TPMT status determination. The patient became severely ill, was hospitalized for 15 days, and needed treatment for 6 weeks. TGN level in the range of 10-fold higher than recommended levels was detected in addition to genotype TPMT*3A/*3A. These kinds of events can, with today's knowledge of pharmacogenetics, be prevented and save both suffering and costs by simply ordering an TPMT status test before the start of treatment.

6.2 Discrepancies and factors influencing the activity

Recently, two large GWAS studies confirm that no factors other than TPMT genotype are connected to TPMT enzyme activity [145, 146]. However, other factors influencing TPMT enzyme activity are proposed now and then and sometimes prove to be significant. Many TPMT researchers state that there could be additional factors affecting TPMT enzyme activity, mostly because of the large variability in enzyme activity (five-fold in individuals defined as wildtype [147]) and several patients' analysis results that do not comply [41, 145].

In the results of [Paper I](#), **Figure 17** (p. 59) it is obvious that other factors influence TPMT enzyme activity due to the overlap between wildtype, heterozygous and homozygous groups. Of the samples from 12,663 individuals, 695 (5.5%, before extended sequencing) were not correlating in their genotype and phenotype. Both too high and to low TPMT enzyme activity in accordance to genotype was found, **Figure 22** (p. 65).

In addition to previously described factors, blood transfusions are established to possibly cause false enzyme activity due to the donors' TPMT enzyme activity [148]. Levels of the cofactor SAM have been shown to correlate with TPMT enzyme activity [149, 150], most pronounced in heterozygotes [151], and variations in a gene involved in SAM production (MTHFR) [152-154] have been connected to TPMT enzyme activity.

In other DMEs, extensive metabolizer alleles and ultra-rapid phenotypes exist, depending on several copies of the enzymes arranged as tandem repeats [11, 13, 155]. In our data cohort, also found in other studies [41, 156-158], a few individuals have nearly four times higher TPMT than normal (up to 40 U/ml pRBC). It has been discussed if these individuals may have advantages of higher than standard thiopurine doses, not risking undertreatment [28]. Other regulatory mechanisms causing varying enzyme activity are variations in the promotor or enhancer regions [11, 159, 160]. In TPMT, variations in the promotor region [33, 158, 161] VNTR have sometimes been correlated to TPMT enzyme activity.

Age and gender have been discussed as additional factors influencing TPMT enzyme activity and studies showing both significant differences as well as none are reported [125, 145, 157, 162-165]. In [Paper I](#) we were able to find correlation between TPMT enzyme activity and gender, where males have significantly higher activity than females. Further,

when dividing the results according to genotypes, there was also a connection between activity and age. As seen in **Figure 23** (p. 66) the largest differences within age and gender occur in wildtype individuals. However, the differences are still small, differing up to approximately 2 U/ml pRBC between young and old individuals and 1 U/ml pRBC between males and females. The reasons behind these associations not being detected in other studies could depend on small differences between the factor groups in combination with small sample populations. It is doubtful whether these small differences are of clinical importance.

So far, no alleles other than exon SNPs and a few intron SNPs close to intron/exon borders are established to have significant effect on TPMT enzyme activity and to be of clinical importance, although it is likely that other kinds of factors affect. One could speculate that the undiscovered factors causing the discrepancies probably derive from different rare variants outside of the exons and possibly, they are more or less pronounced in combination with other factors, making them very hard to detect.

Even though many thiopurine treatments today are adjusted to TPMT status, there are still individuals with ADRs and unexplained reactions [19]. TPMT is likely not the only gene influencing thiopurine treatment. When this PhD project started, a few studies concerning varying enzyme activity or the connection between varying genotypes and adverse reactions in other thiopurine metabolizing enzymes had been published. Throughout these years, the complexity of thiopurine metabolism has been more extensive with reports of several polymorphisms within the metabolism of thiopurines (ITPA [166, 167], HGPRT [142, 168], IMPDH [168-170], XO [60, 171], GST [172-174], GMPS [168], Rac1 [175], NT5C2 [176, 177], PACSIN2 [178], genes in the mismatch repair system (MMR) [179], folate cycle [152-154] and transporters [56, 175, 180-182]) and recently, pharmacogenetic testing of NUDT15, whose variants are more common in Asian populations, has been implemented in the guidelines of the Clinical Pharmacogenetics Implementation Consortium (CPIC) for thiopurine treatment [19, 183]. Otherwise, so far, no other polymorphism has had the same extensive effect on thiopurine treatment as the genetic variants of TPMT.

6.3 Biophysical studies of low activity mechanisms

Most studies regarding TPMT have focused on the clinical aspects like frequency of genetic variants, population studies of TPMT enzyme

activity, TPMT in relation to outcome, and adverse reactions. In the clinic, a TPMT allele is often described as functional or not. However, the enzymatic activity of individual variant TPMT alleles varies from 0-100% [184, 185] which influences the treatment response. An additionally important area, that is less studied than the clinical part, is the mechanisms or causes of the low TPMT enzyme activity. These studies are even more important when phenotyping methods are replaced by genotyping. To be able to recommend thiopurine dose adjustments of rare TPMT variants, its resulting function must be known [186].

The most common and studied effect of SNPs found in TPMT are single amino acid substitutions disturbing TPMT enzyme structure and stability [105, 187, 188]. Decreased quantities of TPMT protein have often been the explanation for low enzyme activity of defective TPMT alleles [15] and correlations between protein amount and enzyme activity for most TPMT*1-13 have been reported [184]. TPMT enzyme activity measurement is not only a measurement of the efficiency of the singular TPMT enzyme, it is also a combined effect of the number of TPMT enzyme molecules available. Therefore, stability measurements are of importance to detect deviating half-life of a TPMT enzyme variant. Other suggested effects on the TPMT enzyme caused by SNPs are increased aggregation formation, lower mRNA or enzyme expression and post-translational stability [149, 189-193].

In addition, all mechanistical studies increase knowledge of the enzyme structure and function and the connection there in between. This knowledge is important in future research for drugs and the usage, production and modification of recombinant enzymes which today are used for DNA technology, energy and environmental issues, food and pharmaceutical industries [194, 195].

In this thesis, four different SNPs were described and characterized. What first looks like a simple nucleotide substitution could affect the enzyme in several different ways and interestingly, all four genetic TPMT variants described in this thesis had their own unique mechanism causing decreased function, **Table 10**.

We know that TPMT has a large and flexible active site since several substrates and inhibitors have been identified. This fact might also strengthen the so far established hypothesis of TPMT's natural function, to neutralize toxic substances in the body (**chapter 1.3.3**). However, a flexible active site also implies that the risk of drug interactions is

increased. In addition to pharmacogenetics, drug interactions are influencing the metabolism and treatment effect of drugs.

Table 10. Summary of characterized TPMT single nucleotide polymorphisms and their consequences.

TPMT allele	Enzyme activity ^a	Nucleotide substitution	Protein consequence	Cause of low enzyme activity
*41	7.7	c.719A>C	p.Y240S	Decreased protein stability and weakened interaction with cofactor SAM
*42	0.5- 0.6	c.95_96insA	p.K32KfsX58	Insertion of extra A caused nonsense amino acid sequence and premature stop codon
*43	7.5 - 8.5	c.243G>T c.262G>A	del. exon V	Deviating splicing pattern, deletion of exon V caused an unfunctional enzyme
*44	2.0 - 2.5	c.497A>G	p.Y166C	Single amino acid substitution decreased the stability of the resulting protein

^a U/ml pRBC

6.4 Drug interaction - Methotrexate and 6-MP

In the NOPHO-ALL treatment protocol, thiopurines are often administered together with other chemotherapy drugs or antibiotics (due to infections caused by the suppressed immune system). The drugs 6-MP and MTX, are combined during the 2.5-year maintenance treatment and have been used successfully for almost 70 years [20]. However, the mechanism behind the synergistic effect of 6-MP and MTX is still today not fully understood. However, the focus of this thesis was mainly to study the effect on 6-MP metabolism in presence of MTX, rather than the synergistic cytotoxic treatment effect of the combination.

In Paper III, TPMT and thiopurine metabolites were measured before and after HD-MTX infusions *in vivo*. Levels of TGN metabolites decreased (-18%) and levels of meTIMP were not changed after HD-MTX. Additionally, TPMT enzyme activity was decreased (-9%) during this time. In the same paper, these findings were confirmed in

lymphoblastic MOLT-4 cells treated with HD-MTX (90 μ M), where TPMT enzyme activity was decreased after 48 and 72 hours. In addition, MTX binds to rTPMT in a clinically relevant concentration ($K_d=24 \mu$ M) and decreases the enzyme activity of rTPMT.

However, even though the same amount of total protein was used in the cell line study, [Paper III](#), a large proportion of dead cells was observed and to decrease the risk of the cell death influencing the results in [Paper IV](#), only living cells after incubation were used for studies regarding TPMT enzyme activity, gene expression, and metabolite levels. In addition, both T- and pre-B-ALL cell lines (MOLT-4 and NALM-6) were treated with the combination of 6-MP and MTX in the range of LD-MTX (0.01-0.5 μ M) and the same number of cells and/or total protein or RNA were used during sample preparation.

Using the LD-MTX concentrations and incubation times of 16 and 26 hours, there was no MTX effect on TPMT enzyme activity. Interestingly, gene expression of TPMT was increased during the combined incubation. The effect on thiopurine metabolite levels (TIMP, TXMP, TGN and meTIMP) was pronounced. All metabolite levels decreased with increasing MTX concentrations, **Figure 33** (p. 78). However, no visual difference was observed between only 6-MP treatment and the addition of the lowest MTX concentration (0.01 μ M). This was in line with the findings of cell growth curves (before sorting out dead cells), **Figure 32** (p. 77), where the growth of MOLT-4 and NALM-6 in presence of the lowest MTX concentration were similar to the growth of only 6-MP treated cells and CV, while the growth was decreased by adding higher MTX concentrations.

What was particularly interesting and new in [Paper IV](#) were the levels of the not commonly studied intermediate metabolites TIMP and TXMP, whose pattern of decrease was contradictory to previous theories of 6-MP – MTX interaction, **Figure 34**. MTX's inhibition of XO should lead to increased metabolite levels. Lack of ATP due to inhibition of PDNS and thereby decreased GMPS activity (cofactor ATP) [56] should accumulate TXMP [55, 87]. Previous studies have found elevated levels of PRPP after combined 6-MP+MTX treatment [68, 127, 196, 197] (explained by inhibited PDNS) and proposed increase of HGPRT conversion (cofactor PRPP), thereby increasing the levels of TIMP. However, none of these expected increases in thiopurine metabolite levels were visible in our results, **Figure 33** (p.78). Other mechanisms, as for instance the previously reported MTX inhibition of HGPRT [198, 199], may deserve further attention. In addition, meTIMP is detected

Figure 34. Simplified figure of the thiopurine (6-MP) metabolism and established mechanisms of action of MTX. In the figure are the four measured thiopurine metabolites included; TIMP, meTIMP, TXMP and TGN. For abbreviations, see the last page. Figure adapted from [Paper IV](#).

together with meMP (**chapter 4.12.3**) in our, and most other, methods used for thiopurine monitoring, which could make these results harder to interpret.

Our data regarding the effect on 6-MP metabolism in presence of MTX are interesting and encourage further research to elucidate all the effects of the successful combination treatment. Why the decrease in TGN is beneficial to the treatment remains to be explained and it seems to be in conflict with reports of low levels of TGN in patients being connected to worse outcomes in ALL treatment [55, 76, 200, 201]. Individuals with low TPMT enzyme activity due to heterozygosity have lower rates of relapse than TPMT wildtype individuals, and are shown to have higher intracellular TGN levels and lower risk of positive MRD after the consolidation phase of the treatment [202]. Higher TPMT enzyme activity has been reported to be connected to increased risk of relapse [200, 202] and speculated to depend on high meTIMP levels inhibiting the PDNS and making lymphoblasts rest in the S-phase and regrow later after treatment has finished. However, MTX and MTXPG are also strong inhibitors of the PDNS. The disadvantages of decreased thiopurine levels

could be compensated by the beneficial combination of inhibiting both PDNS and the purine salvage pathway [203] and all effects that MTX exerts by itself (inhibition of PDNS, formation of MTXPG with even higher inhibitory effects and disruption of the folate cycle). In addition, the decrease in thiopurine metabolite levels seems to recover rapidly after MTX infusion [85].

Interestingly, the results in Papers III and IV are contradicting. The hypothesis about this is that the difference is a matter of concentration of LD- and HD-MTX, and maybe also treatment duration. It could be that the MTX doses in Paper IV were too low to affect TPMT enzyme activity. It is also possible that the MTX-inhibition of TPMT enzyme activity in MOLT-4 cells in Paper III appeared to be more pronounced due to cytotoxic effects. Another proposed mechanism for TPMT inhibition, involving inhibition of XO, is 6-MP conversion into TX by aldehyde oxidase [204]. TX is known to inhibit TPMT ($K_i=329 \mu\text{M}$ [204]), although it is weaker than K_d of MTX in Paper III. If MTX only binds to rTPMT under perfect conditions and not *in vivo*, this could be an alternative mechanism explaining our *in vivo* results of decreased TPMT enzyme activity in Paper III.

More research regarding MTX's effect on thiopurine metabolism and the difference between LD- and HD-MTX is needed to elucidate all its effects. Both ours and other studies indicate that MTX could have several more mechanisms than we know of today [196, 198, 205].

6.5 Method considerations of study matrices

Study matrix is another factor that could influence the result of pharmacogenetic studies. In this thesis, *in vivo* studies with measurements using RBC, cell line studies of lymphoblastic cells *in vitro*, and recombinant expressed protein were used to study our research issues. *In vivo* studies are the closest to the real situation in the human body. However, depending on in which matrix the mechanism of action takes place, the availability could be more or less easy to obtain and to ethically motivate sampling.

TPMT enzyme activity in several matrices, whole blood, RBC, liver or kidney tissue, or leukemic blasts, reflect one other [206-211] even if the expression levels differ with higher TPMT enzyme levels in liver tissue. Cell lines are a commonly used substitute, including in this thesis, where

the metabolism could be executed by the cell and the mechanisms could be studied in the main target, which in ALL research is the lymphoblasts.

However, the metabolism of thiopurines is mainly performed in the liver [145], and here some complicated issues arise since different cells or tissues do not express the same levels of enzymes or do not express all enzymes needed for thiopurine metabolism. For instance, RBC lack IMPDH [100]. Deviating intracellular levels of competing substrates could also affect metabolism. Still, the measurement of TGN *in vivo* is performed in RBC and these metabolites are claimed to mainly come from the hepatic metabolism. The levels of metabolites in RBC are consequently additionally dependent on the transportation system, making RBC levels more likely to be displaying the integration of metabolites rather than the effect on target tissue [100]. In any case, this could be valuable information as well.

In addition, different ways of drug administration could affect metabolism *in vivo*. XO is expressed in large extent in the liver and intestine and is mainly responsible for the first pass metabolism of 6-MP. Due to high amount of XO in intestine, bioavailability of 6-MP after oral administration is low [47] and is increased with intravenous administration. In addition, the expression of XO in lymphoblasts is limited [56].

With that in mind, the results of cell line studies are complicated. On one hand, lymphoblasts express enzymes needed for the conversion of thiopurines into active metabolites and it is the target matrix in leukemia. Therefore, it is an excellent model of how these cells handle the metabolism and cytotoxic effects from metabolites. On the other hand, lymphoblastic cell lines lack the complex interplay with all surrounding matrices with different capacities and transports between cells. The *in vivo* and *in vitro* situations could therefore be greatly deviating, thus not saying that cell lines are not strong and useful tools in drug research. Studies on pre-B- and T-lymphoblasts have for example given valuable information about why these cells respond differently to treatment by investigating deviating gene expressions and pathways [212]. Expression of TPMT variant alleles in COS-1 cells has shown possible mechanisms of low quantification of TPMT protein by proteasome-mediated degradation [190, 191, 193] or aggregate and aggresome formation [192]. However, the limitations of cell line studies are important to consider.

Studying recombinant enzymes is even more focused on the single enzyme and its properties and interactions. This method can give us functional information, which is not possible to get from cell systems where many factors interact. Here it is important to remember where the enzyme occurs naturally, what substances are available in the matrices, and in what concentrations. The interaction between MTX and TPMT had a K_d of 24 μM . This could be compared to K_m of 6-MP 10.6 μM , 6-TG 18.1 μM and TIMP 25.7 μM , studied in yeast expressed rTPMT [122], and with our K_d of SAM 2.7 μM , Paper II. More valid comparisons are made within the same expression system. Since TPMT has several substrates and inhibitors, there could be competitive factors making MTX not bind *in vivo*. By modifying the recombinant TPMT according to the amino acid substitutions found *in vivo*, the interaction with substrates could be thoroughly studied, as described in Paper II.

7. Conclusions

From the results of Papers I-IV, the conclusions are:

- ❖ The concordance between genotyping and phenotyping was high (94.5%). Despite this, the combination of genotyping and phenotyping is recommendable in clinical TPMT status determination, in order not to exclude rare or novel defective TPMT alleles (Paper I).
- ❖ The mechanisms causing decreased TPMT enzyme activity varies between the different defective alleles (Papers I, II).
- ❖ Factors such as gender, age, and hematological disease affects TPMT enzyme activity levels (Papers I, III).
- ❖ MTX binds to recombinant TPMT and increases TPMT gene expression *in vitro* and decreases TPMT enzyme activity *in vitro* and *in vivo*. However, the interaction seems to be dose- and time-dependent (Papers III, IV).
- ❖ Thiopurine metabolism is influenced by the combined incubation of 6-MP and MTX. The presence of MTX makes TGN levels decrease *in vivo* and levels of TIMP, TXMP, and TGN decrease *in vitro*. These results challenge the established metabolism mechanisms of thiopurines and MTX (Papers III, IV).

8. Future aspects

Clinical usage of pharmacogenetics is a powerful tool to both decrease the individual's suffering and costs for the society. Treatment according to genotype will be more common in the future. In addition, the costs of whole genome sequencing have dramatically decreased in recent years, opening for standard investigation of patients.

Research on TPMT is today focused on the exon variation of TPMT, probably because it has been enough for a huge positive effect when implemented in the clinic. Further investigation of promotor sites and enhancer regions, intron regions and variance in splicing and expression levels is needed in the search for explanations of the discrepancies. If patients' whole genomes are sequenced routinely in the future, there will be more information regarding TPMT and also rare and novel TPMT alleles will be found, making phenotype and clinical information regarding these alleles important. The data from not exon coding parts of TPMT would be even more interesting to compare with cDNA analyses to be able to see the transcription consequences of splicing and mRNA levels. Since this is also possible to study in a simple blood sample from healthy individuals, there should be much information available in the coming years. However, more data regarding other polymorphic enzymes in the metabolism and combinations of these will be challenging to interpret.

Concerning the combined treatment of 6-MP and MTX, we know that it works excellently and the positive outcomes after ALL-treatment is very good. Continuously, much research is performed to further optimize the protocols. To further investigate and understand mechanisms of combined 6-MP and MTX treatment would be valuable for further individualized treatment.

Of course, other common drug combinations used during thiopurine treatment are of importance to evaluate. Except for *in vivo* and *in vitro* cell line and recombinant studies, animal models or liver microsomes may be used for this purpose.

The cause of the skewed metabolizers, most occurring as problems during IBD treatment (**chapter 1.3.6**), needs to be explained. Also, here, whole genome studies will give valuable pieces to this mysterious metabolism phenomenon. If its cause is found, other mechanisms of the

thiopurine metabolism will probably follow, perhaps questioning some of the today's established metabolism pathways.

Studies of DNA-TGN, the levels of incorporated TGN in DNA, have so far been promising and it will be very interesting to follow the development of DNA-TGN as a target factor for thiopurine treatment. This is also a tool for other interesting studies, like drugs' effect on incorporation levels as well as polymorphisms in enzymes regulating this.

9. References

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11. Appendix

- ❖ Paper I
- ❖ Paper II
 - Supplemental data (Paper II)
- ❖ Paper III
- ❖ Paper IV
 - Supplemental data (Paper IV)
- ❖ Summary of the thiopurine pathway

Papers

The papers associated with this thesis have been removed for copyright reasons. For more details about these see:

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→ *The author's summary of the thiopurine metabolism and its interactions with the purine salvage pathway and the methotrexate targets; the folate cycle, the methionine cycle and the purine de novo synthesis.*

Yellow/orange: drugs; blue: enzymes; green: cofactors; dotted lines indicate inhibition. **Abbreviations:** **6-MP**, 6-mercaptopurine; **6-TG**, 6-thioguanine; **AICAR**, 5-Aminoimidazole-4-carboxamide ribonucleotide; **ADA**, Adenosine deaminase; **AMP**, adenosine monophosphate; **ATIC**, aminoimidazolecarboxamide ribonucleotide formyltransferase; **ATP**, adenosine triphosphate; **AZA** azathioprine; **d**, deoxy; **DHF**, dihydrofolate; **DHFR**, dihydrofolate reductase; **FGAR**, formyl GAR; **FAICAR**, formyl AICAR; **GAR**, glycinamide ribonucleotide; **GART**, glycineamide ribonucleotide transferase; **GMP /GDP/GTP/dGDP/dGTP**, (deoxy) guanosine-mono/di/triphosphate; **GMPR**, guanosine monophosphate reductase; **GMPS**, guanosine monophosphate synthetase; **GST**, glutathione S-transferase; **Hcy**, homocysteine; **HGPRT**, hypoxanthine-guanine phosphoribosyl transferase; **Hx**, hypoxanthine; **IMPDH** inosine monophosphate dehydrogenase; **ITP**, inosine triphosphate; **ITPA**, inosine triphosphatase; **me**, methyl; **meMP**, methyl mercaptopurine; **me(T)IMP**, methyl (thio) inosine monophosphate; **Met**, methionine; **MTHFR**, methylene tetrahydrofolate reductase; **MTR**, methionine synthase, **MTRR**, methionine synthase reductase; **MTX**, methotrexate; **NAD⁺** nicotinamide adenine dinucleotide; **NT5C2**, 5'-nucleotidase cytosolic II; **NUDT15**, nudix hydrolase 15; **PPAT**, phosphoribosyl pyrophosphate aminotransferase; **PRPP**, phosphoribosyl pyrophosphate; **SAH**, S-adenosyl-L-homocysteine; **SAM**, S-adenosyl-L-methionine; **Rac1**, Ras-related C3 botulinom toxin substrate 1; **T**, thio; **THF**, tetra-hydrofolate; **TdGMP / TdGDP / TdGTP / TGMP / TGDP / TGTP**, thio-(deoxy)-guanosine-mono/di/triphosphate (=TGN, thioguanine nucleotides); **dTMP/dTDP/dTTP**, deoxythymidine mono/di /tri phosphate; **TPMT**, thiopurine-S-methyltransferase; **(T)UA**, (thio) uric acid; **TYMS**, thymidylate synthetase; **dUMP**, deoxyuridine monophosphate; **(T)XMP**, (thio)xanthine monophosphate; **XO**, xanthine oxidase.

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