Pharmacogenetic studies of thiopurines – focus on thiopurine methyltransferase

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To my family

Johan
Mamma & Pappa
Magnus, Tina, Josefine & Viktor
- En del avhandlingsprojekt är som en straffspark utan målvakt....... 

.......men kräver ju ändå en duktig straffläggare och tre domare -

"Anonymous"

- Det du gör, gör fullt och helt, icke styckevis och delt-
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ABSTRACT

Pharmacogenetics represents the study of variability in drug response due to genetic variations. The thiopurines (6-mercaptopurine, 6-thioguanine and azathioprine) are prodrugs which require metabolic transformation to exert effect. Thiopurines are used in inflammatory bowel disease, as maintenance treatment of childhood acute lymphoblastic leukaemia, and for immunosuppression after transplantation. The metabolism is complex and one important enzyme involved is thiopurine methyltransferase (TPMT). Inherited variation in TPMT activity is one factor responsible for individual differences in susceptibility to thiopurine-induced toxicity or in the therapeutic response to thiopurines. The enzyme activity is under control of a genetic polymorphism. The frequency distribution of TPMT activity in Caucasians is trimodal, with 89% having high enzyme activity, 10% having intermediate activity and 1 of 300 having almost undetectable activity. The TPMT gene has been characterised and several single nucleotide polymorphisms (SNPs) identified causing decreased enzyme activity. The most common SNPs are TPMT*2, TPMT*3A and TPMT*3C.

In the investigations for this thesis we have studied the pharmacogenetics of thiopurines with focus on TPMT. A real-time RT-PCR method was developed for quantification of TPMT gene expression, and a pyrosequencing method was developed for genotyping of TPMT SNPs. TPMT gene expression correlated to enzyme activity in individuals with high enzyme activity. The allele frequencies of TPMT*3A and TPMT*3C in samples from 800 Swedish individuals were in agreement with those in other Caucasian populations, although TPMT*3B was more common and TPMT*2 was rarer.

We investigated the concordance between genotype and phenotype and found discordance between genotype and phenotype in two unrelated patients. In these patients, we detected two new sequence variants,
*TPMT*14 and *TPMT*15, which lead to a non-functional TPMT enzyme. The *TPMT* genotype and phenotype were also determined in the parents of the two patients and the inheritance of these alleles was investigated.

Sixty patients with inflammatory bowel disease following a standardised dose escalation schedule of azathioprine or 6-mercaptopurine were closely monitored over the course of 20 weeks. During treatment, the *TPMT* gene expression decreased. In contrast, TPMT enzyme activity did not change. *TPMT* heterozygous patients had a lower probability of remaining in the 20-week study. Forty-five percent of the patients were withdrawn due to adverse events, but 67% of these tolerated a lower dose of thiopurines. The inosine triphosphate pyrophosphatase polymorphism (*ITPA* 94C>A) was not associated with occurrences of adverse events.

Konstruktionsansvisningen för enzymer och andra proteiner ligger i vår arvsmassa (DNA). Man brukar säga att DNA kodar för olika enzymer och proteiner. Man kan mäta aktiviteten av enzymet TPMT, detta kallas fenotypning. Om man har en ärfilig defekt i genen för TPMT, så får man mindre mängd TPMT-enzym än andra människor. En sådan defekt finns hos ungefär 10 % av befolkningen och dessa människor får vid behandling med tiopuriner en ökad risk för biverkningar. De förändringar man hittar i gener kallas mutationer. De vanligaste mutationerna i TPMT-genen kallas TPMT*2, TPMT*3A och TPMT*3C.

I våra studier har vi utvecklat en molekylärbioligisk metod för att i detalj studera TPMT-genen, så kallad genotypning. Vi har undersökt TPMT-genen i DNA från 800 svenskar och sett att de mutationer som är vanliga i andra befolkningar i Europa och Nordamerika också är vanliga i Sverige. Vi har även hittat två helt nya mutationer; TPMT*14 och TPMT*15, vilka leder till sänkt TPMT-enzymaktivitet. Hos 90 % av befolkningen kan man inte finna några genetiska defekter i TPMT-genen. De har hög enzymaktivitet, men ändå skiljer sig aktiviteten åt mellan olika
människor inom denna grupp. För att undersöka om detta berodde på
omskrivningen från TPMT-genen till enzym, utvecklade vi en
molekylärbiologisk metod för att mäta budbärar-RNA (mRNA). Vi
studerade bland annat hur TPMT-mRNA nivåer förändrades under
behandling med tiopuriner hos patienter med inflammatorisk
tarmsjukdom, och fann att nivåerna sjönk under behandlingens gång. Däremot kunde vi
inte se att TPMT enzymaktiviteten förändrades under behandlingen. Vi
fann att patienter med sänkt enzymaktivitet som hade en TPMT-mutation
(TPMT*3A), fick biverkningar tidigare under behandlingens gång. Dessa
patienter blev tvungna att avsluta sin tiopurinbehandling eller att få dosen
sänkt.

Sammanfattningsvis lägger avhandlingen en grund för bättre möjligheter
att individualisera behandlingen av patienter med inflammatorisk
tarmsjukdom och barnleukemi.
This thesis is based on the following papers, which will be referred to by their Roman numerals:


ABBREVIATIONS

The most important abbreviations used in this thesis are listed below:

AZA  Azathioprine
DNA  Deoxyribonucleic acid
cDNA  complementary DNA
HGPRT Hypoxanthine guanine phosphoribosyl transferase
HPLC High-performance liquid chromatography
huCYC Human cyclophilin
IBD  Inflammatory bowel disease
IMPDH Inosine monophosphate dehydrogenase
ITPase Inosine triphosphate pyrophosphatase
ITPA gene Inosine triphosphate pyrophosphatase gene
6-MP  6-Mercaptopurine
meMP methyl mercaptopurine
meTG methyl thioguanine
meTIMP methyl thioinosine monophosphate
MTX  Methotrexate
PCR  Polymerase chain reaction
PDNS Purine de novo synthesis
RBC  Red blood cells
RT-PCR Reverse transcription polymerase chain reaction
RNA  Ribonucleic acid
RIN  RNA integrity number
SAM  S-adenosyl methionine
SNP  Single nucleotide polymorphism
cSNP coding single nucleotide polymorphism
6-TG  6-Thioguanine
TGMP Thioguanosine monophosphate
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>TGDP</td>
<td>Thioguanosine diphosphate</td>
</tr>
<tr>
<td>TGTP</td>
<td>Thioguanosine triphosphate</td>
</tr>
<tr>
<td>TGN</td>
<td>Thioguanine nucleotides</td>
</tr>
<tr>
<td>TIMP</td>
<td>Thioinosine monophosphate</td>
</tr>
<tr>
<td>TPMT</td>
<td>Thiopurine methyltransferase</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number of tandem repeats</td>
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<td>XO</td>
<td>Xanthine oxidase</td>
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INTRODUCTION

It is well recognised that different patients respond in different ways to the same medication. Many non-genetic factors influence drug effects, including age, kidney and liver function, as well as will concomitant therapy interactions and the nature of the disease. However, there are now numerous examples of cases in which inter-individual differences in drug response are due to sequence variants in genes encoding drug-metabolising enzymes, drug transporters or drug targets (Ingelman-Sundberg, 2001a; 2001b; Evans & McLeod, 2003). Pharmacogenetics represents the study of variability in drug response due to genetic variations or heredity (Vogel & Motulsky, 1986; Weinshilboum, 1989b; Eichelbaum & Evert, 1996; Linder et al., 1997).

Genome variation

A genetic polymorphism defines a monogenic trait that exists in the population in at least two phenotypes (and presumably two genotypes), neither of which is rare, i.e., the rarest phenotype still occurs at a frequency of more than 1% (Vogel & Motulsky, 1986; Meyer, 1991). Most genetic polymorphisms involve single nucleotide differences and are commonly referred to as single nucleotide polymorphisms (SNPs, Kruglyak & Nickerson, 2001). SNPs in the coding regions (cSNPs) are rare, and have been estimated to 1/50000-100000 bases (Ingelman-Sundberg, 2001a). SNPs which alter the DNA sequence involved in gene regulation or the amino acid sequence of a protein are likely to contribute significantly to a number of diseases, as well as inter-individual variations in response to drug therapy (Meyer & Zanger, 1997; Cargill et al., 1999).
Genetic polymorphisms in drug-metabolising enzymes

Genetic polymorphisms affecting pharmacotherapy were first discovered in the 1950s by incidental observations that some patients or volunteers experienced unpleasant and disturbing adverse events when given standard doses of drugs (Vogel & Motulsky, 1986; Meyer, 2000; 2004). For example, haemolysis caused by anti-malarials was recognised as being caused by inherited variants of glucose-6-phosphate dehydrogenase (Meyer, 2000).

Genetic polymorphisms in drug-metabolising enzymes give rise to distinct phenotypes in their ability to metabolise drugs or foreign compounds, often called poor, extensive or ultrarapid metabolisers, the latter having increased enzyme activity relative to the normal (extensive) metaboliser. The poor metaboliser phenotype is monogenetically inherited as an autosomal recessive trait and is due to a complete absence or marked decrease in the amount and/or activity of the enzyme. The clinical consequences of genetic polymorphisms in a drug metabolising enzyme can be either toxicity in poor metabolisers, or inability of reaching therapeutic plasma concentrations in the case of ultrarapid metabolisers, due to fast elimination of the drug (Bertilsson et al., 1993; Dahl et al., 1995).

Genetic polymorphisms have been described for several drug-metabolising enzymes, for example the debrisoquine (or CYP2D6) polymorphism, the acetylation (NAT2) polymorphism, and the mephenytoin (CYP2C19) polymorphism (Meyer & Zanger, 1997; Evans & Relling, 1999). Thiopurine methyltransferase (TPMT) is a polymorphic enzyme (Weinshilboum et al., 1978; Weinshilboum & Sladek, 1980), of great clinical importance regarding effects and occurrences of adverse events in individuals treated with thiopurines (Lennard et al., 1989; Evans et al., 1991; Krynetski et al., 1996). Methyl conjugation is an important pathway in the biotransformation of many drugs, neurotransmitters and xenobiotic compounds (Weinshilboum, 1988; 1989b). The TPMT polymorphism has been considered to be one of the best models for the translation of genomic information to guide treatment of patients (McLeod & Siva, 2002).
Phenotyping and genotyping

Phenotype and genotype determinations are pharmacogenetic tools for the detection of variability in drug metabolism (Linder et al., 1997; Iyer, 1999). Phenotyping has traditionally been determined in vivo by administration of a probe drug (the metabolism of which is solely dependent on a specific enzyme) and subsequent measurements of metabolites in plasma or urine (Innocenti et al., 2000). Phenotyping can also be performed in vitro, as is used for phenotyping of the methylating enzyme TPMT investigated in this thesis. Phenotyping in vitro is accomplished by direct measurement of enzyme activity, generally in surrogate tissues or peripheral blood cells (Weinshilboum et al., 1978; Innocenti et al., 2000).

An alternative methodology to phenotyping is genotyping, which is the genetic analysis of functionally important polymorphisms in the gene encoding a specific protein (Linder et al., 1997). Genotyping has many practical advantages over phenotyping, since it only has to be performed once and requires a small volume of DNA-containing material, such as blood or saliva. Many types of PCR-based methods for genotyping exist, such as allele-specific polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (PCR-RFLP), single-strand conformation polymorphism (SSCP), etc. Newer methods that require less sample preparation and have higher sensitivity and increased throughput have been developed and optimised.

Even if genotyping shows many advantages, phenotyping might be more representative of the current protein function in the patient. On the other hand, for determination of enzyme activity in red blood cells (RBC) from individuals that have received blood transfusions, genotyping is the golden standard, as the phenotype measured reflects both the patient and the blood donor (Yates et al., 1997). The genotype is not affected by other factors such as concomitant therapy or blood transfusions. However, one must always consider the possible existence of unknown sequence variants.
Thiopurines

History

In the mid 1940s, the knowledge about the nucleic acids and the helical structure of DNA was still rather poor. The theory was that there were two purines and two pyrimidines building the DNA strand, strung together in some unknown fashion. George H. Hitchings theorised that, since all cells required nucleic acids, it might be possible to stop the growth of rapidly growing cells (for example bacteria and tumours) with antagonists of the nucleic acid bases (Elion, 1989). Methodology using the bacteria Lactobacillus (L.) casei was developed which made it possible to determine whether a compound could substitute for the natural purines or if it was an inhibitor of its utilisation (Elion, 1989).

In 1948, Gertrud B. Elion, together with Hitchings, found that 2, 6-diaminopurine (later known as thioguanine, 6-TG, Figure 1) inhibited the growth of L. casei and tumours in mice. 6-TG is the thio-analogue of endogenous guanine. It produced good clinical remission in chronic granulocytic leukaemia in two adults, but produced severe nausea and vomiting, as well as bone marrow depression (Burchenal et al., 1951).

It was concluded that the substitution of oxygen by sulphur at the 6-position of guanine and hypoxanthine (for example 6-mercaptopurine, 6-MP) produced inhibitors of purine utilisation. After testing over 100 purines in an L. Casei screen, 6-MP and 6-TG were found to be active against a wide spectrum of rodent tumours and leukaemias. 6-MP was especially interesting (Clarke et al., 1953), and rapidly introduced in clinical trials in children with acute leukaemia (Burchenal et al., 1953). At that time, methotrexate (MTX) and steroids were the only drugs available for the treatment of these terminally ill children. The addition of 6-MP, increased the median survival time from 3 to 12 months. Already two years after the synthesis and initial anti-microbiological investigations, 6-MP was approved by the U.S Food and Drug Administration to be used in the treatment of acute lymphoblastic leukaemia in children (Elion, 1989). With the use of combination chemotherapy with three or four drugs to produce
and consolidate remission, plus years of maintenance therapy with 6-MP and MTX, almost 80% of children with acute lymphoblastic leukaemia could be cured in the late 1980s (Elion, 1989).

During the initial investigations, 6-MP was found to be rapidly metabolised after administration. In an attempt to prolong the half life of 6-MP, the sulphur on 6-MP was protected from oxidation and hydrolysis by introducing blocking groups that might be removed in the body to release 6-MP. The 1-methyl-4-nitro-5-imidazolyl derivative (later known as azathioprine, AZA) was the most successful compound to emerge from these attempts. AZA showed a better immunosuppressive effect than 6-MP when examined on kidney transplants in dogs (Calne et al., 1962) and was further evaluated in humans (Murray et al., 1963), which led to the use of AZA, rather than 6-MP, when an immunosuppressive effect is required. In 1988, Gertrud Elion and George Hitchings received the Nobel Prize in Physiology or Medicine for their discoveries of important principles for drug treatment.
Clinical use

**Acute lymphoblastic leukaemia in children**

Childhood acute lymphoblastic leukaemia (ALL) is the most common malignancy of childhood. Between 175 and 200 children are diagnosed with ALL each year in the five Nordic countries Denmark, Finland, Iceland, Norway and Sweden. Treatment of children with ALL is stratified on the basis of various combinations of clinical and lymphoblastic characteristics in standard, intermediate and intensive therapy groups (NOPHO-ALL 2000 treatment protocol). Patients with higher risk are treated more aggressively, whereas less toxic treatment is used for patients with lower risk. Childrens ALL is unique among human cancers in that an important component of successful therapy is a long period of two to three years of maintenance treatment with low dose oral 6-MP and weekly oral MTX after induction and consolidation therapy (Lilleyman & Lennard, 1994; Pui, 1995).

**Inflammatory bowel disease**

The idiopathic inflammatory bowel diseases (IBD, ulcerative colitis and Crohn’s disease) share many similarities in epidemiology, symptoms and therapy but they are two distinct entities. The highest incidence and prevalence for both Crohn’s disease and ulcerative colitis have been reported from Northern Europe, the United Kingdom, and North America (Loftus, 2004). In Europe, incidence rates range from 1.5 to 20.3 cases per 100,000 person-years for ulcerative colitis and 0.87 to 9.8 cases per 100,000 person-years for Crohn’s disease (Loftus, 2004). It is estimated that in Europe, 50,000 to 68,000 new cases of ulcerative colitis are diagnosed annually, and between 23,000 and 41,000 new cases of Crohn’s disease. There seem to be slight gender-related differences in IBD incidence. In general there is a female predominance in Crohn’s disease, though in certain low-incidence areas a male predominance exists (Loftus, 2004).

Ulcerative colitis is characterised by a colonic inflammation, whereas inflammation in Crohn’s disease can occur in both the small intestine and
the colon. Crohn’s disease and ulcerative colitis result from an inappropriate response of the mucosal immune system to the normal enteric flora in a genetically susceptible individual (Ahmad et al., 2004). The strongest identified environmental risk factors for IBD are cigarette smoking (increased risk for Crohn’s disease, reduced risk for ulcerative colitis) and appendectomy, with reduced risk for ulcerative colitis (Loftus, 2004).

Susceptibility loci for IBD that have been replicated in independent studies have been identified on seven chromosomes (IBD1-7). Some loci have been shown to be specific to either ulcerative colitis (e.g. IBD2) or Crohn’s disease (IBD1/CARD15), whereas others confer a general susceptibility to develop IBD (Ahmad et al., 2004). For Crohn’s disease, the first susceptibility locus was mapped to chromosome 16 (Hugot et al., 1996), and three main mutations in the gene NOD2 (CARD15) were subsequently described (Hugot et al., 2001). NOD2/CARD15 mutations confer susceptibility to Crohn’s disease by altering inflammatory signals in monocytes (Hugot et al., 2001), including both cytokine production and apoptosis.

Drug treatment for inducing and remaining in remission in ulcerative colitis and Crohn’s disease is similar, corticosteroids and aminosalicylates are most often used (Schwab & Klotz, 2001). In addition, immunomodulators (6-MP, AZA, MTX, cyclosporin) and antibiotics are used (Schwab & Klotz, 2001). Infliximab, a monoclonal IgG antibody directed against tumour necrosis factor alfa (TNFα) is a new approach to inhibit the production of proinflammatory cytokines (Schwab & Klotz, 2001; Ahmad et al., 2004). However, 20% to 40% of patients fail to respond to this drug (Ahmad et al., 2004).

The therapeutic advantages of 6-MP in the USA and AZA in Europe are well documented for induction and maintenance of remission in corticosteroid-dependent or corticosteroid-resistant IBD and are the most important immunosuppressors used in Crohn’s disease (Pearson et al., 1995). Thiopurines also have a place in the management of fistulising Crohn’s disease both in adult and paediatric patients (Schwab & Klotz, 2001). Thiopurine treatment is the medical treatment of choice in patients with ulcerative colitis who fail to respond to 5-minosalicylates and
corticosteroids and who are not candidates for immediate surgery (Feagan, 2003). Up to 40% of IBD patients treated with thiopurines fail to benefit from the treatment (Dubinsky et al., 2002), which underlines the need to individualise the treatment by identifying patients at risk for adverse events and patients who will not at all benefit from the treatment. To accomplish this individualisation, a better understanding of the mechanisms of drug action is acquired.

**After transplantation and in other autoimmune diseases**

Since the initial observation of the immunosuppressive effect of AZA (Murray et al., 1963), AZA has been used to reduce the risk of graft versus host reactions after transplantation (Chocair et al., 1992; Chrzanowska & Krzymanski, 1999). AZA is also used in the treatment of rheumatoid arthritis, but mostly as a steroid-sparing agent (Gaffney & Scott, 1998; Clunie & Lennard, 2004). AZA is also used for immunosuppression in systemic lupus erythematosus (Mosca et al., 2001).

**Metabolism**

The bioavailability of 6-MP is only 16% (a range of 5-37%, Zimm et al., 1983), because of high first-pass metabolism. As earlier mentioned, AZA was originally synthesised as a “slow-release” form of 6-MP, in the hope that the imidazolyl group would protect the thiopurine group of 6-MP from rapid methylation and thereby inactivation. AZA is, however, rapidly converted to 6-MP in the presence of glutathione in the liver and red blood cells (deMiranda et al., 1973; Elion & Hitchings, 1975; Odlind et al., 1986). AZA is cleaved by chemical attack by sulphydryl groups on the nitroimidazol ring with no enzyme involved (Elion & Hitchings, 1975).

The metabolism of 6-MP is complex and involves many enzymatic reactions (Figure 2). Oxidation of 6-MP in the liver via xanthine oxidase (XO), generates thiouric acid (TUA), an inactive metabolite that is eliminated in the urine (Parks & Granger, 1986; Guerciolini et al., 1991). XO activity is absent in circulating blood cells (Parks & Granger, 1986; Lennard & Lilleyman, 1996).
6-MP is converted by TPMT (EC 2.1.1.67) to the inactive metabolite methyl mercaptopurine (meMP, Lennard, 1992). In a third metabolic route, 6-MP as well as 6-TG are initially converted by hypoxanthine guanine phosphoribosyl transferase (HGPRT, EC 2.4.2.8) to 6-thioguanosine monophosphate (TIMP) and thioguanosine monophosphate (TGMP), respectively. Further conversion of TIMP to TGMP via thioxanthine monophosphate (TXMP) is catalysed by two enzymes, IMP dehydrogenase (IMPDH, EC 1.2.1.14) and GMP synthetase (GMPS, EC 6.3.4.1), respectively (De Abreu et al., 1995).

Subsequently, TGMP is converted in consecutive steps to TGTP and deoxy (d) TGTP, which are incorporated into RNA and DNA, respectively (Tidd & Paterson, 1974). Ribonucleotides and deoxyribonucleotides of TG are usually collectively referred to as thioguanine nucleotides (TGN, Lennard & Maddocks, 1983; Lennard, 1992). Thus, 6-MP and 6-TG form identical phosphorylated metabolites.

Besides methylation of 6-MP to the inactive metabolite meMP, TPMT methylates TIMP to meTIMP, a metabolite that has been shown to inhibit purine de novo synthesis (PDNS, Tay et al., 1969; Bökkerink et al., 1993; Dervieux et al., 2001).

In contrast to 6-MP, XO is not the primary catabolising enzyme for 6-TG. 6-TG must first be converted to thioxanthine (TX) by guanase and then further by XO, before eliminated as TUA. Another elimination pathway for 6-TG is by methylation by TPMT to meTG (Kitchen et al., 1999).

**Mechanisms of action**

**Cytotoxicity by incorporation into nucleic acids**

Already in 1974 it was demonstrated that 6-MP was incorporated as TGN into DNA and RNA (Figure 2), and that a relationship existed between the extent of incorporation and the delayed cytotoxic reaction of 6-MP (Tidd & Paterson, 1974; Lennard & Maddocks, 1983). The thiopurine metabolite dTGTP is used as a building block in DNA synthesis, and can replace guanine in the DNA strand. Although incorporation into DNA is required for these agents to exert their cytotoxic effects, the mechanism(s)
by which DNA incorporation translates into cytotoxicity were for long unknown.

Studies have now shown that the cytotoxicity partly is a delayed effect associated with inhibition of cell cycle progression through the S and G$_2$ phases, subsequent to the first cell division after incorporation of dTGTP into DNA. It has also been shown that incorporation of dTGTP into DNA inhibits the activity of RNase H, a critical enzyme involved in DNA replication (Maybaum & Mandel, 1983; Krynetskaia et al., 1999).

**Inhibition of purine de novo synthesis**

meTIMP, the predominantly methylated metabolite formed by metabolism of 6-MP, is a potent inhibitor of phosphoribosyl pyrophosphate (PRPP) amidotransferase (EC 2.4.2.14, Figure 2), the first enzyme of PDNS (Tay et al., 1969; Bennett & Allan, 1971; Bökkerink et al., 1993). In PDNS, PRPP amidotransferase catalyses the conversion of PRPP to PRA (Figure 2). This inhibition by meTIMP can induce cytotoxicity by several mechanisms; inhibition of PRPP amidotransferase results in accumulation in PRPP. Since PRPP is a co-substrate for conversion of 6-MP to TIMP, increase of PRPP induces an increased conversion of 6-MP to TIMP (Bökkerink et al., 1993). Secondly, a depletion of endogenous nucleotides is observed due to the decreased PDNS by meTIMP. Cells with a high cell proliferation rate are highly dependant on PDNS. Depletion of purine nucleotides results in decreased RNA and DNA synthesis. It has also been suggested that decreased purine synthesis leads to increased pyrimidine synthesis which would lead to imbalanced cell growth and contribute to cell death (Bökkerink et al., 1993).

**Immunosuppressive effects**

T-lymphocytes play a primary role in many autoimmune disorders and in allograft rejection (Maltzman & Koretzky, 2003). Recently, it has been suggested that the immunosuppressive effects of thiopurine drugs are due to the competition by TGTP on the GTP-binding protein Rac1. This will result in suppression of Rac1 activation and induction of apoptosis. This mechanism is thought to control elimination of activated T-lymphocytes.
It has also been shown recently that thiopurines selectively inhibit inflammatory gene expression in activated T-lymphocytes (Thomas et al., 2005).

**Thiopurine methyltransferase (TPMT)**

**Tissue localisation and inheritance**

TPMT is a cytoplasmic enzyme that catalyses the S-methylation of aromatic and heterocyclic sulfhydryl compounds, such as 6-MP and 6-TG (Remy, 1967; Woodson & Weinshilboum, 1983; Weinshilboum, 1989a) with S-adenosyl methionine (SAM) as a methyl donor (Remy, 1963; Elion, 1967). Although extensively studied, the physiological role of TPMT in the human body has not yet been fully elucidated (Evans & Relling, 1999).

TPMT was first found in a variety of tissues in experimental animals (Remy, 1963). In humans, TPMT has been found and quantified in red blood cells (Weinshilboum et al., 1978), peripheral leukocytes, bone marrow cells, kidney, liver and the brain (Van Loon & Weinshilboum, 1982; Pacifici et al., 1991; Szumlanski et al., 1992; Van Loon et al., 1992; McLeod et al., 1995; Coulthard et al., 1998). TPMT is typically measured in the easily obtainable red blood cell, as the level of TPMT activities in human liver, kidney, leukaemic blasts and normal lymphocytes have been shown to correlate with that in red blood cells (Van Loon & Weinshilboum, 1982; Szumlanski et al., 1992; McLeod et al., 1995; Coulthard et al., 1998).

The first indication that TPMT activity in humans might be influenced by inheritance was obtained by measuring the enzyme activity in blood from a large, randomly selected, Caucasian population (Weinshilboum & Sladek, 1980). The frequency distribution of red blood cell TPMT activity is trimodal in Caucasians, ~89% have high activity, ~10% have intermediate enzyme activity and 1 of 300 has almost undetectable activity (Weinshilboum & Sladek, 1980). The distribution has been studied in many populations, including a Swedish population of 219 individuals (Pettersson...
et al., 2002), showing the same distribution as other Caucasian populations (Figure 3). The trimodal distribution is that predicted by the Hardy-Weinberg theorem for a monogenetically inherited trait controlled by a single gene locus with two alleles (Weinshilboum & Sladek, 1980).

![Figure 3](image.png)

**Figure 3.** Frequency distribution histogram of red blood cell TPMT enzyme activities in a Swedish population (n=219, Pettersson et al., 2002). Reprinted with permission.

### The *TPMT* gene

The structure of the *TPMT* gene was first described in 1993, when a *TPMT* cDNA from human liver was isolated (Honchel et al., 1993). The complete structure of the gene, including exons and introns as well as the promoter region of the gene, was later described (Szumlanski et al., 1996; Krynetski et al., 1997; Fessing et al., 1998). The *TPMT* gene is approximately 34 kB in length, consists of ten exons (eight of which encode protein, Figure 4), and it maps to chromosome 6p22.3 (Szumlanski et al., 1996). Exon 2 is not uniformly expressed within the 5’ untranslated regions (Honchel et al., 1993; Szumlanski et al., 1996), probably as a result of alternative splicing with “exon skipping” (McKeown, 1992).

A processed pseudogene on chromosome 18 has been detected (Lee et al., 1995), which has a nucleotide sequence similarity of 96% of the *TPMT* cDNA. This complicates the use of DNA-based tests for the determinations
of TPMT polymorphisms. Reverse transcription (cDNA synthesis) must be applied to TPMT with the understanding that amplifications performed in the presence of even minute amounts of genomic DNA contamination will result in amplification of the processed pseudogene (Otterness et al., 1997). Use of intronic primers abolishes the risk for amplification of the pseudogene, since the processed pseudogene by definition (Strachan & Read, 1996) only includes sequences corresponding to exons of the gene.

The molecular basis for the genetic polymorphism of TPMT has been explained by the existence of several SNPs. The most common alleles after TPMT*1 in all populations studied are TPMT*2, TPMT*3A and TPMT*3C (Krynetski et al., 1995; Szumlanski et al., 1996). TPMT*2 denotes a G>C transition at nucleotide 238 (exon V). TPMT*3A consists of two nucleotide substitutions, 460G>A and 719A>G, located in exons VII and X, respectively. These SNPs can occur independently, on separate alleles, and are then referred to as TPMT*3B (640G>A) and TPMT*3C (719A>G), respectively (Figure 4).

**Figure 4.** The structure of the TPMT gene (TPMT*1, wild-type), as well as the most common variant alleles TPMT*2, TPMT*3A and TPMT*3C, and the rare TPMT*3B. Black boxes indicate exons within the open reading frame and white boxes indicate exons that are not translated. Lines in-between the exons represent introns (not proportional to their relative sizes).
Enhanced ATP-dependent proteasomal degradation has been identified as the mechanism for TPMT enzyme deficiency inherited by TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C (Tai et al., 1999).

Besides the more common variant alleles, rare sequence variants have been detected defining the alleles TPMT*3D, *4, *5, *6, *7, *8 and *10 (Otterness et al., 1997; Otterness et al., 1998; Spire-Vayron de la Moureyre et al., 1998b; Hon et al., 1999; Colombel et al., 2000). Interethnic differences exist in the prevalence of variant TPMT alleles.

The overall variant allele frequencies are equal in Caucasians and African-Americans (3.7% and 4.6% of alleles, respectively, Hon et al., 1999). However, TPMT*3A is the most prevalent allele (75-86% of variant alleles) in Caucasians (Tai et al., 1996; Hon et al., 1999) whereas it only represents 17% in African-Americans. In South-west Asians (of Indian, Pakistani, Sri Lankan and Nepali origin) TPMT*3A is the only variant allele found (Collie-Duguid et al., 1999; Hongeng et al., 2000). TPMT*3B is rare and has only been found in a few individuals (Otterness et al., 1997).

TPMT*3C is the predominant allele in African-Americans, whereas the frequency in Caucasians is much lower (Hon et al., 1999). In Ghanaians, TPMT*3C is found in 4.8% of the population, whereas TPMT*2, TPMT*3A and TPMT*3B are not found at all (Ameyaw et al., 1999; McLeod et al., 1999).

In Chinese and Japanese, TPMT*3C is the only present allele (Hiratsuka et al., 2000; Kubota & Chiba, 2001; Kumagai et al., 2001). The distribution of enzyme activity in Chinese is bimodal (Lee & Kalow, 1993). TPMT*2 is more common in African-Americans as compared to Caucasians (8.7% and 4.8% of mutant alleles, respectively, Hon et al., 1999).

Interethnic difference in the level of TPMT activity exists as well. Sami populations have 29% higher activity than white subjects (Klemetsdal et al., 1992). African-Americans have 17-33% lower activity as compared to white Americans (Jones et al., 1993; McLeod et al., 1994). Chinese have a lower activity than whites (Tinel et al., 1991; Klemetsdal et al., 1992; McLeod et al., 1994).

TPMT enzyme activity is bimodal (Lee & Kalow, 1993) or trimodally distributed in all these populations, with 73-94% high activity (Weinshilboum & Sladek, 1980; Tinel et al., 1991; Klemetsdal et al., 1992;
Jones et al., 1993; Lee & Kalow, 1993), and 6-27% are phenotypic intermediates. In Koreans the enzyme activity is unimodally distributed within the population (Jang et al., 1996; Park-Hah et al., 1996; Otterness et al., 1997).

From an anthropological point of view, it seems that the 719A>G transition was the first TPMT mutation to arise in humans, as this is the only mutation found in Ghanaian, Kenyan and Chinese populations (Ameyaw et al., 1999; Collie-Duguid et al., 1999; McLeod et al., 1999), and this mutation is also found in Caucasians, either alone or in combination with 460G>A creating the TPMT*3A allele (Tai et al., 1996).

The known cSNPs of the TPMT gene accounts for only two-thirds of the total variance in activity measured in red blood cells (Vuchetich et al., 1995). The variance within the group considered as TPMT wild type varies over twofold (Figure 2), and family studies have shown that the variance within the group significantly correlates within families (Vuchetich et al., 1995). These results suggest that additional genetic factors exist that modulate TPMT activity.

A polymorphism in the 5'-flanking promoter region has been identified due to a variable number of tandem repeats (VNTR) ranging from four to eight repeats (Krynetski et al., 1997; Spiere-Vayron de la Moureyre et al., 1999). Each repeat consists of 17-18 bp units and contains potential binding sites for the transcription factors Sp1, E2F and KROX24/Zif268 (Szumlanski et al., 1996; Krynetski et al., 1997; Spiere-Vayron de la Moureyre et al., 1999).

**Clinical significance of the TPMT polymorphism**

Inherited variation in TPMT activity is one factor responsible for individual differences in susceptibility to thiopurine induced toxicity or in the therapeutic response to these drugs. Numerous studies have shown that patients with very low enzyme activity are at high risk for severe, and sometimes fatal, haematological toxicity (Evans et al., 1991; Schutz et al., 1993; Evans et al., 2001), and patients who are TPMT heterozygous have an intermediate risk of haematological toxicity (Black et al., 1998; Relling et al., 1999). TPMT activity has been inversely correlated to TGN
concentrations in red blood cells from children with ALL treated with 6-MP (Lennard & Lilleyman, 1987).

TGN have been proposed as a target for monitoring the efficiency and toxicity of the treatment. A correlation has been demonstrated between TGN levels and haematologic toxic effects (neutropenia). Children with ALL with low TGN levels, have a higher probability of relapse (Lennard et al., 1983; Lennard et al., 1990). In IBD, clinical effects have been correlated to TGN-levels, and hepatotoxicity has been associated with methylated metabolites (Cuffari et al., 1996; Dubinsky et al., 2000).

Care has been taken to study and clarify the role of meTIMP (Dervieux et al., 2001). It has been shown that individuals with TPMT deficiency tolerate higher concentrations of TGN than do patients with wild-type TPMT (Lennard et al., 1987; Lennard et al., 1990; Relling et al., 1999), and in vitro data reveal that incorporation of TGN into DNA in various cell lines does not correlate with their sensitivity to 6-MP (Krynetskaia et al., 1999).

Together, these in vivo and in vitro data suggest that methylation contribute to cytotoxicity (Dervieux et al., 2001) and that if metabolite determinations are to be used for therapeutic drug monitoring, the meTIMP levels might be equally as important as TGN levels.

**Drugs interacting with thiopurine metabolism**

The administration of one drug can alter the action of another by pharmacokinetic or pharmacodynamic interaction.

Aminosalicylates have been shown to influence TPMT enzyme activity (Lennard, 1998; Weinshilboum, 2001; Dewit et al., 2002). In vitro kinetic studies with recombinant TPMT enzyme have demonstrated that sulphasalazine, 5-aminosalicylic acid, balsalazide, and olsalazine (Szumlanski & Weinshilboum, 1995; Lewis et al., 1997; Lowry et al., 1999) inhibit the enzyme in a non-competitive manner. Therefore, simultaneous treatment with thiopurines and these drugs has been postulated to lead to a low activity of TPMT.
Studies in clinical samples have not been able to confirm this inhibitory effect on TPMT. However TGN concentrations increased during coadministration with aminosalicylates, whereas TPMT activity measured in vitro was not affected (Lowry et al., 2001; Dewit et al., 2002). It has recently been shown (Shipkova et al., 2004), that the inhibitory effect of concomitantly administered aminosalicylates cannot be measured on TPMT in vitro, due to the washing steps included in the preparation of red blood cells for TPMT analyses.

An important 6-MP interacting drug is allopurinol (Keuzenkamp-Jansen et al., 1996). Allopurinol is used for treatment of hyperuricemia and in gout (Elion, 1989) and is an effective inhibitor of XO, the quantitatively largest catabolic route for thiopurines. Inhibition of XO leads to increased amounts of 6-MP to be converted into active metabolites, which results in an increased cytotoxic effect (Elion et al., 1963; Elion, 1989).

The drug interaction between 6-MP and MTX is synergistic, meaning that cotreatment produces an effect which neither could produce alone, or an effect that is greater than the total effect of each agent acting by itself (Giverhaug et al., 1999). MTX increases the plasma concentrations of 6-MP by inhibiting hepatic and intestinal XO (Innocenti et al., 1996). Both drugs inhibit PDNS (Giverhaug et al., 1999). In vitro and in vivo, cotreatment with 6-MP and MTX, casuses increased levels of both TGN and mcTIMP as compared to 6-MP alone (Giverhaug et al., 1998; Pettersson et al., 2002). In vivo, cotreatment with 6-MP and MTX has been shown to inhibit PDNS (Dervieux et al., 2002). Diuretics inhibit TPMT both in vitro (Lysaa et al., 1996) and in vivo (Klemetsdal et al., 1993).
AIMS OF THE STUDY

The general aim of this thesis was to study the pharmacogenetics of thiopurines with focus on the polymorphic enzyme TPMT.

Specific aims:

1. To develop methodology for quantification of TPMT gene expression, and to investigate the correlation between gene expression and enzyme activity.

2. To develop a pyrosequencing method for TPMT genotyping, and to investigate the allele frequencies of SNPs in a Swedish population. The aim was further to investigate the concordance between genotype and phenotype.

3. To investigate the non-concordance between genotype and phenotype in two individuals genotyped as heterozygous for one non-functional TPMT allele.

4. To study the influence on TPMT gene expression and TPMT enzyme activity during initiation of thiopurine treatment in patients with IBD, and to investigate other pharmacogenetic and pharmacokinetic effects of the thiopurine treatment.
MATERIALS AND METHODS

Patients and healthy volunteers

The patients included in the present investigation were diagnosed with inflammatory bowel disease. A summary of all subjects investigated in this thesis is presented in Table I. The relationship between TPMT gene expression and TPMT enzyme activity was investigated in two studies (papers I and IV). For isolation of RNA, blood was collected in EDTA tubes (paper I) or PAXgene™ RNA blood collection tubes (paper IV).

Table I. Summary of IBD patients, healthy individuals and reference population investigated in the thesis

<table>
<thead>
<tr>
<th>Paper</th>
<th>Ulcerative colitis</th>
<th>Crohn’s disease</th>
<th>Other diagnosis</th>
<th>Healthy individuals</th>
<th>Total</th>
<th>Reference population</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>19</td>
<td>15</td>
<td>1</td>
<td>4</td>
<td>39</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>16</td>
<td>7</td>
<td>1</td>
<td>6</td>
<td>30</td>
<td>800</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>6</td>
<td>200</td>
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<td>33</td>
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<td>55</td>
<td>3</td>
<td>14</td>
<td>135</td>
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</tbody>
</table>

A total of 39 individuals were enrolled in the study for paper I (35 IBD patients and 4 healthy volunteers). In paper IV, consecutive IBD patients with ulcerative colitis or Crohn’s disease (n=60) in whom thiopurine treatment was indicated were included. Primary indications for thiopurine treatment were steroid-dependent and steroid-resistant chronic active disease, frequent relapses ($\geq 2$ per year), and fistulising disease. Secondary
indications were maintenance of medically or surgically induced remission. The target dose was for AZA 2.5 mg/kg body weight, and for 6-MP 1.25 mg/kg body weight. All patients followed a predetermined dose escalation schedule (Tables IIA and IIB). The target dose was reached after three weeks. Patients visited the outpatient clinic at baseline (week 0), and then 1, 2, 3, 4, 5, 6, 7, 8, 12, 16 and 20 weeks after start of treatment.

Note: The daily dose was administered in the morning during week 1, divided into morning and evening doses in weeks 2 to 4 and then administered once daily in the evening from week 5 on. The target dose was 2.5 mg per kg body weight for azathioprine and 1.25 mg per kg body weight for 6-mercaptopurine.

For investigation of the allelic frequencies of reported TPMT SNPs and the ITPA 94C>A SNP, DNA samples from a Swedish regional population based biobank were used (papers II, III and IV; Table I). The biobank consisted of DNA from 800 individuals living in the South-east region of Sweden (400 males, 400 females). Individuals were selected randomly from the Swedish population register and anonymously included in the biobank after informed consent. The concordance between TPMT genotype and phenotype (paper II) was investigated in 30 individuals who were chosen from their earlier determined TPMT enzyme activity either for clinical purposes or from their participation in paper I. Six of the individuals were healthy volunteers and 24 were patients with IBD. The non-concordance between genotype and phenotype were further investigated in two non-related individuals and their parents (paper III).

The Ethics committee at the Faculty of Health Sciences, Linköping University has approved the studies.
Methodology overview

Detailed methodological descriptions are given in the individual papers (Table III). Therefore only a summary and principal aspects will be given here.

**Table III.** Methodology used in this thesis

<table>
<thead>
<tr>
<th>Technique</th>
<th>Papers</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA isolation</td>
<td>I and IV</td>
</tr>
<tr>
<td>cDNA synthesis</td>
<td>I and IV</td>
</tr>
<tr>
<td>Real-time RT-PCR</td>
<td>I and IV</td>
</tr>
<tr>
<td>DNA isolation</td>
<td>II, III and IV</td>
</tr>
<tr>
<td>Manual DNA sequencing</td>
<td>I and III</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>II, III and IV</td>
</tr>
<tr>
<td>TPMT enzyme activity determination</td>
<td>I-IV</td>
</tr>
<tr>
<td>Determination of TGN and meTIMP</td>
<td>IV</td>
</tr>
</tbody>
</table>

Stability of RNA in PAXgene™ blood collection tubes

Blood was collected in the PAXgene™ RNA tube, which contains a cationic reagent and additives to stabilise RNA (Rainen *et al.*, 2002). According to the manufacturer, RNA stored in the tubes are stable for up to five days at room temperature (PreAnalytix product circular: PreAnalytix RNA tube) and for weeks if refrigerated (Rainen *et al.*, 2002). After preliminary observations of instability of RNA collected in the PAXgene™ tube (*B. Kågedal, personal communication*), we decided to investigate the stability and integrity of the total RNA and the *TPMT* mRNA.

Thirty-six tubes were taken from one blood donor at one occasion. All tubes were allowed to incubate at room temperature for two hours, and were then stored in one of the following: -20°C freezer, +4°C refrigerator or at room temperature (22°C). RNA was isolated according to the instructions given by the manufacturer immediately after two hours (=day 0), and after one, three and seven days. The total RNA integrity and concentration was determined using Agilent 2100 Bioanalyzer and the
RNA 6000 Nano LabChip® kit (Agilent Technologies), in conjugation with the Agilent 2100 Expert software. The specific stability of the TPMT mRNA was evaluated in real-time RT-PCR experiments.

**Real-time RT-PCR**

A real-time reverse transcription (RT)-PCR method was developed for quantification of TPMT gene expression. For the real-time RT-PCR, PCR primers were designed to amplify a short region (151 bp) of the TPMT cDNA and between them a dual labelled fluorogenic hybridization TaqMan® probe. The probe was labelled with the fluorescent dye 6-carboxyfluorescein (FAM) and an internal dark quencher. During the PCR reaction (Figure 5), the probe is cleaved by the 5’ to 3’ nuclease activity of Taq polymerase and the fluorogenic reporter is separated from the quencher (Holland et al., 1991; Livak et al., 1995; Heid et al., 1996). The fluorescence increases proportionally to the amount of PCR product. Reactions are characterised by the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a number of fixed cycles. The reactions were performed and the fluorescence detected using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

A threshold cycle (C<sub>T</sub>) value was determined for every sample at the beginning of the amplification. The relative quantification was accomplished using the standard-curve method, comparing with the gene expression of the housekeeping gene cyclophilin (huCYC) as described in user bulletin no. 2 (Applied Biosystems Inc).
Figure 5. Stepwise representation of the polymerisation-associated 5’ to 3’ nuclease activity of Taq DNA polymerase acting on the TaqMan® probe during one extension phase of PCR. Two fluorescent dyes, a reporter (R) and a quencher (Q), are attached to the 5’ and 3’ ends of the TaqMan® probe. The quencher can also be a dark quencher positioned within the probe sequence. When both dyes are attached to the probe, reporter dye emission is quenched. During each extension cycle, the Taq DNA polymerase cleaves the reporter dye from the probe. Once separated from the quencher, the reporter dye emits its characteristic fluorescence. Copyright and provided courtesy of Applied Biosystems (an Applera Corporation Business). All rights reserved.
DNA sequencing

Manual DNA sequencing of the real-time RT-PCR fragment (paper I) and the individual TPMT exons (paper III) was performed using the dideoxy chain termination method (Sanger et al., 1977). Identification of known SNPs in the TPMT and ITPA genes was performed using pyrosequencing.

The Sanger sequencing method is based on the incorporation of dideoxynucleotides in the elongating DNA molecule during PCR. In contrast to deoxynucleotides, dideoxynucleotides have a hydrogen atom attached to the 3’ carbon atom instead of a hydroxyl group which prevents chain elongation and thereby terminates the growing DNA chain (Sanger et al., 1977).

The pyrosequencing technique (Ronaghi et al., 1996) is based on polymerase primer extension, and detection occurs by the release of pyrophosphate (PPI) from the primer extension reaction (Figure 6). A sequencing primer is hybridized to a single-stranded, PCR-amplified DNA template, and incubated with enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) and substrates (adenosine 5’ phosphosulfate, APS and luciferin). Deoxynucleotide triphosphates (dNTPs) are added according to a user-defined nucleotide order. DNA polymerase incorporates the nucleotides if there is a complementary base on the DNA strand. If incorporated, PPI is released; ATP sulfurylase converts PPI to ATP in the presence of APS. Luciferase utilises the formed ATP and catalyses luciferin to oxyluciferin that generates light, which is detected by a charge-coupled device camera. The light generated is proportional to the number of incorporated nucleotides. Unincorporated dNTPs and ATP are degraded between each cycle by apyrase. The result is displayed in a Pyrogram (Figure 6).
Figure 6. In the pyrosequencing reaction, a DNA polymerase incorporates dNTP complementary to the template strand. Pyrophosphate (PPi) is released in the reaction. Adenosine 5’ phosphosulfate (APS) and PPi are enzymatically converted to ATP by sulfurylase. ATP, which is a substrate for luciferase, is converted to light and detected by a charge-coupled device camera. The result is displayed in a Pyrogram and the fluorescence is proportional to the amount of dNTP incorporated.
TPMT enzyme activity determination

TPMT enzyme activity was determined in red blood cells by a radiochemical method originally described by Weinshilboum et al. (1980), modified by Klemetsdal et al. (1992). The method measures the formation rate of $^{14}$C-methylmercaptopyrimidine from 6-MP using S-adenosyl-$L^{-14}$C-methyl-methionine as methyl donor (Figure 7). Product formation is measured by a liquid scintillation counter. The result is expressed as units TPMT per ml of packed red blood cells (pRBC) per hour of incubation. The intra- and inter-assay coefficients of variations were 3.3% and 4.7%, respectively. We used cutoffs of 9.0 U/ml pRBC to distinguish high TPMT enzymatic activity from intermediate activity and 5.0 U/ml pRBC to distinguish intermediate from low TPMT enzymatic activity (Pettersson et al., 2002).

![Diagram of TPMT reaction](image-url)

**Figure 7.** TPMT reaction with 6-MP as a substrate and S-adenosyl-$L^{-14}$C-methionine (SAM*) as a methyl donor, SAH; S-adenosyl homocysteine.
Determination of TGN and meTIMP

The nucleotides (TGN and meTIMP) were determined in red blood cells by reversed-phase high performance liquid chromatography (HPLC) as purine bases after acid hydrolysis and an extraction procedure (Lennard & Singleton, 1992). For TGN, the intra- and inter-assay coefficients of variation at 75 pmol/8x10^8 RBC were 5.7% and 12.2%, respectively. For meTIMP, the intra- and inter-assay coefficients of variation at 1500 pmol/8x10^8 RBC were 16.7% and 17.4%, respectively.

Statistics

For all variables, distribution was tested for normality. The correlation between gene expression and TPMT enzyme activity was evaluated using Spearman’s rank correlation coefficient (paper I). The Mann Whitney’s U test was used to compare groups in papers II and IV.

In paper IV, TPMT enzyme activity, gene expression and levels of TGN and meTIMP were described as median (quartile 1; quartile 3). However, changes in TPMT enzyme activity and gene expression over time were analysed using two-way ANOVA, after testing that the residuals were close to normally distributed. In a subsequent post-hoc test (Dunnet’s t-test), one group of measurements was used as a control (values at baseline) and other groups of measurements (values at different weeks) were compared against this control. The Wilcoxon signed rank test was used to test differences between paired observations. The generalised Fisher’s exact test was used when testing for associations between \( ITPA \) 94C>A alleles and occurrence of adverse events. Fisher’s exact test was used to test the association between \( TPMT \) genotype and occurrence of adverse events. A Kaplan-Meier plot and Log Rank test was used to visualise and to test differences in probability of remaining in the study between \( TPMT \) genotypes. The increase in TGN and meTIMP concentrations from weeks 1 to 5 was calculated as a slope (regression coefficient) for each person, and the slopes were compared between groups by two-tailed t-test. P values of <0.05 were considered as statistically significant. SPSS® for Windows release 11.5 and Minitab version 13 were used to analyse the data.
Bioinformatics

- The *TPMT* gene was identified by use of the UCSC genome server (http://genome.ucsc.edu) and PCR primers were designed with Primer 3, available through the Biology WorkBench (http://workbench.sdsc.edu).
- Primers and probe for real-time RT-PCR were designed using ABI PRISM Primer Express software (Applied Biosystems).
- Sequence-specific primers for pyrosequencing were designed using software supplied by Biotage AB (http://www.biotage.com).
- Primers were checked for specificity by use of the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/blast/).
- The NCBI ORF finder, a graphical annotation tool (http://www.ncbi.nlm.nih.gov) was used to investigate the theoretical outcomes of new sequence variants. The *TPMT* mRNA sequence (accession number U12387) was used and analysed after modification to correspond to the new sequence variants.
RESULTS AND DISCUSSION

Real-time RT-PCR methodology for quantification of TPMT gene expression

(Paper I)

Non-functional TPMT alleles account for the majority of the variation of TPMT enzyme activity. However, TPMT SNPs in the coding region fail to explain considerable differences in TPMT enzyme activity among subjects with apparently identical open reading frame sequences, differences that also show a high degree of familial aggregation (Vuchetich et al., 1995; Yan et al., 2000; Alves et al., 2001). VNTR have been identified in the promoter region of the TPMT gene, and they have been suggested to modulate TPMT activity (Spire-Vayron de la Moureyre et al., 1998a; Spire-Vayron de la Moureyre et al., 1999; Yan et al., 2000; Alves et al., 2001).

In paper I, we developed a real-time RT-PCR method to quantify the variation in TPMT gene expression. We set out to investigate if the variability within individuals with high enzyme activity and wild-type TPMT alleles was a result of variability in gene expression (papers I and IV). We tested a set of 11 housekeeping genes and selected human cyclophilin (huCYC) as an internal control, as the huCYC gene expression was stable and in the same range as TPMT (paper I). Five serial dilutions (1:4) were used to plot standard curves for both huCYC and TPMT in the real-time RT-PCR experiments (Figure 8). The design of the primers and
probe for *TPMT* was performed, in order not to amplify of the processed pseudogene at chromosome 18 (Lee *et al.*, 1995). The identity of *TPMT* cDNA amplified during the real-time RT-PCR reaction was confirmed by sequencing and showed specificity when compared to the pseudogene. Further, standard curves for *TPMT* and *huCYC* showed PCR efficiency close to 100%. The slopes of the standard curves were $-3.38$ and $-3.34$, respectively (Figure 8). The intra- and inter-assay coefficient of variation was 8% and 25%, respectively, for the ratio between *TPMT* and *huCYC*.

![Figure 8](image.png)

**Figure 8.** A 1:4 dilution series of Molt-4 cell line cDNA sample in triplicate was used as standard in the real-time RT-PCR reaction experiments. The real-time RT-PCR amplification plot is as presented by the ABI PRISM 7700 SDS program for the standard curve of *TPMT* (A). The C$_T$-values obtained from the amplification plots for *TPMT* and *huCYC* were used to plot standard curves (B) for the quantification of unknown samples.
Evaluation of PAXgene™ blood collection tubes for stabilisation of RNA

Gene transcription analysis of blood samples is most often carried out on leukocytes isolated from venous blood samples, where the blood has been collected in Vacutainer® tubes containing an anticoagulant such as EDTA. These additives do little to stabilise RNA, which is degraded within a few hours \textit{in vitro} if not stabilised (Rainen \textit{et al.}, 2002). To enable sending of samples and to simplify the procedures and routines concerning RNA isolation in paper IV, a recently developed system, the PAXgene™ RNA tube, was used for blood collection (Rainen \textit{et al.}, 2002). In our study (paper IV) PAXgene™ RNA tubes were sent at room temperature from other hospitals or stored immediately in the refrigerator if samples were taken at Linköping University Hospital. The RNA integrity was evaluated using the Agilent Bioanalyser and subsequent software which evaluates the RNA by calculating RNA integrity numbers (RIN). The RIN considers the total trace of RNA fragments of different sizes. A RIN value of 10 indicates RNA of excellent quality, whereas a RIN value of 5 indicates a partly degraded RNA (Marx, 2004).

The total RNA showed good integrity for all samples stored at +4°C or -20°C (RIN values over 7.9) However, the RIN values were lower for tubes stored at room temperature for more than three days, with RIN values from 3.3 to 6.5.

Compared to \textit{TPMT} gene expression measured in tubes processed immediately after blood collection, we found that \textit{TPMT} gene expression was only 40% after three days’ storage of the PAXgene™ tube at room temperature. After storage of tubes at +4°C, the \textit{TPMT} gene expression was 58% after one day, 61% after three days and 53% after seven days, compared to \textit{TPMT} gene expression measured in tubes processed immediately after blood collection (Table IV). The \textit{TPMT} mRNA was more stable when stored in the freezer for up to seven days. We conclude that even if the integrity of the RNA was good when the PAX gene™ tubes were stored in refrigerator or freezer temperatures, the stability of the \textit{TPMT} mRNA was affected under these conditions.
As mRNA only constitutes 3-5% of total RNA (Alberts, 1994), it is important to investigate the stability of the transcript of interest, and not to rely solely on the integrity of the total RNA. We therefore suggest that investigators should evaluate the PAXgene™ tubes for particular mRNA species of interest, before using the tubes in the study design. In our study (paper IV), we decided only to analyse samples stored for one to seven days in the refrigerator. Thus, samples isolated on the day of sampling were not analysed.

### TPMT gene expression and enzyme activity

**- In healthy individuals and patients not treated with thiopurines**

(Papers I and IV)

Measurement of *TPMT* gene expression and enzyme activity in 39 individuals showed that in individuals with high enzyme activity (*TPMT ≥ 9.0 U/ml pRBC*), there was a statistically significant correlation between mRNA level and enzyme activity ($r_s=0.66$, $p=0.0001$, paper I, Figure 9A).

In paper IV, we did not find the same correlation (Figure 9B) using PAXgene™ tubes to collect the RNA and categorising individuals by TPMT genotype. We did not find any significant correlation in individuals with lower enzyme activity ($\leq 8.9$ U/ml pRBC) or when analysing the whole population, either in paper I or paper IV.
Figure 9. TPMT enzyme activities and mRNA in A): 39 individuals from paper I, classified by TPMT enzyme activity, and B): 24 patients in paper IV, classified by TPMT genotype.

Individuals with intermediate or low enzyme activity harbour SNPs in the open reading frame of the gene. It has been shown that the reason for decreased activity caused by the most common SNPs in the TPMT gene (460G>A, 719A>G and 238G>C) is an enhanced proteasomal degradation of the protein, thus the mRNA level is not affected by the SNPs, whereas the protein is (Tai et al., 1999), in accordance with our results.

To fully investigate the relationship between mRNA levels and enzyme activity of TPMT, a large population of healthy individuals without medication should be used. Patients included in our studies (papers I and IV) were not taking thiopurines, but some patients were taking aminosalicylates or corticosteroids as well as other medications at the time of sampling.

The results from our investigations suggest that, in individuals with two wild-type alleles and high enzyme activity, factors other than cSNPs may contribute to the variation in enzyme activity. The characterisation of the TPMT promoter has shown a polymorphism consisting of VNTR regions in the 5’ region of the gene (Spire-Vayron de la Moureyre et al., 1998a; Spire-Vayron de la Moureyre et al., 1999; Yan et al., 2000; Alves et al., 2001) that may account for differences in TPMT gene transcription. Although
some of these studies have found correlations between the existence of different subtypes or combinations of VNTR and enzyme activity, they have failed to explain the large variations within the high TPMT enzyme activity group.

Thus, the reason for the large variation within the high TPMT enzyme activity group remains unclear. However, our data indicate that it may be due to quantitative differences in gene transcription.

- In patients with inflammatory bowel disease during initiation of thiopurine treatment

(Paper IV)

In paper IV, we followed 60 patients with ulcerative colitis or Crohn’s disease during initiation of either AZA or 6-MP treatment, following the dose escalation schedule as described in Materials and Methods. The characteristics of the patients are summarised in Table V. One patient was excluded because her TPMT activity was lower than 3 U/ml pRBC, and five were withdrawn during the first three weeks because of non-compliance. Of the remaining 54 patients, 27 followed the 20-week study per protocol (PP group) and 27 were withdrawn due to side effects (AE group). Of the 54 patients, 43 were treated with AZA and 11 with 6-MP. Forty-five percent (n=27) of patients included in this study (n=60), discontinued thiopurine treatment (n=22) or required a dose reduction (n=5). The proportion of patients discontinuing treatment would probably have been lower, if dose escalation during a period longer than three weeks had been used. Thus, 13 patients that discontinued treatment restarted thiopurine treatment at a lower dose within the 20-week period. In total, 67% (18/27) of the withdrawn patients were on thiopurines at week 20. Myelotoxicity (n=9) was the most common cause for withdrawal from the study, and led to discontinuation of therapy (n=6) or a reduction in dose (n=3).
Table V. Patient characteristics (n=60, paper IV)

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (Male/Female)</td>
<td>32/28</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>41.5 (30.5; 53.5)</td>
</tr>
<tr>
<td>Disease</td>
<td>33/27</td>
</tr>
<tr>
<td>Withdrawals</td>
<td>33</td>
</tr>
<tr>
<td>TPMT enzyme activity &lt; 3 U/ml pRBC</td>
<td>1</td>
</tr>
<tr>
<td>Protocol violation weeks 0-3</td>
<td>5</td>
</tr>
<tr>
<td>Thiopurine-related adverse events</td>
<td>27</td>
</tr>
<tr>
<td>Myelotoxicity (leucopenia/thrombocytopenia)</td>
<td>9</td>
</tr>
<tr>
<td>Abdominal pain/nausea/vomiting</td>
<td>7</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>4</td>
</tr>
<tr>
<td>Myalgia/arthralgia</td>
<td>3</td>
</tr>
<tr>
<td>Hepatotoxicity</td>
<td>2</td>
</tr>
<tr>
<td>Other (palpitations, headache, sleeping disturbances, depression)</td>
<td>2</td>
</tr>
</tbody>
</table>

Drug therapy with thiopurines has been reported to lead to a variable increase (Lennard et al., 1990; McLeod et al., 1995; Thervet et al., 2001) or decrease (Capdeville et al., 1994) in TPMT enzyme activity, which could alter the optimal drug dose and the therapeutic outcome.

The (TPMT/huCYC) gene expression ratio decreased during the treatment from 4.8 at baseline to 3.5 at week 20 (p=0.02, Figure 10A) which could reflect an inhibition of PDNS by the thiopurines, leading to less purines available for mRNA synthesis. This finding needs to be confirmed, and the mechanisms behind this observation need to be clarified. Recently, it has been shown that thiopurines inhibit expression of several inflammatory genes in activated T-lymphocytes (Thomas et al., 2005).
In contrast to the TPMT gene expression, the enzyme activity did not change during treatment (Figure 10B). In the PP group, the median enzyme activity did not differ at subsequent weeks compared to baseline (p>0.05). We found, however, large inter-individual differences in the change of TPMT during treatment. In four patients, there was a discrepancy between phenotype (TPMT activity $\leq 8.9$ U/ml pRBC) and genotype ($TPMT^{*1/*1}$). In these misclassified patients, the enzyme activity increased 12-61% during treatment (Figure 11).

Figure 10. Median (quartile 1; quartile 3) of TPMT gene expression (A, n=10) and TPMT enzyme activity (B, n=27) during the first 20 weeks of thiopurine treatment.

Figure 11. TPMT enzyme activity during the 20-week course of study in four TPMT wild-type patients with a baseline TPMT activity below the cutoff for high enzyme activity ($\leq 8.9$ U/ml pRBC).
TPMT genotyping

In paper II, we established a pyrosequencing method for genotyping of ten TPMT SNPs, including the common 460G>A, 719A>G and 238G>C as well as seven less frequently occurring SNPs. Pyrosequencing is a flexible and convenient methodology for genotyping, enabling a high throughput approach, and gives easily interpreted results presented in Pyrograms (Figure 12).

![Pyrogram](image)

**Figure 12.** Pyrosequencing of the 460G>A SNP included in the TPMT*3A, TPMT*3B and TPMT*3D alleles. Representative Pyrograms illustrate; A) individuals with a wild-type G/G; B) a heterozygous G/A, or C) a homozygous A/A genotype. The sequence (G/A)CATTAGTTG was analysed.
A limitation with pyrosequencing, as with most genotyping methodologies used (McDonald et al., 2002) is that it does not discriminate between the two genotypes $TPMT^*/3A$ and $TPMT^*/3C$. Methods that can distinguish between the two genotypes have been developed, but they are much more labour intensive (McDonald et al., 2002; von Ahsen et al., 2004).

Prospective TPMT enzyme activity determination in red blood cells is often used as a routine safety measure before initiation of thiopurine treatment. However, the phenotyping method is time consuming and has a number of limitations. Firstly, children with newly diagnosed ALL often receive transfusions of erythrocytes. A TPMT-deficient patient receiving erythrocyte transfusion from a $TPMT$ wild-type individual would be phenotyped as an intermediate activity phenotype. TPMT enzyme activity cannot be reliably determined within 30-60 days after transfusion (Schwab et al., 2001; Cheung & Allan, 2003). Secondly, circulating red blood cells do not constitute a homogenous cell population. Red blood cells have a life span of 120 days and during that time they undergo progressive ageing. Deficient red blood cell production is the cause for anaemia in children with ALL, and the relative excess of older red blood cells, which has lower TPMT activity than younger, often leads to these children displaying lower activity at diagnosis than a control group (Lennard et al., 2001). In these circumstances, $TPMT$ genotyping would be a better alternative for determination of the inherited capacity to metabolise thiopurines (Schaeffeler et al., 2004).

**TPMT allele frequencies in a Swedish population**

(Paper II)

The frequencies of the three SNPs 238G>C, 460G>A and 719A>G were investigated in 800 DNA samples from a biobank from south-eastern Sweden. Of the 800 samples, 730 displayed none of these SNPs. Seventy individuals were heterozygous for one non-functional $TPMT$ allele. In total
91% of the population were genotyped as \textit{TPMT}*1/*1, 7.5% were \textit{TPMT}*1/*3A, 0.88% were \textit{TPMT}*1/*3C, 0.25% were \textit{TPMT}*1/*3B, and 0.13% (one individual) was genotyped as \textit{TPMT}*1/*2. The allele frequencies are summarised in Table VI. None of the 800 individuals was identified as a carrier of two non-functional alleles, although previous estimates indicate an occurrence of 1 in 300 individuals (Weinshilboum & Sladek, 1980). However, in our laboratory 15 homozygous TPMT-deficient individuals of about 2500 individuals totally have been identified during routine prospective TPMT activity determinations.

\begin{table}[h]
\centering
\begin{tabular}{|l|c|}
\hline
\textbf{Genotype} & \textbf{Allele frequencies} \\
\hline
\textit{TPMT}*3A (460G>A, 719A>G) & 3.75 \\
\textit{TPMT}*3C (719A>G) & 0.44 \\
\textit{TPMT}*3B (460G>A) & 0.13 \\
\textit{TPMT}*2 (238G>C) & 0.06 \\
\hline
\end{tabular}
\caption{	extit{TPMT} variant alleles identified in a Swedish population (n=800)}
\end{table}

The allele frequencies of \textit{TPMT}*3A and *3C in the Swedish population are in agreement with results obtained in other Caucasian populations (Collie-Duguid \textit{et al}., 1999; Loennechen \textit{et al}., 2001; Rossi \textit{et al}., 2001). The allele \textit{TPMT}*3B was more common and the \textit{TPMT}*2 allele less frequent compared to other populations. \textit{TPMT}*3B has to our knowledge only been detected in a few individuals (Otterness \textit{et al}., 1997). Still, only genotyping for \textit{TPMT}*3A and \textit{TPMT}*3C will identify 99% of non-functional alleles in a population.
Concordance between TPMT genotype and phenotype and identification of TPMT*14 and TPMT*15

(Papers II and III)

To determine the concordance between genotype and phenotype, we selected 30 individuals based on their TPMT activity. Characteristics of these individuals are summarised in Table VII.

Table VII. Findings in 30 subjects selected according to their TPMT phenotype and genotyped with pyrosequencing

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Treatment at sampling</th>
<th>Phenotype U/mL pRBC</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>UC</td>
<td>AS, GC</td>
<td>0.3</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>Crohn’s</td>
<td>TP</td>
<td>0.3</td>
<td>*3A/*3C</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>UC</td>
<td>NT</td>
<td>0.4</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>UC</td>
<td>NT</td>
<td>0.4</td>
<td>*3A/*3A</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>UC</td>
<td>TP, AS</td>
<td>0.4</td>
<td>*3A/*3A</td>
</tr>
<tr>
<td>6</td>
<td>29</td>
<td>UC</td>
<td>GC</td>
<td>0.8</td>
<td>*3A/*3A</td>
</tr>
<tr>
<td>7</td>
<td>59</td>
<td>Crohn’s</td>
<td>TP, AS</td>
<td>0.8</td>
<td>*3A/*3A</td>
</tr>
<tr>
<td>8</td>
<td>59</td>
<td>UC</td>
<td>NT</td>
<td>0.8</td>
<td>*3A/*3C</td>
</tr>
<tr>
<td>9</td>
<td>33</td>
<td>UC</td>
<td>GC</td>
<td>6.5</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td>HV</td>
<td>NT</td>
<td>6.9</td>
<td>*1/*2</td>
</tr>
<tr>
<td>11</td>
<td>21</td>
<td>UC</td>
<td>NT</td>
<td>7.2</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>12</td>
<td>35</td>
<td>OD</td>
<td>TP, GC</td>
<td>7.2</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>13</td>
<td>46</td>
<td>Crohn’s</td>
<td>AS</td>
<td>7.3</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>14</td>
<td>56</td>
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<td>AS</td>
<td>7.4</td>
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<tr>
<td>15</td>
<td>22</td>
<td>UC</td>
<td>AS</td>
<td>7.6</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>16</td>
<td>50</td>
<td>UC</td>
<td>AS</td>
<td>7.7</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>17</td>
<td>52</td>
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<td>TP</td>
<td>7.8</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>18</td>
<td>49</td>
<td>UC</td>
<td>AS, GC</td>
<td>8.1</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>19</td>
<td>35</td>
<td>Crohn’s</td>
<td>AS, GC</td>
<td>8.5</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>20</td>
<td>58</td>
<td>UC</td>
<td>AS</td>
<td>8.6</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>21</td>
<td>46</td>
<td>UC</td>
<td>TP</td>
<td>8.9</td>
<td>*1/*3A</td>
</tr>
<tr>
<td>22</td>
<td>60</td>
<td>HV</td>
<td>NT</td>
<td>9.8</td>
<td>*1/*1</td>
</tr>
<tr>
<td>23</td>
<td>61</td>
<td>Crohn’s</td>
<td>AS</td>
<td>11.0</td>
<td>*1/*1</td>
</tr>
<tr>
<td>24</td>
<td>51</td>
<td>HV</td>
<td>NT</td>
<td>11.6</td>
<td>*1/*1</td>
</tr>
<tr>
<td>25</td>
<td>18</td>
<td>UC</td>
<td>TP, AS</td>
<td>12.4</td>
<td>*1/*1</td>
</tr>
<tr>
<td>26</td>
<td>61</td>
<td>HV</td>
<td>NT</td>
<td>13.2</td>
<td>*1/*1</td>
</tr>
<tr>
<td>27</td>
<td>28</td>
<td>HV</td>
<td>NT</td>
<td>14.8</td>
<td>*1/*1</td>
</tr>
<tr>
<td>28</td>
<td>59</td>
<td>HV</td>
<td>NT</td>
<td>15.9</td>
<td>*1/*1</td>
</tr>
<tr>
<td>29</td>
<td>18</td>
<td>Crohn’s</td>
<td>TP, AS, GC</td>
<td>17.1</td>
<td>*1/*1</td>
</tr>
<tr>
<td>30</td>
<td>12</td>
<td>Crohn’s</td>
<td>AS</td>
<td>19.6</td>
<td>*1/*1</td>
</tr>
</tbody>
</table>

TP; Thiopearine, AS; aminosalicylate, GC; glucocorticoid, NT; no treatment, UC; Ulcerative Colitis, Crohn’s; Crohn’s disease, HV; Healthy volunteer, OD; Other diagnosis
Nine individuals with enzyme activity ≥ 9U/ml pRBC, at the locally established cutoff for intermediate/high enzyme activity carried none of the ten investigated SNPs, and they were therefore considered as wild type (TPMT*1/*1, Table VII).

Thirteen individuals displayed intermediate TPMT activity (6.5-8.9 U/ml pRBC). These were genotyped as TPMT*1/*3A (n=12) or TPMT*1/*2 (n=1). Of the 30 individuals, eight showed very low enzyme activity (0.3-0.8 U/ml pRBC); four were genotyped as TPMT*3A/*3A and two as TPMT*3A/*3C (Table VII).

Two patients with 0.3 and 0.4 U/ml pRBC in enzyme activity were carriers of the 460G>A and 719A>G nucleotide substitutions, the two most commonly reported SNPs in the TPMT gene. These SNPs are commonly situated on the same allele, giving the genotype TPMT*1/*3A (Figure 4, Table VII). However, this genotype was in conflict with their low enzyme activity. The low enzyme activity therefore indicates that they were carriers of two non-functional alleles. There is a possibility that they were compound heterozygous TPMT*3B/*3C. We were not able to discriminate between the two possible genotypes (TPMT*1/*3A or TPMT*3B/*3C) by pyrosequencing. During the course of our studies, TPMT*11, TPMT*12, and TPMT*13 (Hamdan-Khalil et al., 2003; Schaeffeler et al., 2003) representing rare sequence variants were identified and thus not genotyped for.

To clarify the genetic basis for the TPMT deficiency in the two patients, sequencing of exons III to X of the TPMT gene, including exon-intron borders, was carried out in the patients and their parents (paper III) and the TPMT enzyme activity was determined in the parents (Figure 13). The mother and father in family 1 had enzyme activities of 5.0 and 6.3 U/ml pRBC, respectively, indicating a heterozygous genotype. This was an indication, that the patient might have inherited one non-functional allele from each of the parents. The parents were genotyped for the ten SNPs by pyrosequencing, as described in paper II and the mother revealed to be a carrier of both 460G>A and 719A>G, whereas the father had none of the investigated SNPs, indicating that the patient was a carrier of the TPMT*3A allele, in combination with a hitherto unknown allele inherited from the father. Manual sequencing of the TPMT gene was performed and resulted
in identification of an A>G transition in the start codon (exon III) of the TPMT gene (TPMT*14, Figure 13). This new sequence variant was found in the patient as well as in the father.

The male patient (family 2) displayed a TPMT activity of 0.4 U/ml pRBC and the parents revealed enzyme activities of 6.9 and 6.0 U/ml pRBC, respectively, indicating heterozygosity. In this family, the TPMT*3A allele was identified in the father. The mother was wild type for the ten investigated SNPs. Manual sequencing showed that the proband and the mother were carriers of a G>A transition at the splice site between intron VII and exon VIII (TPMT*15, Figure 13).

The two variants were deposited in the NCBI SNP database (dbSNP), TPMT*14 (1A>G, Met>Val, ss12709560) and TPMT*15 (IVS7-G>A, ss12709561). The novel sequence variants were located in highly conserved regions of the TPMT gene and resulted in decreased enzyme
activity both in the heterozygous form (parents) and in combination with \textit{TPMT*3A} (children).

\textit{TPMT*14} abolishes the start codon of the protein (Met>Val). We investigated the theoretical consequences of the new alleles, and a bioinformatics prediction with the NCBI ORF finder reveals the use of an in-frame downstream ATG as a start codon and a truncated protein (170 amino acids) as a result instead of the wild-type TPMT protein that has 245 amino acids.

\textit{TPMT*15} potentially alters the splice site between intron VII and exon VIII. One possible consequence of \textit{TPMT*15} is a deletion of exon VIII in the final protein, resulting in a frame shift and a premature stop codon in exon IX. The length of the truncated protein would therefore be 173 amino acids instead of the 245-aminoacid wild-type protein. Pyrosequencing for \textit{TPMT*14} and \textit{TPMT*15} in 200 DNA samples from a Swedish biobank detected no individuals with any of the new alleles. We conclude that \textit{TPMT*14} and \textit{TPMT*15} represent rare \textit{TPMT} alleles (paper III).

Our results show that employing a genotyping approach as an alternative to routine phenotyping may miss rare genetic variants affecting the inherited capacity of an individual to metabolise thiopurines, and the existence of so far uncharacterised sequence variants must be considered, for example by using methodology that detects unknown sequence variants.

So far, many studies in rather small groups (<200) have been made concerning the correlation between genotype and phenotype (Otterness \textit{et al.}, 1997; Yates \textit{et al.}, 1997; Spire-Vayron de la Moureyre \textit{et al.}, 1998b; Hon \textit{et al.}, 1999; Alves \textit{et al.}, 2001; Loennechen \textit{et al.}, 2001; Rossi \textit{et al.}, 2001; Indjova \textit{et al.}, 2003; Larovere \textit{et al.}, 2003; Reis \textit{et al.}, 2003). Recently, a large genotype-phenotype investigation in a German population of 1214 individuals was performed (Schaeffeler \textit{et al.}, 2004). The overall concordance in that study between \textit{TPMT} genotype and phenotype was 98.4%, with a sensitivity and specificity for genotyping compared to enzyme activity, of 90% and 99%, respectively.

However, that study identified by genotyping 6 of 111 heterozygous individuals, with a TPMT activity in the high range, and 17 of 1096 presumed carriers of two functional alleles with intermediate activities.
These seventeen individuals with intermediate enzyme activity were genotyped as TPMT*1/*1, showing a discrepancy between genotype and phenotype. By denaturing HPLC and DNA sequencing, new sequence variants in four individuals were found in exons III, IV, V and VII, all leading to amino acid changes. These new variants were designated TPMT*16, TPMT*17, TPMT*18 and TPMT*19, respectively (Schaeffeler et al., 2004; Hamdan-Khalil et al., 2005). In the remaining 13 individuals, no further variants were found (Schaeffeler et al., 2004). An earlier identified but not published allele, TPMT*9, was also detected.

Interestingly, a subpopulation of ultrarapid metabolisers was identified by Schaeffler et al.(2004). This large-scale study demonstrates the robustness and accuracy of TPMT genotyping for genetic polymorphisms in the TPMT gene, but these observations also indicate the existence of still unrecognised modulators of TPMT activity, which could be genetic, epigenetic or non-genetic in origin (Krynetskiy & Evans, 2004).

From our results we suggest that phenotyping should be used for determining the metabolic capacity of the patient before start of thiopurine treatment. However, genotyping should be considered as an important alternative, especially in patients who have received blood transfusions recently or in ALL patients at diagnosis.
Pharmacogenetics during initiation of thiopurine treatment – *TPMT*, *ITPA* genotypes and adverse events

(Paper IV)

Of 60 patients with IBD (paper IV), 1 was genotyped as *TPMT*3A/*14 (Table VIII), 7 were heterozygous for one non-functional *TPMT* allele (*TPMT*1/*3A), and 52 were wild type (*TPMT*1/*1). As previously described, the patient with very low enzyme activity (*TPMT*3A/*14) was excluded and five non-compliant patients were withdrawn. Median TPMT enzyme activity at baseline was comparable in the two study groups; for the group completing the study per protocol (PP group) it was 12.1 (10.7; 13.2) U/ml pRBC, and for the group withdrawn due to adverse events (AE group), 11.8 (9.35; 14.0) U/ml pRBC (p=0.45).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n (%)</th>
<th>biobank</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TPMT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPMT*1/*1</td>
<td>52 (87%)</td>
<td></td>
</tr>
<tr>
<td>TPMT*1/*3A</td>
<td>7 (12%)</td>
<td></td>
</tr>
<tr>
<td>TPMT*3A/*14</td>
<td>1 (1.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>ITPA 94C&gt;A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITPA 94C/C</td>
<td>53 (88%)</td>
<td>174 (87%)</td>
</tr>
<tr>
<td>ITPA 94C/A</td>
<td>6 (10%)</td>
<td>25 (12.5%)</td>
</tr>
<tr>
<td>ITPA 94A/A</td>
<td>1 (1.7%)</td>
<td>1 (0.5%)</td>
</tr>
</tbody>
</table>

The frequency of the *ITPA 94C>A* polymorphism was investigated in 200 DNA samples from a biobank, as well as in the 60 patients (Table VIII) included in paper IV. Allelic frequencies for the C and A allele in the biobank were 0.93 and 0.07, respectively. Allele frequencies were in agreement with other studies in Caucasian populations (Cao & Hegele, 2002; Sumi *et al.*, 2002; Marsh *et al.*, 2004).

It has been suggested that patients carrying the *ITPA 94C>A* transition in the *ITPA* gene could experience adverse events due to accumulation of thio-ITP (Marinaki *et al.*, 2004). In paper IV, we did not find any
association between the *ITPA* 94C>A allele and occurrences of adverse events overall (p=0.35), or with subgroups of adverse events; leucopenia (p=1.0), abdominal pain/nausea/vomiting (p=1.0), pancreatitis (p=0.39), myalgia/arthralgia (p=0.39) or hepatotoxicity (p=1.0). Our results are in accordance with a recently published study (Gearry *et al.*, 2004).

Five of six *TPMT* heterozygous patients failed to complete the study, *TPMT* heterozygous patients (*TPMT*1/*3A) were withdrawn earlier from the study because of adverse events as compared to wild-type patients (p=0.039, Figure 14).

![Figure 14](image.png)

**Figure 14.** Kaplan-Meier analysis of estimated probabilities to remain in the study subdivided for *TPMT* genotypes. The P value, derived from the log-rank test, refers to the overall trend for remaining in the study.

In our study, a heterozygous *TPMT* genotype led to higher TGN values during the first five weeks than a wild type genotype, but the meTIMP levels differed significantly only at week 3 (Table IX).

The AE group formed higher concentrations of TGN and meTIMP (Figure 15); however, the PP group and the AE group did not differ significantly in concentrations except at week 6 for meTIMP. Neither was there any statistical difference from week 1 to week 5 in the formation rate of TGN or meTIMP between the two groups (p=0.08 and 0.3, respectively). The decrease in metabolite levels in the AE group from week 7 (n=10) must be regarded with caution, due to the small numbers of patients at week 8 (n=5) and week 12 (n=3).
Table IX. Differences in thiopurine metabolite concentrations according to TPMT genotype

<table>
<thead>
<tr>
<th>TGN (pmol/8x10⁸ RBC)</th>
<th>meTIMP (pmol/8x10⁹ RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>TPMT*1/*1</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>43.6 (26.4; 60.3)</td>
</tr>
<tr>
<td>2</td>
<td>93.4 (74.1; 115)</td>
</tr>
<tr>
<td>3</td>
<td>145 (119; 207)</td>
</tr>
<tr>
<td>4</td>
<td>173 (111; 222)</td>
</tr>
<tr>
<td>5</td>
<td>184 (139; 264)</td>
</tr>
<tr>
<td>6</td>
<td>187 (149; 247)</td>
</tr>
</tbody>
</table>

TGN=Thioguanine nucleotides, meTIMP=methyl thioinosine monophosphate, W=Week number, n=number of individuals. Median (quartile 1; quartile 3). P for Mann Whitney U test between groups; p<0.05 was considered significant.

There was no difference in metabolite formation comparing patients cotreated with aminosalicylates or corticosteroids and patients not treated with these medications. Our study shows that adverse events leading to treatment cessation or dose reduction are due to other factors in addition to variable TPMT enzyme activity. The importance of other polymorphic enzymes such as ITPase, needs to be further evaluated before being employed as a routine investigation before start of thiopurines.

![Figure 15](image_url)  
Figure 15. Median (quartile 1; quartile 3) of A) TGN and B) meTIMP levels during the 20 weeks in the per protocol (PP) group and adverse events (AE) group.
Based on the findings in papers I-IV, the following conclusions can be made:

- A sensitive, specific, and robust real-time RT-PCR methodology to quantify TPMT gene expression was developed. In our study of immediately isolated RNA, we found a correlation between gene expression and TPMT enzyme activity within the high enzyme activity group showing that the variation in enzyme activity is due to variations in gene transcription.

- If blood was collected in PAXgene™ blood collection tubes and stored either at room temperature, or in a refrigerator or freezer before RNA isolation, the TPMT mRNA was not as stable as stated by the manufacturer. We conclude that other stabilisation techniques should be used before sending samples for isolation of RNA and investigations of the TPMT mRNA.

- TPMT gene expression decreased during thiopurine treatment, which could be a result of decreased PDNS mediated by meTIMP. In contrast, the TPMT enzyme activity was stable during treatment. However, in TPMT wild-type patients misclassified by phenotyping, a 12-61% increase was found during the 20-week treatment, indicating that for some individuals, baseline TPMT activity is not representative for activity during treatment with thiopurines.

- Pyrosequencing represented a flexible, easy and fast methodology for TPMT genotyping. The allele frequencies of TPMT*3A and TPMT*3C were in agreement with other studies in Caucasians, but the frequency of
TPMT*2 was lower and TPMT*3B higher than previously found in Caucasians.

- Two new non-functional TPMT alleles were found (TPMT*14 and TPMT*15) in two individuals from two different families.

- TPMT heterozygous IBD patients had a lower probability of tolerating a standardised dose regimen of thiopurines in comparison to TPMT wild-type patients. The TPMT genotype was, however, not predictive for the occurrences of subgroups of adverse events when compared to wild-type patients. Neither was the ITPA 94C>A polymorphism predictive of adverse events during thiopurine treatment.
It is anticipated that pharmacogenetics will be used more frequently in the future to identify patients at a higher risk of developing specific adverse events due to deficiencies in drug metabolism and response. Further, pharmacogenetics can lead to the development of new treatment and prevention approaches as well as provide guidance for individualisation of treatment choice.

TPMT is a clinically relevant example of the utility of pharmacogenetics. However, uncertainty remains regarding the regulation of TPMT enzyme activity in individuals with the wild-type allele, and the predictive value of measurement of metabolites formed during treatment with thiopurines. Many challenges exist due to the complexity of thiopurine metabolism. To employ measurement of metabolites as a therapeutic drug monitoring tool, future research should be focusing on a better understanding of the contribution to the clinical effects of phosphorylated and methylated metabolites (i.e., TGN and meTIMP). Today, thiopurine metabolites are often measured in patients treated with thiopurines, although the knowledge about clinically relevant reference intervals for these metabolites is inadequate. It has been proposed that a ratio between meTIMP and TGN is indicative of efficacy/toxicity of the treatment, however, yet conflicting results exist. It has also been proposed that separate measuring of the different ribonucleotide and deoxyribonucleotide forms of TG instead of the sum of these nucleotides (TGN) would better correlate to the toxicity.

Measuring of metabolites in combination with TPMT phenotype and/or genotype, as well as other enzymes involved in the metabolism of thiopurines, would be a valuable tool for future monitoring of thiopurine
therapy. However, the importance of, and relation between the different metabolites formed from different enzymatic reactions must first be clarified.
ACKNOWLEDGEMENTS

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