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Azathioprine Pharmacogenetics: The Relationship between 6-Thioguanine Nucleotides and Thiopurine Methyltransferase in Patients after Heart and Kidney Transplantation

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Dedicated to Prof. Dr. Dr. J. Büttner on the occasion of his 65th birthday

Summary: The commonly used immunosuppressive regimen after solid organ transplantation consists of cyclosporine A, azathioprine and steroids. Azathioprine, which is known to carry the risk of severe myelosuppression, is catabolized in vivo by xanthine oxidase and thiopurine methyltransferase, an enzyme which exhibits a common genetic polymorphism; 11% of Caucasians are heterozygous and 0.3% are homozygous with respect to thiopurine methyltransferase deficiency. Toxicity and immunosuppressive effects have been attributed to the 6-thioguanine nucleotides generated from azathioprine. We have studied thiopurine methyltransferase activity and 6-thioguanine nucleotide concentrations in erythrocytes from 39 heart and kidney recipients. Erythrocyte thiopurine methyltransferase was determined by a radioenzymatic assay and erythrocyte 6-thioguanine nucleotide concentration with HPLC. Thiopurine methyltransferase activity [median (range, 10^{th}–90^{th} percentile)] was significantly ($p < 0.05$) higher in patients ($n = 39$) receiving azathioprine [285 (218–362) vs. 262 (160–352) mU/l erythrocytes] than in healthy blood donors as controls ($n = 120$). When stratified according to thiopurine methyltransferase phenotype, one patient homozygous for the low allele exhibited an excessive erythrocyte 6-thioguanine nucleotide concentration (2210 pmol/0.8 · 10^9 erythrocytes). Heterozygous patients had significantly higher 6-thioguanine nucleotide concentrations (median: 435 pmol/0.8 · 10^9 erythrocytes) compared with concentrations in patients homozygous for the high allele (median: 86 pmol/0.8 · 10^9 erythrocytes; $p < 0.01$), although the azathioprine dosage did not differ ($p = 0.66$). Erythrocyte thiopurine methyltransferase determination therefore identifies patients at high risk of accumulating 6-thioguanine nucleotides. The monitoring of this enzyme may contribute to the safer management of immunosuppressive therapy with azathioprine. Alternative regimens such as cyclosporin A/mycophenolate mofetil or tacrolimus should also be considered for this patient group.

Introduction

In immunosuppressive cyclosporin-based triple therapy after organ transplantation, azathioprine is often used as an antiproliferative agent. Azathioprine represents the S-imidazole derivative of 6-mercaptopurine and is rapidly cleaved to this drug in vivo by glutathione-S-transferase (1). It is generally agreed that 6-thioguanine nucleotides, which are synthesised from 6-mercaptopurine through several enzymatic steps including hypoxanthine-guanine-phosphoribosyl transferase (2), are the major toxic species of 6-thiopurine derivatives (fig. 1) (3, 4). The determination of 6-thioguanine nucleotides in erythrocytes is therefore an accepted means of estimating toxicity and efficacy of 6-mercaptopurine treatment in patients with acute leukaemia (5–7) and after solid organ transplantation (8–10). 6-Thiopurines are catabolised by two enzymes, xanthine oxidase and thiopurine methyltransferase. Xanthine oxidase is not genetically polymorphic and if not pharmacologically blocked this pathway leads to 6-thiouric acid. In contrast, thiopurine methyltransferase displays a common genetic polymorphism (11–13). This polymorphism leads in 0.3% of Caucasians to a complete deficiency of the enzyme, and in about 11% to a heterozygous form with an enzyme activity of 40–60%, compared with individuals with the two high alleles (14). An accumulation of 6-thioguanine nucleotides is therefore likely to occur if one of these catabolising pathways is altered. Thiopurine methyltransferase is generally determined in erythrocytes, which are readily accessible, and there is a close correlation between activities measured in erythrocytes and in other cells and organs e.g. liver, kidney and leukocytes (15–17). Erythrocyte thiopurine methyltransferase of

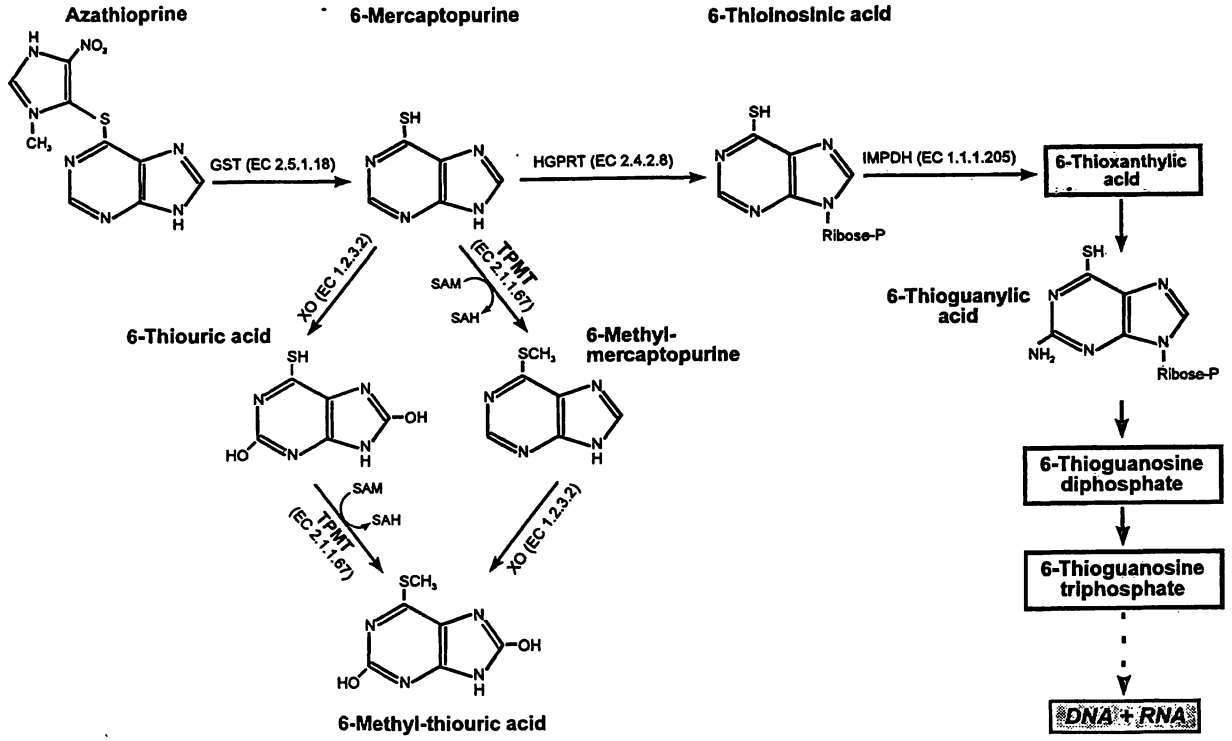

Fig. 1 Metabolic pathway of azathioprine
GST: Glutathione-S-transferase; HGPRT: hypoxanthine-guanine phosphoribosyl transferase; IMPDH:Inosine monophosphate dehydrogenase; XO: Xanthine oxidase; TPMT: Thiopurine methyl transferase; SAH: S-Adenosyl homocysteine; SAM: S-Adenosyl methionine

randomly selected healthy Europeans typically shows a biphasic frequency distribution, consistent with a co-dominant inheritance (12, 13). One single point mutation, which leads to a substantial reduction of catalytic activity has been described (18) and recently a predominant 2-point mutation, which is present in about 70% of thiopurine methyltransferase deficient subjects was discovered (19). Further factors complicating therapeutic management of thiopurines are their widely variable bioavailability (20, 21) and an increase of thiopurine methyltransferase activity during therapy consistent with an induction phenomenon (10, 22). These might be the main reasons for the fact, that until now, only one study has shown a close negative correlation between thiopurine methyltransferase and 6-thioguanine nucleotides (23).

In patients receiving thiopurines for malignancies, i. e. where leukocyte suppression is desired, the dosage is mainly adjusted to achieve low white blood cell count. In contrast, in immunosuppressive triple therapy, azathioprine is most commonly used in a protocol-based standard dosage and only reduced if leukopenia occurs. On the other hand, transplant recipients, compared with acute lymphatic leukaemia and acute myeloic leukaemia patients under maintenance therapy, are at higher risk for complications of leukopenia since they receive a combination of immunosuppressive drugs. They may therefore particularly profit from therapeutic drug monitoring of the toxic 6-thioguanine nucleotides. Furthermore, in these patients it would be of benefit to investigate the correlation of 6-thioguanine nucleotides and thiopurine methyltransferase in vivo, since if thiopurine methyltransferase phenotype is correlated with toxic metabolites, a stratification of each patient according to thiopurine methyltransferase activity prior to therapy may aid in planning subsequent immunosuppressive therapy, particularly with respect to the new immunosuppressants.

We studied erythrocyte thiopurine methyltransferase activity and 6-thioguanine nucleotide concentrations in 39 patients after heart and kidney transplantation under stable clinical conditions at least 3 months after the last blood transfusion, to elucidate the relationship between erythrocyte 6-thioguanine nucleotides and thiopurine methyltransferase activity.

Patients and Methods

Probands

Twenty-one heart transplant recipients and 18 kidney recipients with generally uncomplicated postoperative courses were included in this study. Thirty-eight patients had undergone transplantation at least 3 months prior to the investigation and none had blood transfusions within this time. The only exception was one thiopurine methyltransferase-deficient patient who was tested 2 months after transplantation. The immunosuppressive regimen consisted of cyclosporin A, the dosage being adjusted according to its concentrations in blood (200–350 µg/l in heart recipients, 150–200 µg/l in kidney recipients), steroids, which were tapered during the postoperative period, and azathioprine at a daily dosage median of 1 mg/kg body weight. Some patients received antilymphocyte antibodies as therapy induction for a maximum of 7 days immediately after transplantation. Patients with comedication with drugs which can potentially influence thiopurine metabolism, particularly xanthine oxidase inhibitors, and patients with unstable kidney function (e. g. due to rejection episodes) were excluded. If patients were

tested several times, the latest time point was considered for statistical evaluation unless creatinine concentrations were increased compared with earlier measurements in the same patient.

For comparison, blood samples of 120 healthy blood donors were also analysed for their erythrocyte thiopurine methyltransferase activity.

Methods

Erythrocyte preparation

Erythrocytes were obtained from NH_4-heparinate anticoagulated whole blood by centrifugation at 2000 *g* for 10 min. Two washing steps were performed with *Hank*'s balanced salt solution under the same conditions. Cells were finally rediluted to a haematocrit of about 0.40 and the exact haematocrit and red blood cell count for calculations of thiopurine methyltransferase activity and 6-thioguanine nucleotide concentration were determined with a Coulter STKS cell counting device. Samples were stored at −90 °C until analysis of thiopurine methyltransferase or 6-thioguanine nucleotides.

Thiopurine methyltransferase assay

Erythrocyte lysates were analysed for their thiopurine methyltransferase activity by a modified method according to *Weinshilboum* et al. (24). As proposed elsewhere, S-[*methyl*-^{3}H]adenosylmethionine was used as methyl donor (12) and the chelating step was omitted since erythrocytes lack interfering enzymes requiring bivalent cations (15). Lysates were incubated for 1 h at 37 °C with 6-mercaptopurine, S-[*methyl*-^{3}H]adenosyl-methionine, dithiothreitol, and allopurinol. The formed [*methyl*-^{3}H]6-mercaptopurine was liquid/liquid extracted with 120 mmol/l isoamyl alcohol in toluene and the radioactivity was determined by β-scintillation in a model LS3801 counter (Beckman Instruments, Munich, Germany). The radioactivity of the total reaction mixture was also determined. Results were corrected for quench, chemical and radiochemical purity of S-adenosylmethionine and extraction efficacy (56−65%; determined in each run), and expressed as mU/l packed erythrocytes). These modifications yielded an improvement of between day variation of the erythrocyte thiopurine methyltransferase assay from 9.5% to 5.9%. Within-series imprecision was 4.1−9.0% (100−350 mU/l erythrocytes) in our laboratory with a dynamic range of this procedure of 17−920 mU/l erythrocytes.

6-Thioguanine nucleotide assay

Erythrocyte lysates were hydrolysed in 1.5 mol/l H_2SO_4 at 98 °C for 1 hour, in order to convert the respective nucleotides to 6-thioguanine. A saturated solution of phenyl mercuric acetate in toluene/isoamyl alcohol at alkaline pH was used to extract compounds with sulfhydryl groups. After back-extraction into 100 mmol/l HCl, samples were analysed by C18 reversed phase HPLC (Hypersil ODS 5 µm 250 · 4.6 mm, MZ Analysentechnik, Mainz, Germany, guard column: RP select B 5 µm, Merck, Darmstadt, Germany) under isocratic conditions (25). 6-Thioguanine spiked drug-free erythrocyte lysates were used for construction of a calibration curve with UV detection at 342 nm. 4-Mercapto-1H pyrazolo [3,4-d] pyrimidine was used as internal standard, and was detected at 322 nm (Shimadzu SPD 10 AV, Shimadzu Europe, Großburgwedel, Germany). If deviation from the assigned haematocrit (0.35) of sample preparation was > 0.05, a correction was performed with donor blood or *Hank*'s balanced salt solution prior to the hydrolysis step. This is necessary since extraction efficacy decreases with the number of erythrocytes, with a resulting optimum at an haematocrit of about 0.35. Thioguanine concentrations were expressed as pmol/0.8 · 10^9 erythrocytes. Between-days imprecision was < 7% at concentrations of 100 pmol/0.8 · 10^9 erythrocytes and above, with a detection limit of 20 pmol/0.8 · 10^9 erythrocytes.

All reagents were of highest available purity. S-[*methyl*-^{3}H]adenosyl-methionine was purchased from Amersham (Braunschweig, Germany), isoamyl alcohol from J. T. Baker (Devender, The Netherlands) and toluene, borate, H_2SO_4, HCl and NaOH from Merck (Darmstadt, Germany). All other chemicals were from Sigma Chem. (Deisenhofen, Germany).

Statistical analyses were performed with SAS statistical package, Ver.6.01 (SAS Inst. New York, USA).

This study was approved by the local Ethics Committee.

Results

Erythrocyte thiopurine methyltransferase activity of 120 healthy donors showed a typical biphasic distribution (fig. 2) with a cut-off for the putative heterozygous group at 167 mU/l erythrocytes. The calculated cut-off

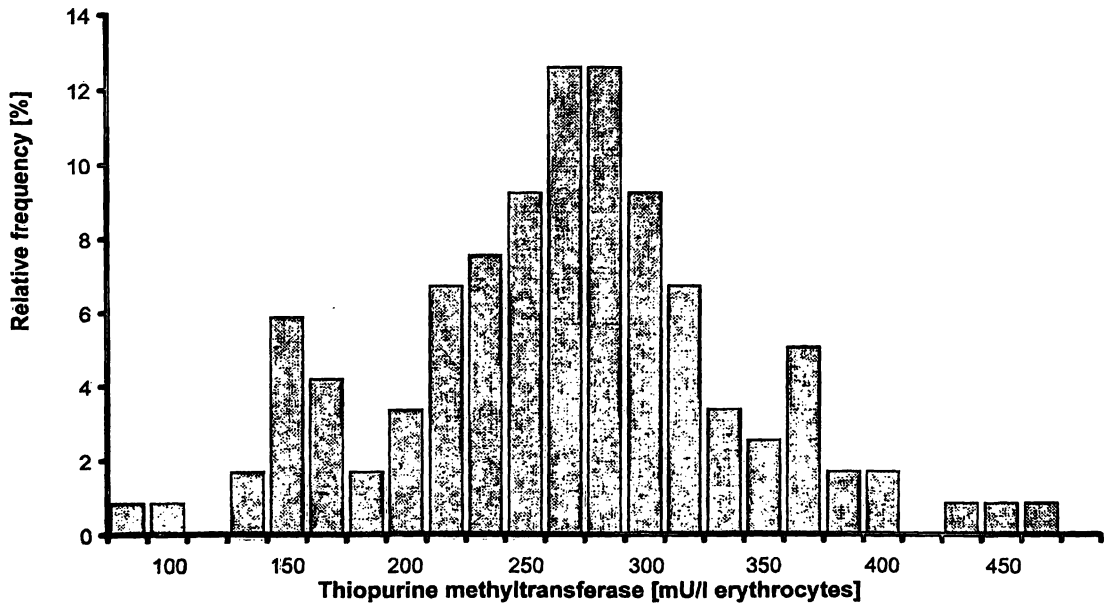

Fig. 2 Frequency distribution of erythrocyte thiopurine methyltransferase in 120 healthy blood donors without thiopurine administration. Groups are defined by their upper limit. Typical biphasic distribution with calculated lower limit for the intermediate group of 162 mU/l erythrocytes

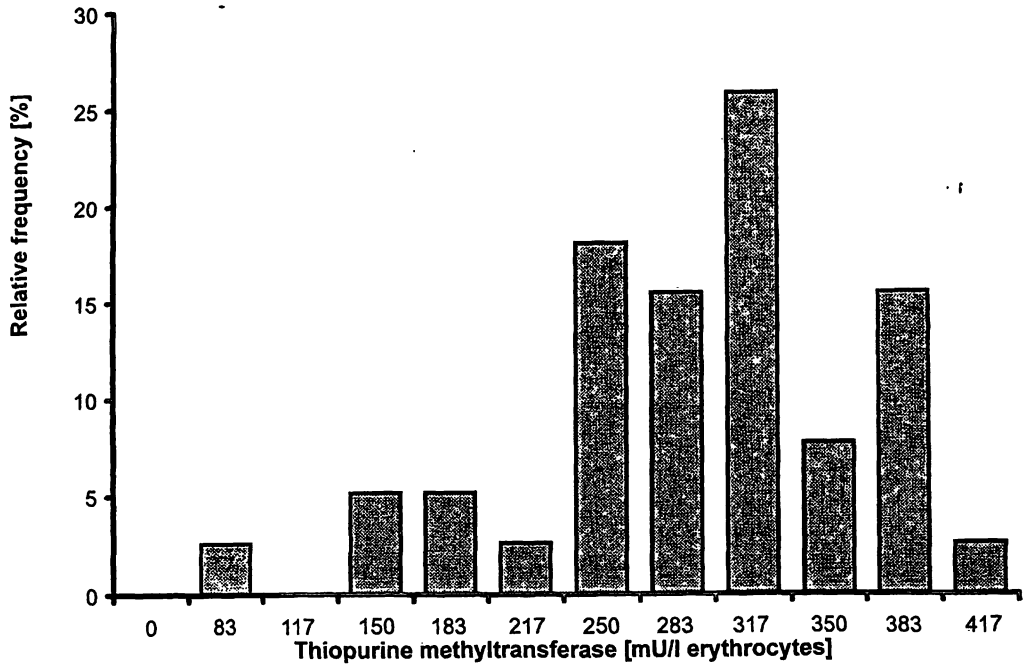

Fig. 3 Frequency distribution of erythrocyte thiopurine methyltransferase in 39 kidney and heart recipients under chronic azathioprine administration. Groups are defined by their upper limit.

point, based on the 11th percentile of this donor group was 162 mU/l erythrocytes. The median for the whole group of healthy donors was significantly lower than in the group of patients under long-term therapy with azathioprine (262 vs. 285 mU/l erythrocytes; $p < 0.05$).

In the patient group, one subject showed a total thiopurine methyltransferase deficiency and five patients were identified with an intermediate phenotype (fig. 3). The upper limit of this intermediate group was 217 mU/l erythrocytes, defined as the 11th percentile of the patient group with the exclusion of the thiopurine methyltransferase deficient patient (since taking this case under consideration would over-represent this group, which has a frequency of 0.3% in a normal population).

Erythrocyte 6-thioguanine nucleotide concentrations displayed a broad distribution from 31 to 599 pmol/0.8 $\cdot 10^9$ erythrocytes in patients with detectable erythrocyte thiopurine methyltransferase activity (fig. 4). The highest 6-thioguanine nucleotide concentration observed was 2210 pmol/0.8 $\cdot 10^9$ erythrocytes in the thiopurine methyltransferase-deficient patient. This was associated with an episode of leukopenia.

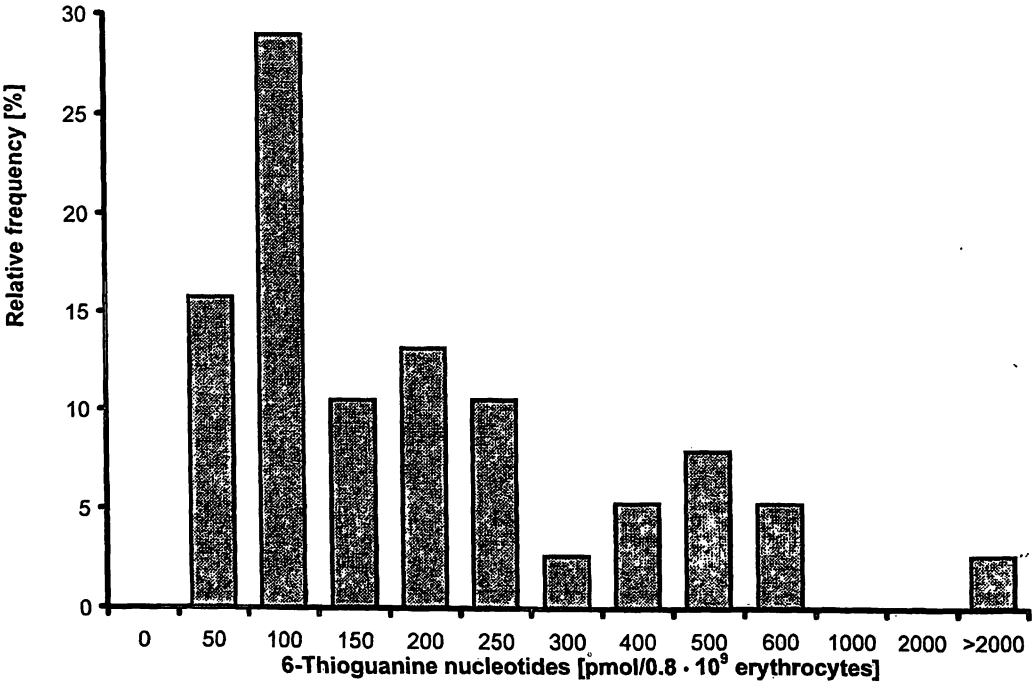

Fig. 4 Frequency distribution of erythrocyte 6-thioguanine nucleotides in 39 kidney and heart recipients under chronic azathioprine administration. Groups are defined by their upper limit.

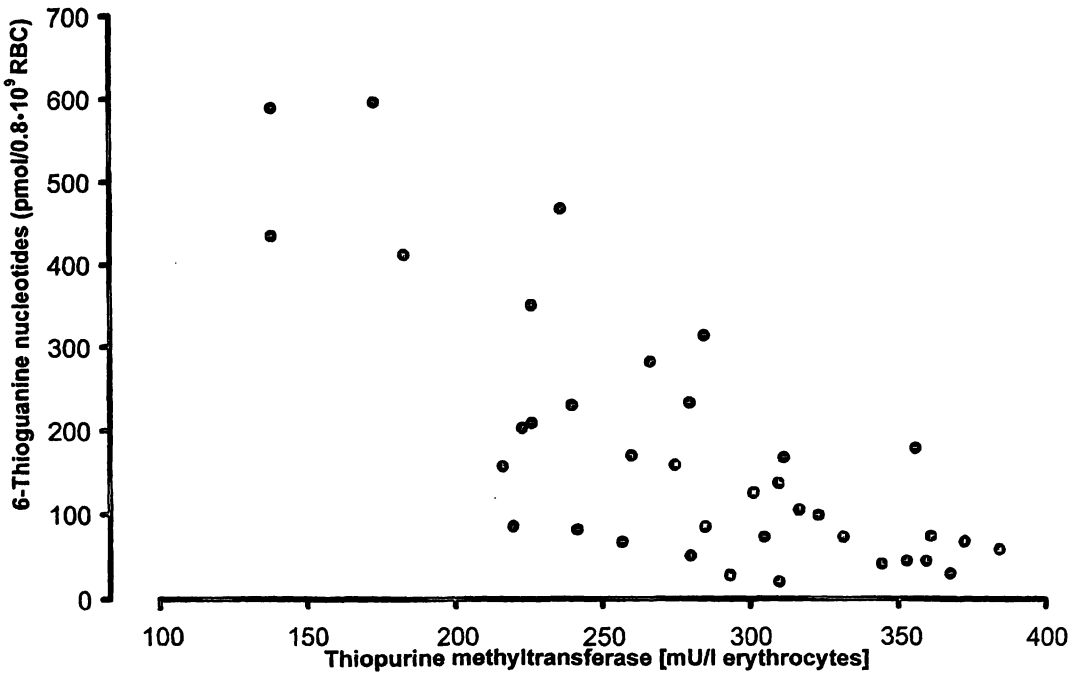

Fig. 5 Scatterplot of 6-thioguanine nucleotides vs. thiopurine methyltransferase in erythrocytes in patients receiving azathioprine. One patient with thiopurine methyltransferase deficiency is not displayed (n = 38; r = −0.785).

Tab. 1 Erythrocyte 6-thioguanine nucleotide concentrations in heart and kidney recipients stratified according to erythrocyte thiopurine methyltransferase activity.

	Thiopurine methyltransferase (mU/l erythrocytes)		
	<17 (n = 1)	17–210 (n = 5)	>210 (n = 33)
	Thioguanine nucleotide concentration (pmol/0.8 · 10^9 erythrocytes)		
Median	2210	436*	99
84th percentile	–	593	228
16th percentile	–	321	46

* $p < 0.05$ vs high thiopurine methyltransferase group

When stratified according to thiopurine methyltransferase activity, significantly higher erythrocyte 6-thioguanine nucleotide concentrations were observed in the groups with low thiopurine methyltransferase activity (tab. 1).

Azathioprine dosage did not differ between these groups (median 75 mg/day; $p = 0.88$). Furthermore there was no correlation between azathioprine daily dose and erythrocyte-6-thioguanine nucleotide concentrations ($r < 0.01$; $p = 0.431$). A highly significant negative correlation was seen between thiopurine methyltransferase activity in erythrocytes and the respective concentrations of 6-thioguanine nucleotides (fig. 5), even when the patient with total thiopurine methyltransferase deficiency was excluded ($r = -0.785$; $p < 0.01$).

Discussion

The detoxification of azathioprine, used in immunosuppressive management after organ transplantation, is dependent on the common genetic polymorphism of thiopurine methyltransferase (11–13, 14, 27). This study showed that thiopurine methyltransferase activity is obviously influenced by thiopurines and shows an increase under therapy. This is in close agreement with results from studies in which thiopurine methyltransferase was measured in patients prior to and under treatment with azathioprine or 6-mercaptopurine, and thereafter (5, 22).

Despite the well known metabolism of thiopurines, only one study has shown a close relationship between thiopurine methyltransferase activity and 6-thioguanine nucleotide concentrations (23). This might be due to several uncertainties of azathioprine therapy, such as the induction phenomenon mentioned above, different dosages, especially if dosage is adjusted according to leukocyte count, and the variable bioavailability of this drug (20, 21) which is influenced by food intake (26). In this study, we observed a close negative correlation of 6-thioguanine nucleotides and thiopurine methyltransferase in patients taking azathioprine. In these heart and kidney recipients, the observed highly significant correlation ($r = -0.785$) was probably due to the relatively constant dosage of 75 mg/day in this patient group.

High concentrations of erythrocyte 6-thioguanine nucleotides are associated with the risk of myelosuppression (5–7, 10), with related complications, such as septicaemia and consecutive multiple organ failure. As reported

(27), the patient with complete thiopurine methyltransferase deficiency and excessive 6-thioguanine nucleotide concentration subsequently died from this complication. Furthermore in a previous study, we showed that patients with 6-thioguanine nucleotide concentrations > 600 pmol/0.8 · 10^9 erythrocytes may develop leukopenia (10). These findings suggest that one might expect an increased risk of toxic side effects in such patients under treatment with higher azathioprine dosage.

In therapeutic drug monitoring for azathioprine after solid organ transplantation the lower limit of erythrocyte 6-thioguanine nucleotides is hard to define, since the immunosuppressive regimen consists of a combination of drugs and there is a lack of simple quantities for assessing the immunosuppressive state of an individual patient. The data of this study demonstrate a close relationship between thiopurine methyltransferase activity and 6-thioguanine nucleotide concentration in transplant recipients under azathioprine therapy. It would therefore seem advisable to measure erythrocyte thiopurine methyltransferase prior to transplantation, to identify those patients with low or absent activity, who obviously carry an increased risk for toxic complications due to elevated 6-thioguanine nucleotides. In patients with lower or absent thiopurine methyltransferase, the concentrations of 6-thioguanine nucleotides should be monitored during azathioprine therapy to avoid toxic effects, and dosage could be adjusted at the start of therapy by taking erythrocyte thiopurine methyltransferase activity into account. Alternatively, determination of erythrocyte thiopurine methyltransferase prior to transplantation could be used to identify patients at high risk of azathioprine toxicity, who might more appropriately be treated with one of the new immunosuppressants such as tacrolimus or mycophenolate mofetil.

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