MONITORING OF CYCLOSPORIN AND AZATHIOPRINE IN ORGAN TRANSPLANTATION

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Cyclosporin A (CsA) is a cyclic undecapeptide that was first isolated from the fungus *Tricoderma polysporum*. The introduction of this potent immunosuppresive compound significantly improved the outcome of solid organ and bone marrow allograft transplantation. There is now a large body of accumulated experience in the monitoring of this drug, and several consensus conferences have addressed this issue /1-3/. Azathioprine in CsA-based triple therapy with steroids is still widely used in organ transplantation even though it is known to carry the risk of severe myelosuppression. Measurement of white blood cell (WBC) and platelet counts is generally used to recognize azathioprine toxicity. However, recent reports /4-6/ have indicated that patients with a genetically determined deficiency in thiopurine methyltransferase (TPMT), an important enzyme in azathioprine metabolism, are at high risk for myelosuppression. Monitoring of active azathioprine metabolites, which are responsible both for the immunosuppressive action and for the toxicity of azathioprine, may be necessary in such cases. It should also be noted that the incidence of posttransplant lymphoproliferative disorder (PTLD) is a function of the intensity of immunosuppression /7,8/. Thus the combination of CsA, azathioprine and steroids is associated with an approximately three-fold higher incidence of PTLD than low dose CsA guided by therapeutic drug monitoring /8/.

KEY WORDS

cyclosporin, azathioprine, transplantation, drug monitoring

CYCLOSPORIN

The immunosuppresive properties of CsA are due to its ability to suppress T-lymphocyte responses. After uptake in lymphocytes cyclosporin binds with high affinity to cytoplasmic protein receptors termed immunophilins. In T-cells the predominant cytoplasmic receptor is a 17 kDa protein termed cyclophilin /9/. The CsA-cyclophilin complex inhibits calcineurin, a serine-threonine protein phosphatase, thereby impairing the dephosphorylation and subsequent nuclear translocation of the transcription factors NF-KB and NF-AT /10/. As a consequence, activation and transcription of the IL-2 gene are reduced. In view of the complexity of these steps, the question arises as to whether whole blood drug levels can predict functional impairment of lymphocyte alloreactivity and the incidence of rejection. Efforts are therefore being made to develop strategies for pharmacodynamic monitoring of cyclosporin, such as measuring the inhibition of calcineurin /11/.

In considering the monitoring of an immunosuppresive drug such as cyclosporin, the critical issues listed in Table 1 must be taken into account. Semiautomated immunoassay techniques involving selective monoclonal antibodies are generally used for routine measurement of cyclosporin. Most laboratories appear to use one of the following three immunoassays: the Incstar CYCLO-Trac SP radioimmunoassay, the Abbott TDx monoclonal antibody fluorescence polarization immunoassay (mFPIA) and the Syva enzyme multiplied immunoassay technique (EMIT). Performance characteristics of analytical methods

TABLE 1

Critical issues in the monitoring of immunosuppressive drugs

- The specificity requirements of the analytical methods need to be defined.
- The necessity for pharmacokinetic monitoring must be considered, i.e. pre-dose trough concentrations versus area-under-the-curve (AUC) determination.
- A time-dependent therapeutic range may be required.
- The question as to whether metabolite concentrations should be determined must be considered.
- An effective quality control program has to be implemented.

for CsA determination, which have been discussed at a recent consensus meeting /3/, are still an important issue.

Precision requirements are by and large fulfilled by all three commonly used immunoassays. According to a recent survey /12/ the following coefficients of variation can be obtained with semi-automated immunoassays at cyclosporin concentrations in the range of 50-350 µg/l: EMIT, 7-15%; CYCLO-Trac SP 5-11%; mFPIA, 3-10%. In our own laboratory using an improved EMIT assay /13/ the between series coefficients of variation are 6.8%, 4.3%, and 5.7% at cyclosporin concentrations of 76, 191 and 420 µg/l respectively. Compared to HPLC all the specific immunoassays overestimate cyclosporin blood concentrations. The mean overestimates for cyclosporin concentrations range from 8 to 30% with the EMIT, from 22 to 30% with the CYCLO-Trac SP and from 24 to 48% with the mFPIA /12/. Particularly in patients who may show an accumulation of cyclosporin metabolites (e.g. liver transplant recipients) results for the same sample can differ by as much as 100% depending upon which of the specific assays is used. This is illustrated by the following case (Fig. 1) in which cyclosporin concentrations were determined by mFPIA, CYCLO-Trac SP, EMIT and the highly specific monoclonal RIA (Sandimmun-Kit) from Sandoz in a retransplanted liver recipient with a deranged cyclosporin metabolite/ parent drug ratio. In this patient with protracted severe cholestasis and increased cyclosporin metabolite concentrations, only the results obtained by EMIT agreed well with those determined by the highly specific Sandimmun-Kit. With the CYCLO-Trac SP and mFPIA larger deviations were found from the values obtained by the specific Sandimmun-Kit. The data confirm that the EMIT shows a higher specificity compared to mFPIA and CYCLO-Trac SP. Since cyclosporin metabolites do not appear to significantly contribute to overall immunosuppression, the use of a less specific immunoassay in patients who accumulate cyclosporin metabolites could cause a physician to underdose the patient.

Whole blood with EDTA as anticoagulant is the preferred matrix for cyclosporin measurement. Currently most centers use trough cyclosporin blood concentrations to guide dosage of this drug. However, patients displaying trough concentrations within a putative therapeutic range are not always spared from either rejection or nephrotoxicity /14/. Because of the marked intra- and interindividual

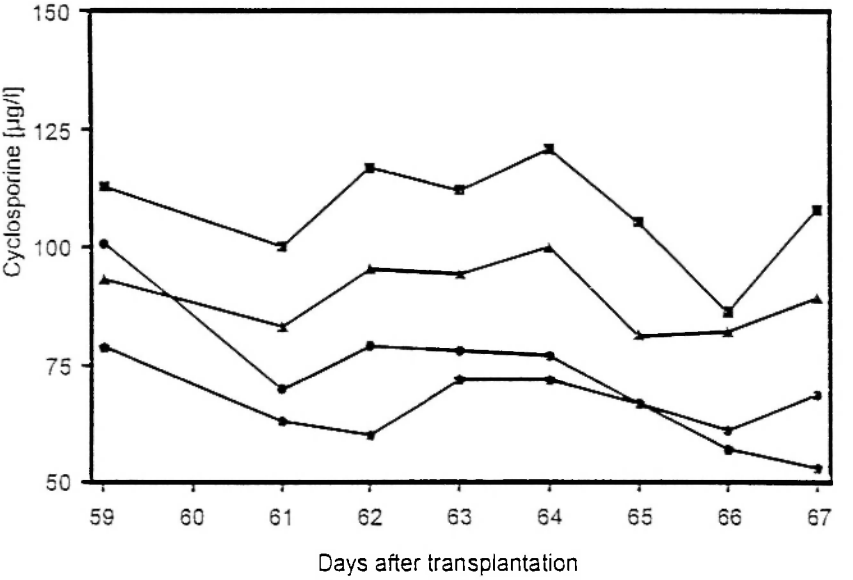

Fig. 1: Whole blood CsA concentrations determined in a liver transplant recipient with severe cholestasis. CsA was measured using the following procedures: (■) mFPIA, (▲) CYCLO-Trac SP, (●) EMIT, (◆) Sandimmun Kit.

variation in cyclosporin pharmacokinetics using conventional oral liquid and gel cap formulations, an area-under-the-curve (AUC) strategy was developed to allow identification of optimal target AUC concentrations /15/. Subsequently it was shown that 2- and 3-point sampling could yield a reasonable estimate of actual AUC /16,17/. Lindholm and Kahan /18/ have carefully investigated the influence of cyclosporin, pharmacokinetics, trough concentrations and AUC monitoring on outcome after kidney transplantation. So far, such comparative studies on AUC vs trough level monitoring have not consistently shown overall superiority of either method. The major practical limitation for AUC monitoring is the necessity for precisely timed blood sampling. According to a recent study of Kahan *et al.* /19/ a much improved correlation between trough levels and AUC is obtained with the new microemulsion formulation of cyclosporin, Neoral ($r = 0.91$) as compared to the standard non microemulsion

formulation (r = 0.71). The use of Neoral is associated with a reduced variability in cyclosporin pharmacokinetics implying single trough levels will be more consistent. At present trough concentration monitoring is the most appropriate and practical method for guiding cyclosporin dosage.

A key problem with cyclosporin monitoring is still the establishment of appropriate time-dependent consensus therapeutic ranges. It is difficult to establish a therapeutic range since there are no simple parameters for the assessment of the immunosuppressive effect. Based on their own and published experience transplant centers have derived therapeutic ranges empirically for the different transplant types. In 1994-1995 a survey was conducted of 35 transplant centers around the world /3/. The centers were asked to report their immunosuppressive protocol and current therapeutic ranges for cyclosporin for the different organ types. Figures 2 and 3 show the CsA therapeutic ranges for liver and heart transplant recipients that are in use at the different centers. As can be seen there is substantial variability in target therapeutic ranges between centers. Most centers use higher target concentrations during the early postoperative period and doses are then tapered to a lower maintenance concentration range 3 to 6 months after transplantation. On the basis of the data obtained in the survey, preliminary recommendations have been drawn up for the therapeutic ranges of cyclosporin in kidney, liver and heart transplantation (Table 2). The ranges refer to the use of cyclosporin in triple therapy and to whole blood levels determined with a specific method such as HPLC or one of the selective immunoassays, for example EMIT or CYCLO-Trac SP. In keeping with the observation that there is a high probability of toxicity at trough concentrations >400 µg/l during maintenance therapy, the therapeutic ranges at all centers do not exceed this value. It is notable that higher cyclosporin concentrations are recommended after heart transplantation during both induction and maintenance therapy (Table 2). This is presumably due to the fact that in the case of an acute rejection, a retransplantation of this organ is generally unsuccessful. The higher cyclosporin blood levels are therefore intended to provide greater security with regard to immunosuppressive efficacy. In general higher cyclosporin levels are also recommended during the induction phase after liver transplantation. However, it should be noted that certain centers /3/ are using lower therapeutic ranges for both liver and kidney

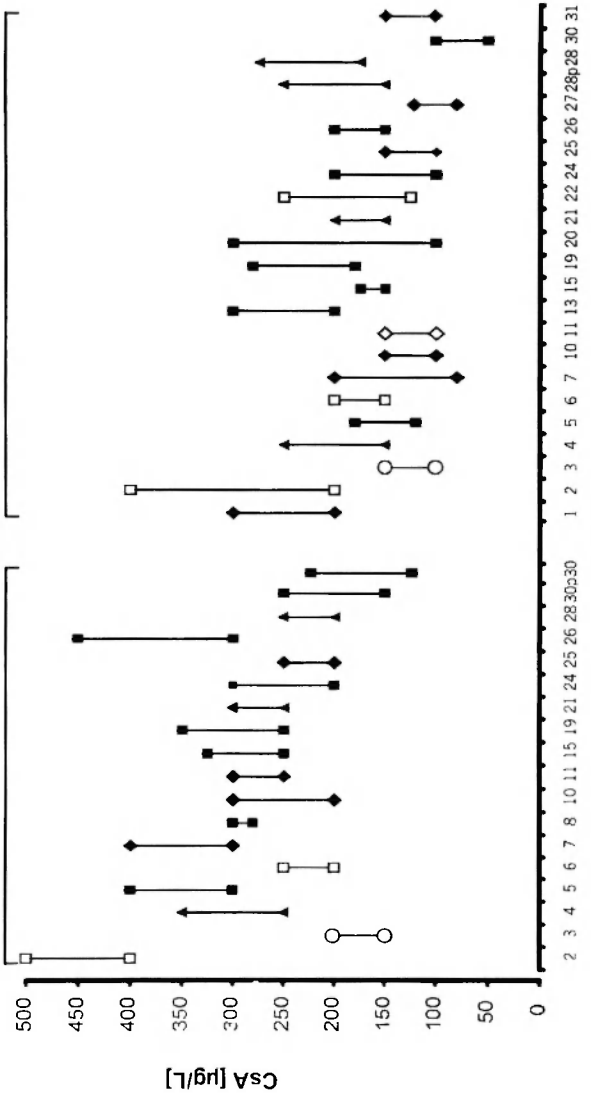

Fig. 2: Comparison of the therapeutic CsA ranges for liver transplant recipients in use at different transplant centers (see 13 for a list of the centers) Initial therapy refers to the first 6 months after transplantation. Closed symbols represent those centers using triple therapy (CsA, steroids, azathioprine); open symbols refer to centers using double therapy (CsA, steroids). Methods: HPLC (◆), mFPIA (■), CYCLO-Trac SP (▲), EMIT (●).

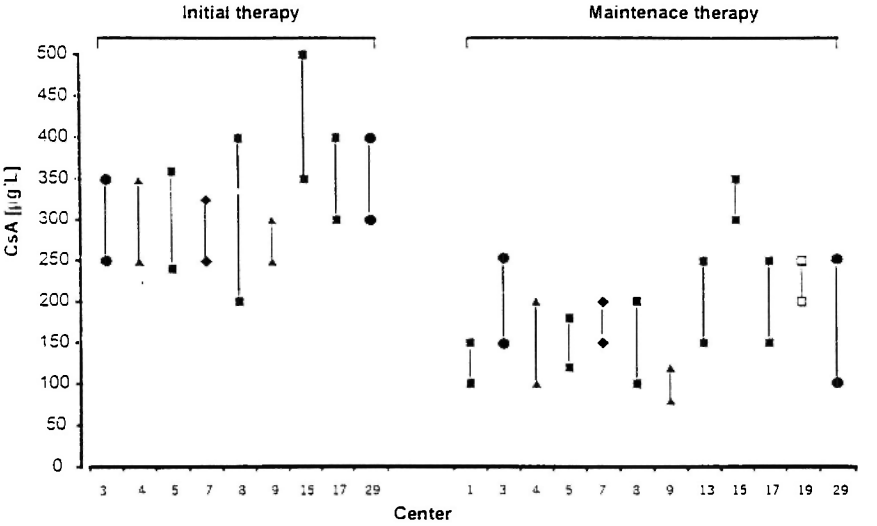

Fig. 3: Comparison of the therapeutic CsA ranges for heart transplant recipients in use at different transplant centers (see /3/ for a list of the centers). Initial therapy refers to the first 6 months after transplantation. Closed symbols represent those centers using triple therapy (CsA, steroids, azathioprine); open symbols refer to centers using double therapy (CsA, steroids). Methods: HPLC (◆), mFPIA (■), CYCLO-Trac SP (▲), EMIT (●).

transplantation without any evidence of an increased incidence of rejection.

The question as to whether the therapeutic ranges for cyclosporin need to be raised or lowered cannot be definitively answered at the moment. There has been increasing emphasis in some centers over the past few years on using higher dosages and maintaining higher cyclosporin blood concentrations. In a recent publication Soin *et al.* /20/ found a significantly lower incidence of chronic rejection in those patients who were maintained on median cyclosporin blood trough levels >175 µg/l in the first 28 days after transplantation. In patients with simultaneous pancreas-kidney transplants /21/ it was found that acute rejection can be virtually eliminated and cyclosporin toxicity minimized by achieving cyclosporin blood concentrations between 300 and 400 µg/l as measured by mFPIA for approximately 4 months

TABLE 2

Preliminary recommendations for therapeutic ranges of cyclosporin

Transplant type	Therapeutic ranges (μg/l)*	
	Induction therapy	Maintenance therapy
Kidney	150 - 225	100 - 150
Liver	225 - 300	100 - 150
Heart	250 - 350	150 - 250

*Ranges refer to use of cyclosporin in triple therapy (with steroids and azathioprine) and to whole blood levels measured with a specific analytical procedure (HPLC, EMIT, CYCLO-Trac SP).

after transplantation. Drug toxicity was observed in patients with cyclosporin levels above 400 μg/l and acute rejection in those patients with cyclosporin levels below 300 μg/l. During maintenance therapy cyclosporin blood levels between 200 and 300 μg/l were recommended.

The guidelines for cyclosporin monitoring as established at the Lake Louise Consensus Conference /3/ are as follows: Trough concentrations of cyclosporin in whole blood should be determined, sampling times being standardized to within 1 hour prior to the next dose. The recommended frequency of monitoring is once every 24 to 48 hours in the immediate posttransplant period. The analytical method used for cyclosporin determination should be specific for the parent drug. To validate and maintain the quality of the method for measuring cyclosporin, participation in an external quality assurance program is essential. Monitoring of cyclosporin metabolites seems not to be warranted in the majority of clinical situations. Cyclosporin concentrations need to be interpreted in conjunction with other laboratory data, clinical considerations and concomitant immunosuppresive therapy. Whether the introduction of the new microemulsion formulation for CsA will affect its therapeutic window

remains to be established. The appropriateness of current therapeutic ranges for CsA when this drug is used in combination with the newer immunosuppressive drugs such as mycophenolate mofetil and rapamycin also needs to be examined.

The clinical relevance of cyclosporin metabolites is still a controversial issue. Some immunosuppressive activity has been found *in vitro* for metabolites AM1, AM9 and AM4N. Furthermore, there is evidence from a study published by Christians *et al.* /22/ that high blood concentrations of metabolites AM1c9 and AM19 are associated with nephrotoxicity in the early postoperative period following liver transplantation. From the study design, however, it was not possible to ascertain whether high metabolite concentrations were the cause or consequence of renal dysfunction. Donatsch *et al.* /23/ investigated the effects of subcutaneous doses of cyclosporin, AM1, AM1A, AM1c and AM4N in a rat model. Statistically significant differences were only observed for serum creatinine and bilirubin concentrations after administration of cyclosporin. Renal or hepatic dysfunction could not be demonstrated after exposure of the animals to the cyclosporin metabolites. However, because of the known differences in the metabolism of cyclosporin in rats and humans and interindividual susceptibilities to cyclosporin toxicity, definite conclusions cannot be made as to the contribution of cyclosporin metabolites to toxicity in humans. Some centers have advocated the parallel use of both non-specific and specific methods in order to gain insight into the ratio between cyclosporin and its metabolites in transplant recipients with severely disturbed liver function. Wonigeit *et al.* /24/ observed that during acute rejection episodes in liver transplant recipients, liver function deteriorated and this led to a marked increase of non-specific cyclosporin levels. Non-specific cyclosporin levels above 1000 µg/l were accompanied by an increase in the serum creatinine concentration. It was suggested that concentrations of parent drug and metabolites exceeding 1200 µg/l as determined with the Incstar non-specific monoclonal RIA should be avoided. For the large majority of clinical situations, however, monitoring of cyclosporin metabolites seems not to be warranted. From the available data it cannot be decided whether the specific measurement of individual cyclosporin metabolites will be of clinical significance.

AZATHIOPRINE

Azathioprine is still commonly used as an antiproliferative agent in immunosuppressive CsA-based triple therapy after organ transplantation. Monitoring of azathioprine therapy is complicated by the fact that this pro-drug is rapidly metabolized to 6-mercaptopurine which is further metabolized to the 6-thioguanine nucleotides (6-TGN). The latter are responsible for the immunosuppressive action of azathioprine, as well as for its toxic properties such as myelosuppression.

There are three major metabolic pathways for the catabolism of 6-mercaptopurine (Fig. 4):

- oxidation to 6-thiouric acid via xanthine oxidase;
- methylation of the aromatic sulfhydryl residue via thiopurine methyltransferase (TPMT);
- formation of 6-thioguanine nucleotides through hypoxanthine-guanine phosphoribosyltransferase.

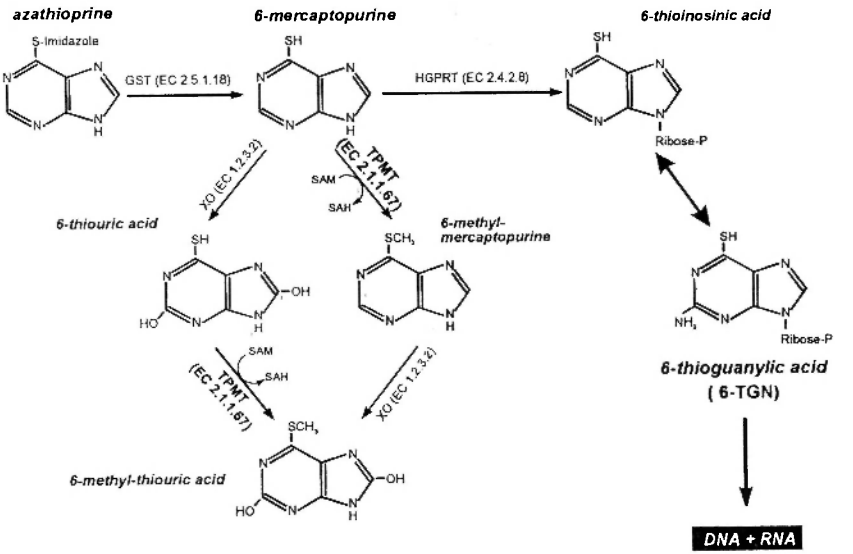

Fig. 4: Metabolic pathways of azathioprine. GST: glutathione S-transferase; HGPRT: hypoxanthine guanine phosphoribosyl transferase; TPMT: thiopurine methyltransferase; XO: xanthine oxidase.

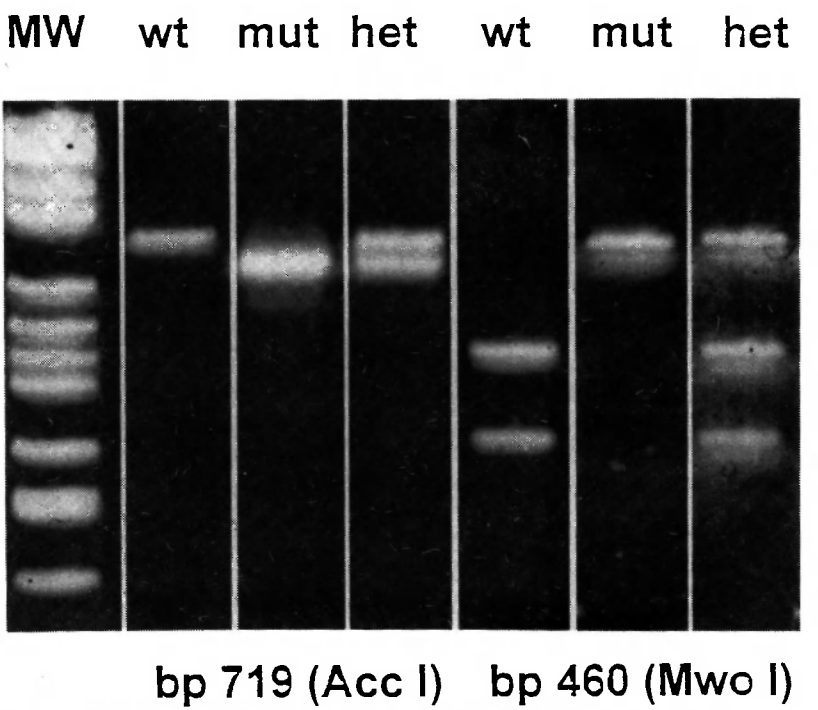

Fig. 5: PCR-RFLP analysis of wild type (wt) DNA and DNA from individuals heterozygous (het) or homozygous (mut) for the TPMT*3 mutation. PCR products were digested by *AccI* for detection of the $A^{719}\rightarrow G$ mutation and with *MwoI* for detection of the $G^{460}\rightarrow A$ mutation. MW: DNA molecular weight markers.

Catabolism of 6-mercaptopurine via either xanthine oxidase or TPMT will consequently reduce the formation of the active 6-TGN. While xanthine oxidase is not genetically polymorphic, large population studies have revealed a polymorphism of erythrocyte TPMT activity /25-27/. Approximately 11% of Caucasians are heterozygous for a deficiency in this enzyme while 0.3% are homozygous for TPMT deficiency. Two functionally inactive alleles have now been characterized in the TPMT gene, the rare TPMT*2 mutation and the commoner TPMT*3 that is present in over 75% of cases. The molecular defect of the TPMT*2 mutation is a $G^{238}\rightarrow C$ transversion which leads to an Ala-Pro substitution /28/. The TPMT*3 mutant

allele contains two nucleotide transition mutations ($G^{460}\rightarrow A$ and $A^{719}\rightarrow G$) leading to the amino acid substitutions $Ala^{154}\rightarrow Thr$ and $Tyr^{240}\rightarrow Cys$ /29,30/. PCR based methods have now been developed to detect these mutations in genomic DNA, either using mutation specific primers, or taking advantage of changes to specific restriction sites. A restriction fragment length polymorphism (RFLP) analysis of individuals heterozygous and homozygous for the TPMT*3 mutant allele is illustrated in Fig. 5.

Recent clinical investigations have demonstrated that cellular accumulation of 6-TGN is inversely related to TPMT activity /31,32/. Patients with homozygous TPMT deficiency accumulate excessively high 6-TGN concentrations in erythrocytes, and presumably in other hematopoetic tissues /4-6/. This accumulation can have dramatic clinical consequences as depicted by our own experience /5/ with a heart transplant recipient in whom a post-hoc diagnosis of TPMT deficiency was made after the patient died of sepsis following three episodes of severe neutropenia. The patient was initially administered conventional doses of azathioprine, but after 4 weeks azathioprine was stopped because of a steady decline in the leukocyte count. After leukocyte levels had recovered, azathioprine therapy was reinitiated but this was followed by a second bout of leukopenia and the drug was discontinued once more. Azathioprine was then given at a very low dose but this again led to a new episode of toxic myelosuppression. The patient developed pneumonia and died on postoperative day 96 with multiple organ failure due to septicemia. Retrospectively, highly elevated red blood cell (RBC) 6-TGN levels (> 2211 pmol/$8x10^8$ RBC) were observed in this case of homozygous TPMT deficiency. Similarly high RBC 6-TGN levels were recently observed in a 14 year-old girl with HLA-B27 associated spondylarthritis who developed pancytopenia in the seventh week of an initially uncomplicated course of azathioprine therapy (3 mg/kg b.wt.). TPMT was found to be below the detection limit of the assay (< 1 nmol/[mlRBCxh]) and RBC 6-TGN was dramatically elevated at 2394 pmol/$8x10^8$ RBC. Bone marrow examination showed complete aplasia. Azathioprine medication was discontinued and the patient was treated with antibiotics, antimycotics and G-CSF. RBC 6-TGN decreased within 5 weeks to a value of 319 pmol/$8x10^8$ RBC paralleled by a normalization of blood counts. Molecular genetic analysis (Fig. 5) revealed that the patient and her mother are homozygous for the

TPMT*3 mutation, whereas her father and two siblings are heterozygous for this mutation.

That the white blood cell count alone is not sufficient for the monitoring of azathioprine therapy is shown by our experience with another heart transplant recipient in whom a fall in the leukocyte count led to a withdrawal of azathioprine /33/. The patient subsequently developed severe rejection and died. Retrospectively we found that this patient did not have TPMT deficiency and that 6-TGN concentrations at the time of azathioprine withdrawal were extremely low. Azathioprine intolerance was apparently not the cause of the myelosuppression seen in this patient.

On the basis of these observations it would appear desirable to screen for TPMT deficiency in those patients who are candidates for azathioprine therapy since in the case of low or absent TPMT activity there is a severe risk of life-threatening myelosuppression under azathioprine treatment. The determination of RBC 6-TGN metabolites may be required to monitor therapy in such patients. According to our experience, 6-TGN levels >430 pmol/8x10^8 RBC should be considered as toxic. Alternatively another immunosuppressive regimen should be considered, such as tacrolimus with low dose steroids or cyclosporin in combination with mycophenolate mofetil.

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