From Therapeutic Drug Monitoring To Drug Management: IMPDH Activity As Novel Surrogate Marker For Dose Adjustment Of Mycophenolate In Immunosuppressive Therapy

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Abstract

Background: Mycophenolate mofetil (MMF) and enteric-coated mycophenolate sodium (EC-MPS) are well established in immunosuppressive therapy after renal transplantation. The active substance, mycophenolic acid, leads to an inhibition of inosine-monophosphate-dehydrogenase (IMPDH) activity in peripheral mononuclear cells. Adverse side effects, most prominently gastrointestinal (GIT) side effects, lead to dose reduction in more than half of the patients, increasing the risk for acute rejection. Therapeutic drug monitoring (TDM) by measuring the trough level shows no relevant correlations with MPA-AUC values, and is consequently obsolete for assessing the risk of graft loss. Therefore the residual IMPDH activity in stable maintenance renal transplant patients was determined in order to suggest trusted intervals allowing monitoring of the level of immunosuppression in the context of dose-reduction. In order to compare the efficacy of the two galenisms, MMF and EC-MPS, respectively, the effect of different dosing patterns of MMF and EC-MPS on IMPDH activity in stable patients after renal transplantation was analyzed.

Materials and Methods: IMPDH activity (pmol/s per pmol AMP) was measured in patients in the maintenance phase after renal transplantation. Besides MMF or EC-MPS, immunosuppressive therapy consisted of calcineurin inhibitor with or without steroids. From 276 maintenance renal transplant patients 2432 individual trough levels and 73 kinetics were obtained for delineation of trusted intervals; for comparing the efficacy of the two galenisms, 260 measurements in 110 patients (82 on MMF, and 28 on EC-MPS) were performed.

Results: Mean patient age range of 43 women and 67 men was 22 to 74 years. Mean serum creatinine in the MMF group was 1.7 ± 1.3 mg/dL compared to 1.48 ± 0.45 mg/dL in the EC-MPS group ($P < .05$). A mean overall residual activity of 38.9 ± 45.9 pmol/s pmol AMP (median = 28 pmol/s pmol AMP) was derived from a total of 2505 individual measurements. The residual IMPDH activity of 10% of measurements was below 6 pmol/s pmol AMP (10th percentile = 5 pmol/s pmol AMP), the 20th percentile was 12 pmol/s pmol AMP, while the 80th and 90th percentile established as 56 and 78 pmol/s pmol AMP, respectively. The median IMPDH activity

in the EC-MPS patients was lower than in the MMF patients (10 vs. 24 pmol/s pmol AMP; *P* < .005). This was especially pronounced in patients on 1440 mg/d EC-MPS compared with 2000 mg/d MMF (*P* < .001).

Conclusion: Measurement of IMPDH activity in renal transplantation patients adds additional information on the degree of immunosuppression. We can assume that any residual activity below the tenth percentile indicates very strong immunosuppression, possibly pointing to an increased risk for infections, while values above the 80th or 90th percentile should not allow for dose reduction. Comparing MMF and EC-MPS efficacy, inhibition of IMPDH activity with EC-MPS seemed more pronounced than MMF despite formally equipotent doses.

Introduction

Mycophenolic-acid (MPA) is a selective, non-competitive inhibitor of Inosine-Monophosphate-Dehydrogenase (IMPDH) leading to the inhibition of the de-novo synthesis of guanosine-nucleotides. In human lymphocytes inhibition of IMPDH results in altered cellular proliferation with arrest in the S-phase of the cell cycle. Due to the absence of a salvage pathway, proliferating activated T-cells are severely affected by the inhibitory effects of MPA (1; 2; 3). For patients after renal transplantation MPA is used either as mycophenolate-mofetil (MMF, Cellcept) or as enteric-coated mycophenolate-Sodium (EC-MPS, Myfortic) in daily doses of 2000 mg respectively 1440 mg per day.

Since its introduction in immunosuppressive therapy more than ten years ago, Mycophenolate-Mofetil (MMF) is an established part of immunosuppressive therapy after renal transplantation. Still in the first publication of the landmark Tricontinental trial because of possibly dose-related side effects of the drug (CMV-infection, gastrointestinal disturbances, and increased cancer risk) the need for individualization depending on clinical course or other factors was mentioned (4).

Side effects of MMF are causing dose reductions in approximately 60% of the patients leading to a cumulative and increasing risk for acute rejection (5). In addition, gastrointestinal (GIT) side effects affect medical adherence of the patients with consecutive risk for graft failure (6). In addition, dose reductions of MMF are related to increased costs, mainly due to frequent hospitalization of the patients (7).

USRDS data of 3589 patients with MMF prescription and GIT complaints revealed that dosage reduction or discontinuation of mycophenolate mofetil in the first 6 months after diagnosis of GI complications was associated with significantly increased risk of graft failure and increased healthcare costs in adult renal transplant recipients (8). Another report from USRDS data of 3675 patients with gastrointestinal complications under MMF and subsequent dose reduction also disclosed an increased risk for graft loss after dose reduction or discontinuation of MMF (9).

Apart from the risk of graft rejection subsequent to dose reduction in response to side-effects, the dose has to be adjusted during the course of therapy since the risk of graft loss declines with time, while toxic effects of the immunosuppressive drugs increase (10).

The usefulness of pharmacokinetic measurements of MMF was shown in early studies stating that the Area-under the curve (AUC) of MMF is predictive of the likelihood of allograft rejection after renal transplantation in patients receiving mycophenolate mofetil (11). The two available preparations of MPA (MMF, EC-MPS) showed equivalent drug exposure measured by MPA-AUC when applied to the patients in equimolar doses. Therefore, both preparations are seen as equipotent (12; 13). A meta-analysis confirmed the bioequivalence of EC-MPS and MMF for both mycophenolate and metabolite exposure and for maximum plasma mycophenolate concentrations (14).

In different clinical trials, MPA drug exposure was correlated with the occurrence of biopsy proven acute rejection (BPAR). In a double blind trial aiming for three predefined target MPA AUC values the incidence of BPAR was lower in patients with MPA AUC values between 30 and 60 µg x h/ml (15).

The APOMYGRE Trial was a study in 137 allograft recipients treated with basiliximab, cyclosporine A, corticosteroids and MMF. Patients were randomized to receive either concentration-controlled doses or fixed-dose MMF. A novel Bayesian estimator of MPA AUC based on three-point sampling was used to individualize MMF doses. At month 12, the concentration-controlled group had fewer treatment failures and acute rejection episodes. Therefore, the authors conclude, that therapeutic MPA monitoring using a limited sampling strategy can reduce the risk of treatment failure and acute rejection in renal allograft recipients 12 months post-transplant with no increase in adverse events (16).

MPA trough levels show relevant inter- and intraindividual variability especially in patients with elevated serum creatinine and proteinuria (17; 18; 19). Clinically important, low trough levels are associated with an increased frequency of rejection (20), whereas elevated MPA trough levels are related to an increased risk for infections (21). Nevertheless, relevant correlations between MPA trough levels and MPA-AUC values could not be detected. In part this is owed to the enterohepatic recirculation 6-12 hours after administration, resulting in increased mycophenolate levels (22).

Furthermore, concomitant immunosuppressive therapy has major influence on MPA pharmacokinetics. Administration of mycophenolic acid concomitantly with Cyclosporine (CsA) or Tacrolimus significantly reduced rejection at trough levels >1.3 ng/ml (23). Since CsA has an inhibitory effect on the reabsorption of mycophenolate metabolites in the intestine, patients on CsA therapy exhibit lower MPA trough levels (24; 25). Consequently, MPA trough levels increased after discontinuation of CsA resulting in almost a doubling of MPA trough concentrations (26). Rath and Küpper give a concise overview of the influence of concomitant immunosuppressive therapy on MPA trough levels (27).

Therefore the usefulness of measuring trough levels in routine care of renal transplant recipients is doubted (28; 29; 30). To facilitate therapeutic drug monitoring, different limited sampling strategies for adult and pediatric patients after renal transplantation were established (22;31-37). Yet,

determination of the AUC is neither feasible in routine care since it requires at least three samples within two hours of administration.

The need for dose adjustment and the described insufficiency of mycophenolate pharmacokinetics make a surrogate marker based on one point t_0 measurement, with the same predictive value as the AUC, highly desirable. This report describes a novel pharmacodynamic approach which assesses the level of immunosuppression as a function of the residual activity of the IMPDH, the target enzyme of mycophenolate. It could already be demonstrated that patients with low IMPDH activity before renal transplantation experienced more MMF dose reductions within the first 3 years. In addition, patients with high IMPDH activity and MMF dose reduction showed the highest rejection rate (38).

The method described here has been applied on a total of 276 patients in the maintenance phase after renal transplantation. The data obtained from this large cohort are ranked into percentiles with the ultimate goal to suggest trusted intervals of IMDPH residual activity as a tool to assist decision-making about possible dose reduction. For a subgroup of 110 patients (82 on MMF, and 28 on EC-MPS), the results of both cohorts are being compared with respect to drug efficacy.

Materials and Methods

IMPDH Activity Assay

Sample Preparation

The samples are processed as described elsewhere (39). Peripheral blood is collected in 5ml tubes with Li-heparin as anti-coagulant and stored at room temperature. Heparin is superior to EDTA as anti-coagulant since it maintains cell viability for longer time. Within four hours after arrival of the sample to the lab, and within no more than two days of collection, the peripheral mononuclear cell fraction is isolated by density centrifugation according to a modified protocol from Glander et al. (40). Li-heparinized blood (2.5mL) is mixed with an equal volume of phosphate-buffered saline (PBS), carefully layered on 4mL Lymphodex (InnoTrain, Germany) density gradient centrifugation medium in a 15mL screw-cap polypropylene tube, and centrifuged at 1200 x g for 15 min without brake at room temperature.

The mononuclear cell fraction is collected from the interphase and transferred into a fresh 15mL screw-cap tube with 5mL PBS for washing. The cells are washed only once with PBS since repeated washing steps might cause diffusion of mycophenolate from the cells, resulting in over-estimation of the residual IMPDH activity. After centrifugation at 1200 x g for 10 min at room temperature, the supernatant is removed quantitatively. This step is crucial with respect to the assay validity, since only a minute fraction of the total mycophenolate is contained within the cells, while the vast majority (estimated 99%) is present in the plasma. Any trace of the supernatant might therefore still contain considerable amounts of mycophenolate, hence leading to a vast underestimation of the residual IMPDH activity. The cell pellet is resuspended in 250µL ice-cold HPLC-grade water, and 125µL of the sample are transferred into each of two 2mL screw-cap vials, one designated as working sample, the second as back-up. The vials are deep frozen at -80°C until assayed. In the same way, control cells

from healthy probands are prepared; these cells will be included in each assay serving as incubation control.

IMPDH activity assay

The residual IMPDH activity is assayed in a cell-free system. After careful thawing at room temperature, the patient samples and control cells are vigorously vortexed for 30 seconds to support cell lysis; insoluble cell fragments are removed by 5-minute centrifugation at 4000 x g at room temperature in a desktop centrifuge.

Cell lysate (50 µL) is added to 100 µL incubation buffer containing 1 mmol/L inosine-monophosphate (IMP) as substrate, 0.5 mmol/L NAD as co-substrate, 72 mmol/L sodium dihydrogen-phosphate, and 180 mmol/L potassium chloride (pH 7.5). After adjusting the volume to 180 µL with distilled water, the samples are transferred to a heating block for incubation at 37°C.

In presence of NAD, IMPDH converts inosine-monophosphate to xanthine 5'-monophosphate. In the subsequent high-performance liquid chromatography (HPLC) assay, the amount of synthesized xanthine 5'-monophosphate is determined together with the amount of AMP, which serves as an internal standard for normalization to the cell count.

After 2.5 hours of incubation, the reaction is stopped by adding 20 µL ice-cold 4 mol/L perchloric acid. Precipitation of denatured protein is enhanced by incubating the samples at -20°C for 10 min. After centrifugation at 13,000 rpm for 2 min in a desktop centrifuge, 170 µL supernatant are transferred to a test tube containing approximately 14 µL of 2.5 mol/L potassium carbonate solution for neutralization. The exact volume of potassium carbonate, required to achieve a final pH between pH 6 and pH 7, has to be determined for each lot of diluted, 4 mol/L perchloric acid and 2.5 mol/L potassium carbonate solution. Prior to HPLC analysis, the samples are deep-frozen at -20°C for at least 30 minutes, thawed, and centrifuged again for 5 min at 13000 rpm in a desktop centrifuge.

HPLC Chromatography

Determination of the amounts of xanthine-monophosphate and adenosine-monophosphate is carried out by ion-pair reversed-phase high-performance liquid chromatography on a computerized isocratic HPLC system from Shimadzu (Kyoto, Japan) consisting of a system controller SCL-10A VP, an HPLC pump LC-10AT VP, an autoinjector SIL-10AF, a column oven CTO-10AS VP, and an UV-VIS detector SPD-10A VP, controlled by Shimadzu LC Solution data collection software.

For the assay 6 µL of the sample are loaded onto a 250 mm x 3.1-mm Prontosil 120 to 5 ODS AQ column (Bischoff Chromatography, Leonberg, Germany). Column oven temperature is set to 40°C. Chromatographic separation is achieved using a mobile phase containing 50 mmol/L potassium-dihydrogen-phosphate, 7 mmol/L tetra-n-butyl-ammonium hydrogen sulfate, and 6% (v/v) methanol at a flow rate of 1 mL/min. The analytes are detected at 254-nm wavelength. Incubation efficacy is verified by including a sample from a healthy volunteer as incubation control in each incubation cycle.

For calibration, two standards containing 500 and 2500 pmol xanthine-monophosphate and adenosine-monophosphate, respectively, in 0.4% BSA solution are processed in several independent

experiments, and repeatedly measured, like the patient specimen: protein denaturation with perchloric acid followed by neutralization with potassium carbonate.

This calibration curve allows deducting the amount of XMP synthesized during incubation and the amount of AMP in the sample. The specific IMPDH activity was then expressed as pmol XMP synthesized per second, which was normalized to 1 pmol of AMP [pmol XMP/(pmol AMP s)].

Patients

IMPDH activity was consecutively measured in a total of 276 maintenance renal transplant patients acquired from several centers as part of the routine follow-up.

Comparison of MMF versus EC-MPS

A subgroup of 110 patients (43 women, 67 men) with an age range of 22 to 74 years with formally equipotent doses of EC-MPS and MMF was divided into two cohorts depending on the use of MMF or EC-MPS, respectively, and the efficacy being compared based on 260 measurements of IMPDH activity (39).

The MMF group of 82 patients including 34 women and 38 men had a mean age of 52 ± 11.9 years. They were treated with immunosuppressive regimens consisting of MMF with or without steroids and Tacrolimus (n = 35) or Cyclosporine (n = 31) or Everolimus/Sirolimus (n = 9). Seven patients were on MMF monotherapy. The mean time after transplantation was 4.7 years (range = 0.5–26 years).

The 28 patients in the EC-MPS group (9 women, 19 men) showed a mean age of 51 ± 11.6 years. They were prescribed immunosuppressive regimens of EC-MPS with or without steroids with Tacrolimus (n = 14) or Cyclosporine (n = 10) or Everolimus (n = 4). Their mean time after transplantation was 3.6 years (range = 0.5–13 years).

Statistical Analysis

Statistical analysis was performed with SPSS 12.0 software. For comparisons between the groups the nonparametric Mann-Whitney U test and Wilcoxon test was used. Linear relationships were described calculating Spearman's rho. In general, a *P* value < .05 was assumed to be significant.

Results

Surrogate Values for Dose Adjustment

During a period of approx. 3 years measurements from 2432 individual trough levels and 73 kinetics were obtained from 276 maintenance renal transplant patients, making a total of 2651 consecutive measurements. IMPDH residual activity shows considerable interindividual variability, going together with data from literature (38; 41), with a mean residual activity of 38.9 ± 45.9 pmol/s pmol AMP (median = 28 pmol/s pmol AMP).

The individual trough level measurements plus the t_0 values of the kinetics, making a total of 2505 values, were utilized to rank the residual activities into percentiles and to derive preliminary cut-off values for evaluating the level of immunosuppression in the context of dose-adjustment (Table 1).

Table 1: Low and high percentiles as preliminary cut-off values with distribution of measurements within the percentiles

	10^{th} percentile	20^{th} percentile	21^{st}- 79^{th} percentile	80^{th} percentile	90^{th} percentile
Activity [pmol/s pmol AMP]	5	12	13-55	56	78
No. of measurements	261	256	1475	257	254

The residual IMPDH activity of 10% of measurements was below 6 pmol/s pmol AMP (10^{th} percentile = 5 pmol/s pmol AMP), the 20^{th} percentile was calculated as 12 pmol/s pmol AMP. Twenty percent of activity values were equal to or above 56 pmol/s pmol AMP (80^{th} percentile), and ten percent of assessed activities ranged above 77 pmol/s pmol AMP (90^{th} percentile 78 pmol/s pmol AMP).

MMF and EC-MPS Dosing

In the MMF group, the mean IMPDH activity was 40.8 ± 78 pmol/s pmol AMP (median = 24 pmol/s pmol AMP), whereas in the EC-MPS group, the mean IMPDH activity was significantly lower (P < .001; Fig 1) with 19.1 ± 20.1 pmol/s pmol AMP (median = 10 pmol/s pmol AMP). There was no difference in IMPDH activity within the groups whether Tacrolimus or Cyclosporine was prescribed in addition to MMF or EC-MPS. In addition, the blood levels of Tacrolimus or Cyclosporine had no influence on IMPDH activity either in the MMF or in the EC-MPS group.

Although IMPDH activity in patients on 720 mg/d EC-MPS was lower than that of patients on 1000 mg/d MMF (median - 18 vs. 23.5 pmol/s pmol AMP), it did not reach significance (P = .27).

Among patients on 1440 mg/d EC-MPS, IMPDH activity was significantly lower than among patients with 2000 mg/d MMF (12.2 ± 10.6 vs. 34.7 ± 33 pmol/s pmol AMP; P = .001). There were no differences between the two groups with regard to concomitant medications, age, sex, time since transplantation, or graft function.

When comparing patients with standard (1440 mg/d) versus low-dose (720 mg/d) EC-MPS, there was no difference in IMPDH activity. The same was true for patients with low (1000 mg/d) versus standard (2000 mg/d) MMF doses.

When relating the suppression of IMPDH activity to the EC-MPS dose per kg body weight, there was a trend to a linear relationship (r = - .344, P = .07), which did not reach statistical significance. For MMF, this was not observed (r =- .06, P = .55).

Graft Function

Patients with EC-MPS showed better graft function measured by serum creatinine than those on MMF (creatinine: 1.47 ± 0.4 vs. 2.0 ± 1.3 mg/dL; $P < .05$; Figure 2). Within the EC-MPS group, there was no difference in kidney transplant function for patients with 720 mg/d or 1440 mg/d, whereas for patients with MMF, the best graft function was observed among patients on a dose of 2000 mg/d MMF (P - .008). In addition, the MMF dose per kilogram of body weight showed a strong inverse linear relationship to graft function (r = - .37, P = .0006). This inverse relationship between drug dose per kilogram of body weight and graft function was also be seen among EC-MPS patients (r = - .41, P = .02).

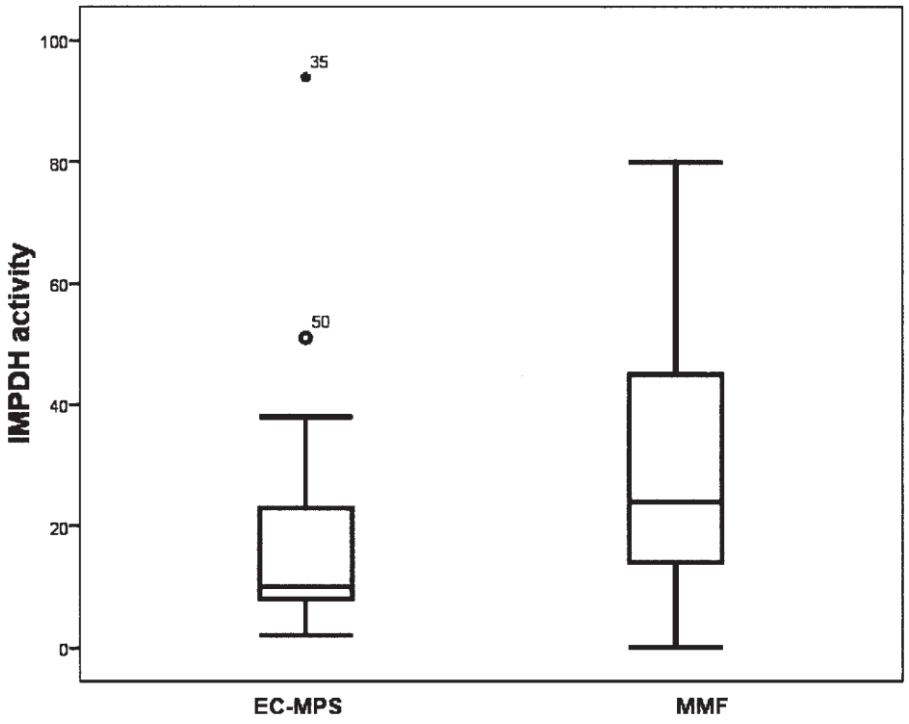

Figure 1: In 110 patients (28 on enteric-coated mycophenolate sodium [EC-MPS] and 82 on mycophenolate mofetil [MMF]), the mean inosine-monophosphate-dehydrogenase (IMPDH) activity was with 19.1 ± 20.1 pmol/s pmol AMP (median 10 pmol/s pmol AMP), significantly lower in the EC-MPS group compared with 40.8 ± 78 pmol/s pmol AMP (median 24 pmol/s pmol AMP) in the MMF group (P < .001).

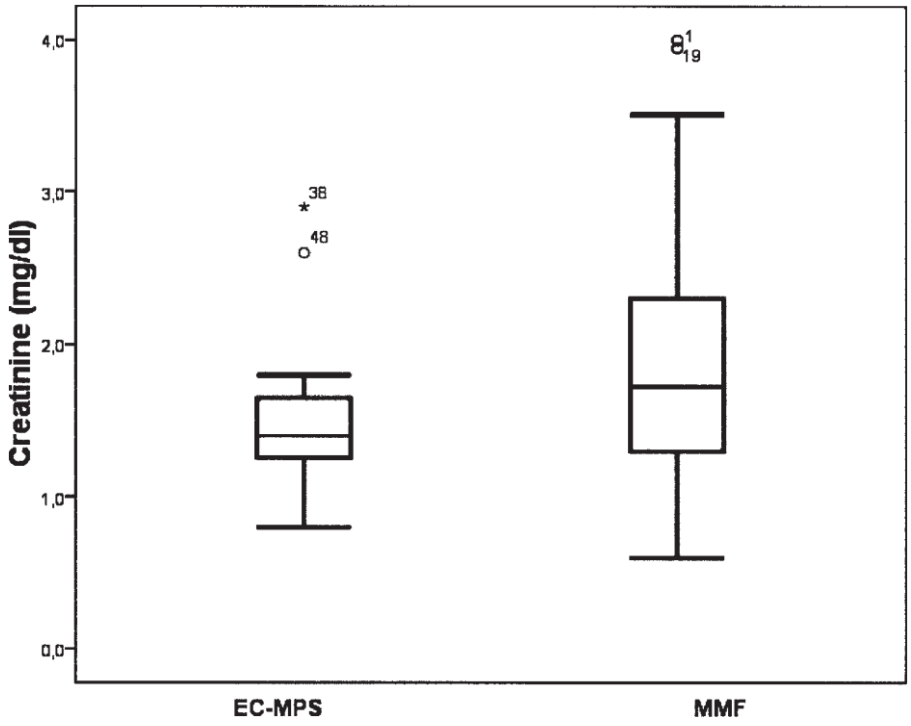

Figure 2: In 110 patients (28 on enteric-coated mycophenolate sodium [EC-MPS] and 82 on mycophenolate mofetil [MMF], the patients on inosine-monophosphate-dehydrogenase (EC-MPS) had better graft function measured by serum creatinine than patients on MMF (creatinine: 1.47 ± 0.4 vs. 2.0 ± 1.3 mg/dL; *P* < .05).

Discussion

One motivation behind this work is the ultimate goal to establish trusted intervals for the residual IMPDH activity which, together with other parameters, would assist any decision concerning dose reduction or dose escalation. Any activity above such interval would indicate insufficient immunosuppression, requiring rather dose escalation, while residual activity below the trusted interval would plead for dose reduction, for possible adverse side effects like GIT, risk of infection or toxicity. For patients with activities within the trusted interval, dose reduction should be possible if indicated, without establishing an increased risk of graft loss.

For the time being, one can assume that any residual activity below the tenth percentile indicates very strong immunosuppression, allowing for immediate dose reduction. In contrast, values above the 90th, possibly already above the 80th percentile should not allow for any dose reduction, or rather demand dose escalation in patients on lose-dose schemes. Especially long term patients, who received dose reduction according to the standard protocol could benefit from this.

Based on our data (n= 2505 measurements), the tenth percentile is defined as 5 pmol XMP/s pmol AMP, while the 80th percentile is marked by 56 pmol XMP/s pmol AMP, and the 90th percentile by 78 pmol XMP/s pmol AMP. If these values are regarded as preliminary cut-offs, they can give valuable

information to the physician in the context of therapeutic drug management. Still, more correct limits of a trusted interval needed to be defined in course of a prospective, multi-centric study.

Comparing the efficacy of EC-MPS vs. MMF, patients with formally equipotent doses showed more pronounced suppression of IMPDH activity measured by HPLC with the former drug. In addition, patients with EC-MPS showed better graft function measured by serum creatinine than those on MMF. These results were not influenced by concomitant immunosuppressive therapy, age, sex, time since transplantation, or graft function.

Although it is known that patients receiving Cyclosporine display lower MPA drug exposure than those on Tacrolimus (42), and although renal function may impair MPA drug exposure (43), these differences were not reflected in this study by changes in IMPDH activity. The stronger suppression of IMPDH activity with EC-MPS compared with MMF, as seen in the present study, may be partially explained by EC-MPS producing higher trough levels and later peak levels compared with patients with MMF (44).

We concluded that patients in the maintenance phase after renal transplantation show more suppressed IMPDH activity with EC-MPS than with MMF, possibly leading to better graft function.

Summary

Therapeutic drug monitoring based on trough levels isn't feasible for mycophenolic acid, since it's not informative neither with respect to the risk of graft loss nor to the risk of adverse side effects. The total drug exposure (AUC) can be deduced from three successive measurements, a procedure which is precise enough, yet not acceptable for clinical routine.

The described method of directly measuring the residual activity of the target enzyme IMPDH reflects the immediate effect of the drug, and hence the level of immunosuppression. These values shall allow judging, whether the suppression of enzymatic activity is insufficient or too stringent, putting the patient at risk of graft loss, or adverse side effects, respectively, or whether it is within a 'safe corridor'. The data presented in this work are preliminary and intended as a guideline; further work, in the ideal case within a prospective, multicentric clinical study, is needed in order to define generally accepted cut-off values.

When comparing patients on MMF vs. those on EC-MPS, the average enzyme activity was lower in the EC-MPS cohort, reaching statistical significance for patients on standard doses for both drugs (2000 mg/d for MMF vs. 1400 mg/d for EC-MPS).

Furthermore, patients on EC-MPS showed better graft function than those on MMF, as determined by serum creatinine.

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