Principles and Practice of Monitoring Immunosuppressive Drugs

Grundsätze und Praxis beim Monitoring von Immunsuppressiva

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Summary: Successful organ transplantation in humans requires administration of pharmacological immunosuppressants for prophylaxis of acute organ rejection. Immunosuppressive drugs, often possess a relatively narrow therapeutic index, and doses must be individualised according to drug concentrations in blood or plasma. While immunoassays are generally used to routinely measure immunosuppressive drug concentrations, they often display variable cross-reactivity with inactive drug metabolites. HPLC based methods with mass spectrometric or tandem mass spectrometric detection are therefore finding increasing application for the specific measurement of the parent immunosuppressive drugs.

Cyclosporine and tacrolimus are the cornerstones of most immunosuppressive protocols. Dosing is usually guided by monitoring pre-dose (trough) blood levels. Measurement of a 2-hour post-dose cyclosporine concentration has been proposed as a more effective monitoring instrument than the trough level for minimising the risk of acute rejection in patients receiving the microemulsion formulation Neoral®. Sirolimus, the most recent immunosuppressive drug to be approved, is given once daily because of its long half-life. Evidence suggests that sirolimus is a critical-dose drug requiring drug concentration monitoring to optimise clinical efficiency and minimise toxicity (thrombocytopenia, hyperlipidemia). Mycophenolate mofetil is a pro-drug used in combination with cyclosporine or tacrolimus that is rapidly hydrolysed *in vivo* to its active constituent mycophenolic acid (MPA). HPLC methods and an EMIT immunoassay can be used to measure MPA in plasma. The EMIT assay also cross-reacts with an active metabolite of MPA. Pharmacokinetic/pharmacodynamic studies have demonstrated a correlation between MPA-AUC and the risk of acute rejection in kidney and heart transplant recipients. Azathioprine, one of the oldest immunosuppressive drugs, is used not only in organ transplantation but also in chronic inflammatory bowel disease. A deficiency in thiopurine S-methyltransferase (TPMT) activity is responsible for azathioprine intolerance. Both phenotype and genotype assays have been described for identifying individuals with TPMT deficiency. The administration of azathipoprine can be further optimised by determining concentrations of its active metabolites the 6-thioguanine nucleotides in red blood cells.

Keywords: drug monitoring; cyclosporine; tacrolimus; sirolimus; mycophenolic acid; azathioprine.

Zusammenfassung: Eine erfolgreiche Organtransplantation beim Menschen bedarf der Verwendung bestimmte Pharmaka, sogenannter Immunsuppressiva, zur Prophylaxe einer akuten Abstoßung. Wegen ihrer engen therapeutischen Indices wird die Gabe solcher Pharmaka auf der Basis von Medikamentenspiegelmessungen im Blut oder Plasma individualisiert. Obwohl Immunoassays eine breite Anwendung für die Routine-Bestimmung gefunden haben, zeigen sie häufig eine variable Kreuzreaktivität mit inaktiven Metaboliten der Muttersubstanzen. Daher kommen häufiger spezifische HPLC Methoden zum Einsatz, vor allem mit massenspectrometrischer bzw. Tandem-massenspektrometrischer Detektion. Ciclosporin und Tacrolimus sind die Eckpfeiler der meisten Immunsuppressionsprotokolle. Zur Steuerung der Dosierung werden üblicherweise Blutkonzentrationen (Talwerte) unmittelbar vor der Verabreichung der nächsten Dosis herangezogen. Die Bestimmung des Ciclosporinblutspiegels 2 Stunden nach Optoral®-Gabe ist als ein effektiveres Monitoring-Instrument vorgeschlagen worden, um die Therapie mit Ciclosporin sicherer zu gestalten.

Sirolimus, ein vor kurzem zugelassenes Immunsuppressivum wird wegen seiner langen Halbwertszeit nur einmal pro Tag verabreicht. Die bisherige Evidenz spricht dafür, dass Sirolimus ebenfalls zur Klasse der "critical dose" Pharmaka gehört. Blutspiegel Bestimmungen (Talwerte) sind daher erforderlich, um die Effizienz der Immunsuppression zu optimieren und die Häufigkeit der Nebenwirkungen (Thrombozytopenie, Hyperlipidämie) zu minimieren.

Mycophenolatmofetil, ein weiteres relativ neues Immunsuppressivum, wird meist in Kombination mit Ciclosporin oder Tacrolimus verwendet. Es ist ein Pro-

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Drug, das *in vivo* rasch zu der aktiven Mycophenolsäure hydrolysiert wird. Sowohl HPLC-Methoden als auch ein EMIT Immunoassay stehen für die Bestimmung des MPA-Spiegels im Plasma zur Verfügung. Der EMIT-Assay weist eine Kreuzreaktivität mit einem aktiven Metaboliten des MPAs auf. Pharmakokinetische/pharmakodynamische Studien haben eine Korrelation zwischen der MPA-AUC und dem Risiko einer akuten Abstoßung nach Nieren- bzw. Herztransplantation nachgewiesen.

Azathioprin, eines der ältesten Immunsuppressiva, findet Anwendung nicht nur in der Transplantation sondern auch bei Patienten mit chronisch-entzündlichen Darmerkrankungen statt. Eine Defizienz des Enzyms Thiopurin S-Methyltransferase (TPMT) führt zu einer Azathioprin-Intoleranz. Sowohl phenotypische als auch genotypische Methoden sind für die Identifizierung von Personen mit einer TPMT-Defizienz beschrieben worden. Die Anwendung des Azathioprins kann weiterhin durch die Bestimmung der 6-Thioguaninnucleotidkonzentration in Erythrozyten optimiert werden.

Schlüsselwörter: Drug-Monitoring; Ciclosporin; Tacrolimus; Sirolimus; Mycophenolsäure; Azathioprin.

Successful organ transplantation in humans is dependent upon administration of pharmacological immunosuppressants for the prophylaxis of acute organ rejection. Detailed knowledge of the immunological mechanisms underlying transplant rejection as well as the ongoing development of novel biological and pharmaceutical agents, which selectively block various steps of the immune response, has enabled a more effective control of graft rejection by the use of combined immunosuppressive therapy. Therapeutic drug monitoring is generally considered essential for those critical-dose drugs which have a narrow therapeutic index, display appreciable inter- and intraindividual variability and have life-threatening consequences of under- or overdosing. Several consensus documents have been published on those immunosuppressive drugs for which drug monitoring is considered to be necessary [1–4]. A joint working group of the scientific Division of the International Federation of Clinical Chemistry (IFCC) together with the International Association for Therapeutic Drug Monitoring and Clinical Toxicology is currently updating these documents [5].

Cyclosporine

Cyclosporine (CsA) is a macrolide immunosuppressant which binds to an intracellular protein, cyclophilin. Its immunosuppressive action is primarily due to the ability of the CsA/cyclophilin complex to inhibit the calcineurin phosphatase signal transduction pathway thereby suppressing the expression of various cytokines including interleukin-2. The result is a specific inhibition of T-cell function. The introduction of this drug into clinical practice in 1983 as a primary immunosuppressant for rejection prophylaxis represented a major advancement in organ transplantation. CsA, however, possesses all the characteristics of a critical-dose drug. Because of its adverse side effects, and in particular its nephrotoxic properties, it was necessary to devise strategies for reducing the risk of acute and chronic nephrotoxicity, while still maintaining effective immunosuppression. CsA dosage is therefore adjusted to maintain trough (pre-dose) blood concentrations in a narrow therapeutic range.

Analytical methods

The recommended matrix for CsA measurement is whole blood with EDTA as anticoagulant. The drug is stable in this matrix for up to 7 days at room temperature or at 2–8 °C [6]. Samples can therefore be shipped at ambient temperature for central processing or for external quality control. In case of prolonged storage, samples should be kept frozen at -20 °C. There is general agreement that the analytical method for monitoring CsA after transplantation should be specific for the parent drug and should display negligible or no cross-reactivity with CsA metabolites [1, 6]. Reversed-phase high-performance liquid chromatography (HPLC) methods with ultra violet [7], mass spectrometric (MS) [8,9] or tandem mass spectrometric (MS-MS) [10, 11] detection have been developed for the specific measurement of this compound. Since HPLC is considered to be the "gold-standard" technique, the increased sensitivity and selectivity offered by MS and in particular MS-MS detection offers the prospect of a more rigorous evaluation of immunoassay performance by comparison with a validated reference technique.

For routine monitoring of the drug, most centres use one of the commercially available semiautomated immunoassay techniques which utilise monoclonal antibodies with a relatively high specificity for the parent compound [1]. Six commercial immunoassays are currently available. The CYCLO-Trac SP® (DiaSorin, Stillwater, MN, USA) is a radioimmunoassay based on the competition between CsA in the sample and a ^{125}I-labelled CsA tracer for a mouse monoclonal antibody with high specificity for CsA. Two immunoassays kits utilise the same mouse anti-CsA monoclonal antibody and fluorescence polarisation immunoassay (FPIA®) technology (Abbott laboratories, Chicago, IL, USA). Another competitive binding assay utilises the enzyme-multiplied immunoassay technology (EMIT®; Dade Behring Corporate, Deerfield, IL, USA). All four immunoassays require a pre-treatment step involving extraction of CsA from the blood sample with an organic solvent. The two most recently introduced commercial assays for CsA do not require pre-treatment extraction. A method has been developed based on the CEDIA® technology (Microgenics Corporation, Fremont, CA, USA). The test principle involves the spontaneous association of a short recombinant NH_2-terminal β-galacto-

Table 1 Mean percentage deviation of the CsA concentrations obtained with immunoassays from the corresponding results determined with specific HPLC procedures.

	Steimer [13]	Schütz *et al* [12]			Hamwi *et al* [14]				Holt *et al* [7]
Transplant type	L/K	K	H	L	K	H-Lu	L	BM	
Sample number	145	100	100	100	49	50	40	40	
CYCLO-Trac	nd	nd	nd	nd	nd	nd	nd	nd	22–30
EMIT	9–12	23.9	20.2	31.2	18.8	15.2	24.6	11.5	8–30
CEDIA	18	22.5	20.5	42.9	14.1	13.4	9.3	22.2	nd
AxSym	29	32.0	33.9	47.5	10	17.5	26.6	18.6	nd
TDx-m	57	nd	nd	nd	50.7	51.6	65.6	55.0	30–55

L = liver
K = kidney
H-Lu = heart-lung
BM = bone marrow
nd = not determined

sidase fragment (α-peptide) and a recombinant β-galactosidase monomer with a deletion near the NH_2 terminus to form active enzyme tetramer. CsA is chemically attached to the α-peptide and an anti-CsA antibody binds to both CsA in the sample and CsA coupled to the α-peptide. Because the latter process interferes with the formation of active enzyme, the amount of CsA in the sample is directly proportional to the residual enzyme activity. The blood sample is mixed with a lysis reagent and this can then be directly placed into the analyser without prior centrifugation. Because of problems with the imprecision and the calibration, the CEDIA was reformulated and re-released as the CEDIA-plus CsA assay. A fully automated CsA method requiring no pretreatment of the whole blood sample has been developed for the Dimension® clinical chemistry analyser (Dade Behring). The EDTA whole blood sample is ultrasonically mixed and combined with a lysing reagent on the Dimension® system itself. A β-galactosidase antibody conjugate is then added to the sample, which binds CsA in the sample to form a CsA-antibody conjugate complex. After incubation, magnetic particles coated with CsA are added to the reaction to bind free (unbound) conjugate reagent. The reaction mixture is separated magnetically and the supernatant, containing the CsA-antibody conjugate complex is transferred to another cuvette where β-galactosidase from the complex converts the chromegenic substrate chlorophenol red beta-D-galactopyranoside to chlorophenol red.

The monoclonal antibodies used in these commercial immunoassays, although selected for their high specificity towards the parent CsA, still display a spectrum of cross reactivity towards different CsA metabolites [7, 12–14]. The impact of this cross reactivity in the clinical situation will depend upon the actual concentrations of these metabolites in patients as well as the relative affinity of the respective antibody for the parent drug and for the metabolite. Several investigators have evaluated the performance of these immunoassays in comparison to specific HPLC procedures in various transplant populations. The results from three recent investigations [12–14] and from earlier studies [7] are summarised in Table 1. The assay system which showed the greatest deviation from HPLC results was the TDx-m. The reason for this discrepancy is probably not only the greater cross-reactivity between the antibody and CsA metabolites, but also a calibration bias. Steimer reported a shift in the bias between TDx-m and EMIT from a relatively small bias in an earlier study performed in 1992 and a much stronger positive bias in 1997 [13]. This shift may reflect variations in the calibrators supplied with some commercial assay kits [15]. When comparing the different transplant types, the greatest overestimations between the respective immunoassays and HPLC were generally observed for liver graft recipients early after transplantation. The time after transplantation and hepatic function will obviously affect the concentrations of CsA metabolites. Substantial variation from the HPLC mean was also apparent for the immunoassays irrespective of the transplant type. None of the selective immunoassays evaluated so far can completely satisfy the criteria demanded by previous consensus conferences [1, 6]. Because of their different metabolite cross-reactivity profiles, it is clear that the different immunoassays will yield different results from each other and from those obtained with a more specific HPLC assay. Of the commercial immunoassays, the TDx-m has been consistently shown to have the lowest imprecision. The AxSym has a lower imprecision than both the EMIT and CEDIA assays [12, 13]. Both the AxSym and EMIT procedures were found to fulfil [12] the between-day requirements of ≤10 % at 50 µg/L and ≤5 % at 300 µg/L as demanded by CsA consensus documents. The imprecision of the CEDIA, however, exceeded these limits. Studies are currently on-going to evaluate the CEDIA-plus and the Dimen-

Table 2 Typical pre-dose (trough) therapeutic ranges for CsA in kidney, liver and heart transplant recipients. These ranges apply to patients receiving a triple-drug immunosuppression protocol and a specific procedure for measurement of CsA.

	Initial therapy*	Maintenance therapy
Kidney	150–225 µg/L	100–150 µg/L
Liver	225–300 µg/L	100–150 µg/L
Heart	250–350 µg/L	150–250 µg/L

* ≤ 3 months post-transplant

sion immunoassays in comparison to HPLC-based procedures.

The commercial immunoassays differ not only in their analytical specificity and precision but also in their dynamic ranges. While these dynamic ranges cover the therapeutic windows that are used for trough level monitoring [1], this will not always be the case for strategies involving blood sampling in the absorption phase when CsA concentrations can be 1000 µg/L or greater. In a proficiency testing survey [16], 125 laboratories were challenged with a blood sample containing 2000 µg/L of CsA. A broad range (1082–3862 mg/L) of results was obtained, although the laboratories had been alerted to the approximate concentration of CsA in the sample before analysis. Carefully validated dilution procedures that are linear across a wide range of concentration are essential if correct CsA concentrations are to be recorded. Furthermore, there is a need for manufacturers of commercial CsA assays to increase the ranges of their assay calibrators. In this respect, the new CEDIA-plus assay offers a two channel calibration system to meet the needs of trough and absorption monitoring.

Trough level monitoring

CsA is usually administered twice daily. Pre-dose (trough) CsA concentrations taken just before the morning dose have proven to be a simple and effective means for adjusting CsA dosage [1]. It should be noted that CsA exhibits diurnal variation, and evening trough concentrations are significantly lower than morning trough concentrations. Most transplant centres have developed target therapeutic concentration ranges based on their own experience and through collaboration with other centres. Such target ranges are dependent upon analytical methodology, type of organ transplant, time post-transplant and immunosuppressive regimen. Typical time-dependent therapeutic ranges based on a specific assay procedure are given in Table 2. It is important to realise that target therapeutic values are used as guidelines only and the decision to adjust drug doses must take into account the complete clinical situation.

Table 3 Provisional target CsA blood concentrations 2 h post-dose in adult transplant recipients.

Type of graft	Time after transplantation	Target C_2 concentration
	(months)	(µg/l)
Liver	0–6	1000
	6–12	800
	> 12	600
Kidney	0–1	1700
	1–2	1500
	3–4	1200
	5–6	1000
	≥ 7	800

Provisional target concentrations ± 20 %; liver and kidney target concentrations based on mFPIA (Abbott TDx) and mRIA (DiaSorin, CYCLO-Trac), respectively. Data from Refs 18 and 19.

C_2 monitoring

CsA was originally introduced as an oil-based oral solution or in the form of soft gelatin capsules under the trade name Sandimmun (Novartis, Basle Switzerland). Despite the success of this drug in reducing the incidence of acute rejection in transplant recipients, substantial intra- and interpatient variability was observed in its pharmacokinetics. To improve the bioavailability of CsA a microemulsion delivery system (Neoral; Novartis, Basle, Switzerland) was developed, which has self-emulsifying properties and spontaneously forms a microemulsion in the aqueous fluids of the gastrointestinal tract [17]. The introduction of this new formulation with its more consistent pharmacokinetic absorption profile has renewed interest in alternative monitoring strategies. In particular, measurement of a 2-hour post-dose CsA concentration appears to be a better surrogate for CsA exposure than the trough level [18, 19]. As with trough level monitoring, target C_2 therapeutic ranges are dependent upon the type of organ and the time post-transplant. Provisional target therapeutic ranges for C_2 concentrations after liver and kidney transplantation based on present clinical experience have been formulated (Table 3)

Support for the concept that the determination of CsA during the absorption phase (0–4 h) might offer a better prediction of CsA immunosuppressive efficacy comes from pharmacodynamic studies. A direct relationship has been shown between CsA blood concentrations and the extent of calcineurin inhibition [20]. Maximum CsA concentrations occurred 1–2 h after the cyclosporine dose, and they were paralleled by a maximum inhibition of calcineurin activity.

Monitoring C_2 may provide a more practical and useful alternative for optimising therapy with Neoral®. The

validity of this strategy compared with trough level monitoring must now be confirmed in randomised multicenter clinical trials for different transplant types.

Tacrolimus

Although the structures of tacrolimus and CsA are very different, their mechanisms of action are similar. After binding to an intracellular binding protein (FKBP-12), the resulting tacrolimus/FKBP-12 dimer engages the calcineurin/calmodulin/calcium complex, thereby inhibiting calcineurin phosphatase activity. Tacrolimus, however, is a much more potent immunosuppressant, with a 10–100 fold greater *in vitro* immunosuppressive activity compared with CsA. Consistent with its greater potency, therapeutic whole-blood trough concentrations for tacrolimus are around 20-fold lower than the corresponding CsA concentrations.

Analytical methods

Whole blood is the preferred matrix for concentration measurement of tacrolimus [2] and samples are stable for up to 7 days at room temperature. Highly specific liquid chromatography/tandem mass spectrometry (LC-MS-MS) methods have been described for the quantification of tacrolimus in whole blood [21, 22]. For routine monitoring of tacrolimus, however, most centres use one of the two commercially available immunoassays, the PRO-Trac II ELISA or the microparticle enzyme-linked immunoassay MEIA II, the latter being by far the most widely used assay at present [23]. Both methods use the same monoclonal antibody that has been shown to cross-react with several metabolites of tacrolimus [24]. A new immunoassay based on the EMIT technology has recently been released.

All three immunoassays cross react with tacrolimus metabolites. A pentamer formation assay has also been described that is based on pharmacodynamic properties of tacrolimus [25]. Thus, the parent drug and its immunologically active metabolites are able to support formation of a pentameric complex between the drug/FKBP12 dimer and the calcineurin/calmodulin/calcium complex. In a comparison with a specific LC-MS-MS procedure and the MEIA-II immunoassay, good agreement was observed between the pentamer assay and the MEIA-II in around 76 % of routine specimens from liver and kidney transplant recipients. In the remaining 24 % of specimens, MEIA-II concentrations displayed a positive bias of 3 µg/l or greater compared to the results from the pentamer formation assay and the LC-MS-MS procedure. Such specimens tended to be from patients with impaired liver function in the early post-transplant phase. These data suggest that patients with impaired liver function have elevated concentrations of inactive tacrolimus metabolites such as M-2, which also cross-react with the antibody used in the MEIA II. In a comparative study of the PRO-Trac II ELISA with LC-MS-MS [26], tacrolimus concentrations from a subset of patients with cholestasis were higher when measured with the ELISA. From these observations, it can be concluded that care must be exercised in interpreting the concentration measurements obtained with the immunoassays particularly for patients with impaired liver function.

Correlation of concentration measurement with clinical efficacy and toxicity

In a retrospective analysis of 13 000 blood samples obtained from 248 patients after kidney or liver transplantation, a correlation was observed between low or elevated tacrolimus whole-blood trough concentrations and rejection or toxicity, respectively [27]. The results from a multicentre open-label concentration-ranging trial of tacrolimus in primary kidney transplantation revealed a significant association between low trough tacrolimus whole blood concentrations and the incidence of acute rejection [28]. In contrast, analysis of three liver transplant studies [29] failed to show any relationship between rejection and tacrolimus blood levels. This may have reflected differences in the design of the studies. In two of the liver studies, a single starting dose of tacrolimus was used. Although the third liver study initially had two randomised dose groups, the doses were titrated early such that, by the end of the second week, there was essentially only a single tacrolimus group. A recent study by Venkataramanan *et al* [30] used the ELISA to measure tacrolimus concentrations in samples from a prospective multicenter study in liver transplant patients. A statistically significant relationship between trough concentrations of tacrolimus and a decreasing risk of acute rejection could be demonstrated; the risk of acute rejection at a tacrolimus concentration of 5 µg/L was approximately twice that at a tacrolimus concentration of 10 µg/L. A recent study in kidney transplant patients has shown similar results for the significance of tacrolimus concentrations and the incidence of rejection.

The correlation between toxicity and tacrolimus trough concentrations is stronger than that for acute rejection. The results from the concentration-ranging trial in primary kidney transplantation, the three liver transplantation trials and the study of Venkataramanan *et al* all found a significant relationship between toxicity and tacrolimus trough levels [28–30]. On the basis of such pharmacodynamic/pharmacokinetic studies and a survey of 36 transplant centres from around the world, therapeutic ranges have been proposed for whole-blood tacrolimus concentrations in kidney, liver and heart transplant recipients [23]. In the initial post-transplant phase (≤ 3 months), the recommended therapeutic ranges are 10–15 µg/L for kidney and liver transplant recipients and 10–18 µg/L for heart transplant recipients. The recommended ranges during maintenance therapy are 5–10 µg/L (liver and kidney) and 8–15 µg/L (heart).

Sirolimus

Sirolimus (rapamycin), a macrocylic triene antibiotic with immunosuppressive properties, was first approved in 1999 for use in kidney transplant recipients. As with CsA and tacrolimus, its cellular activity depends upon binding to specific cytosolic binding proteins. Due to structural similarity in their binding domains, sirolimus and tacrolimus both bind to the same immunophilin, FKBP-12. Whereas tacrolimus and CsA block lymphokine gene transcription, notably of interleukin-2, sirolimus acts at a later stage in the cell cycle by blocking and inhibiting several cytokine- or growth factor-induced signal transduction pathways [31].

Analytical methodology
Whole blood has been recommended as the matrix of choice for the measurement of sirolimus [3]. Currently there is no commercial immunoassay available for measurement of this drug, although an investigational microparticle enzyme immunoassay was used in the Phase III studies. Reversed-phase HPLC procedures with uv detection have been developed for the quantification of sirolimus concentrations in whole blood [31]. These methods generally require a complex sample extraction procedure. For those centres with the necessary equipment, HPLC-based assays with MS [32, 33] or MS-MS detection [34, 35] are available for the sensitive and specific measurement of this drug. The MS detection assays have the added advantage that methods are being developed for the simultaneous determination of several immunosuppressive drugs [33].

Correlation of concentration measurement with clinical efficacy and toxicity
The pharmacokinetics of sirolimus in renal transplant recipients have been shown to vary widely between patients [36]. In a study involving 40 stable renal transplant recipients, Zimmermann and Kahan [37] found a 4.5-fold intersubject variability in dose clearance and steady-state volume of distribution. An excellent correlation was observed between area under the concentration-time curve (AUC) and trough blood concentrations at steady state. In this study, sirolimus did not produce any significant changes in the area under the concentration-time curve of CsA, even though both drugs are metabolised by the same cytochrome P450 A enzyme system.

Target trough concentration ranges will depend on the concomitant immunosuppressive regimen. A range of 5 to 15 µg/L has been suggested as appropriate if CsA is being used at trough concentrations of 75 to 150 µg/L [36]. Weekly monitoring is recommended for the first month and bi-weekly for the next month; thereafter, concentration measurements are necessary only if warranted clinically

Mycophenolate mofetil (MMF)

This drug was approved in 1995 as an adjunctive therapy with corticosteroids and CsA in renal transplantation. It has since been approved for the prophylaxis of acute rejection in heart transplantation. MMF is rapidly metabolised *in vivo* to its active constituent mycophenolic acid (MPA). The latter compound reversibly inhibits inosine monophosphate dehydrogenase (IMPDH) by an uncompetitive mechanism. The inhibition of IMPDH-II in activated lymphocytes by MPA causes a reduction in intracellular guanine nucleotide pools and leads to an arrest of lymphocyte proliferation. The primary metabolite of MPA is the phenolic glucuronide 7–0-MPAG which is eliminated by the kidney. In addition to 7–0-MPAG, two further metabolites have been identified in the plasma of transplant recipients, namely the acyl glucuronide (AcMPAG) and the phenolic glucoside of MPA [38]. Of these three metabolites, only AcMPAG is capable of inhibiting human IMPDH-II [39]. It also displays antiproliferative properties in human mononuclear leukocytes [40].

Analytical methodology
Since > 99,9 % of MPA in blood is retained in the plasma, this matrix is recommended for MPA measurement [4]. Reversed-phase HPLC methods with uv detection have been used to obtain extensive pharmacokinetic data on both MPA and its major metabolite 7–0-MPAG from renal transplant recipients, liver and heart transplant recipients [41, 42]. A recently developed HPLC procedure allows the simultaneous quantification not only of MPA and 7–0-MPAG but also of the AcMPAG [41]. A commercial immunoassay based on the EMIT technology is also available for the determination of MPA. Several studies have revealed a systematic positive bias between the results obtained with the EMIT assay and those found with HPLC methodology. This bias is primarily due to cross-reactivity of the AcMPAG with the antibody used in the EMIT-MPA assay [41]. The EMIT-MPA assay is, therefore, well suited for monitoring MPA since it detects both the parent drug and its active metabolite. MPA is extensively protein bound in plasma, albumin being the major binding protein. The free MPA fraction is presumed to be the pharmacologically active fraction of the drug. An ultrafiltration method has been validated for the determination of the free fraction [43].

Correlation of concentration measurement with clinical efficacy and toxicity
Highly variable inter- and intraindividual pharmacokinetics have been found for MPA in renal transplant recipients [42, 44]. Furthermore, a time-dependent increase in MPA-AUC and pre-dose levels has been observed in adult and pediatric renal transplant recipients on a combination therapy of cyclosporine, MMF and steroids [43, 45]. Evidence is accumulating that the

concomitant immunosuppressive therapy affects the plasma concentrations of MPA. Higher MPA levels have been found in renal transplant patients receiving MMF in combination with steroids or in combination with tacrolimus, as compared to patients on cyclosporine, MMF and steroids. Preliminary evidence suggests that CsA but not tacrolimus may inhibit the excretion of MPAG into bile, thereby attenuating the enterohepatic circulation of MPA [46].

Pharmacokinetic/pharmacodynamic (PK/PD) relationships between MPA-AUC and the risk of acute rejection have been established in pediatric [47] and adult renal transplant recipients [48, 49]. A PK/PD relationship was also documented between the pre-dose MPA concentration and acute rejection in pediatric renal recipients on cyclosporine, MMF and steroids [47], although the correlation was weaker than that seen for MPA-AUC. Several factors have been identified that influence the binding of MPA to plasma proteins. These include renal function, plasma 7-O-MPAG levels and plasma albumin concentration. Impaired renal function, elevated 7-O-MPAG or low albumin are generally associated with an increased free MPA fraction, which may have clinical consequences. Severe leukopoenia was reported in a patient after pancreas/kidney transplantation on 0.75 g MMF bid [50]. Although the total MPA-AUC was in the range observed for stable renal transplant recipients, this patient had an increased MPA free fraction due to impaired renal function. As a result, the free MPA-AUC was substantially elevated. In the multicentre study involving pediatric kidney recipients [47], severe adverse events (leukopoenia, severe infection) were more frequently associated with free MPA-AUC values > 600 µg*h/L.

Based on the data from pharmacokinetic-clinical outcome and concentration-controlled studies in renal transplant recipients on CsA, MMF and steroids, a therapeutic range of 30–60 mg*h/L for MPA-AUC in the early post-transplant phase would appear to be a reasonable target for minimising the risk of acute rejection. In the case of pre-dose MPA concentrations, a range of 1–3.5 mg/L would seem to be appropriate. These ranges are based on MPA concentrations determined by HPLC. Due to the cross-reactivity between the EMIT MPA antibody and the active metabolite AcMPAG, the decision thresholds for the EMIT will be higher. In one comparative study [51] an MPA-AUC of 36 mg*h/L derived from MPA measurements using EMIT was found to have a comparable diagnostic efficacy to an HPLC-derived threshold of 30 mg*h/L. In the case of the pre-dose level, an EMIT MPA value of 1.3 mg/L was comparable to an HPLC value of 1.0 mg/L. Since a full 12-h AUC is difficult to obtain in clinical practice, limited sampling strategies have been proposed. Both a 3-point sampling strategy utilising blood samples drawn prior to, 1.25 and 4 h after the MMF dose [47–52], and a five-point strategy involving sampling over the first 2-h post-dose [48] have been shown to provide a good estimation of the full MPA-AUC

Serial measurements of pre-dose MPA concentrations measured with the EMIT immunoassay have been used to individualise MMF dosage in heart transplant recipients on concomitant therapy with tacrolimus. Based on the data from a retrospective study, 30 heart transplant recipients were enrolled into a prospective study in which MMF doses were adjusted to achieve pre-dose concentrations between 2.5–4.5 mg/L [53]. During the follow-up (range: 175–562 days) only 3 cases (10 %) of acute rejection were observed.

Azathioprine

Azathioprine was one of the first drugs to be used for the prophylaxis of acute rejection in organ transplant recipients. Although it is being replaced by the newer immunosuppressive drugs in immunosuppressant protocols after organ transplantation, it is finding increasing use in the treatment of autoimmune diseases. Azathioprine is converted *in vivo* to 6 mercaptopurine, which is subsequently metabolised to the pharmacologically active 6-thioguanine nucleotides (6-TGN). The latter are also responsible for the cytotoxic side effects associated with this drug. Measuring blood counts has therefore been a routine part of laboratory monitoring during azathioprine therapy. Reliance on blood counts can be misleading, and they do not provide information on immunosuppresive efficacy [54]. More pertinent information can be obtained through the measurement of thiopurine S-methyltransferase (TPMT) activity and the quantification of intracellular 6-TGN concentrations in red blood cells [55].

Thiopurine S-methyltransferase

TPMT, a cytosolic enzyme that catalyses the S-methylation of mercaptopurines, plays an important role together with xanthine oxidase in the catabolism of 6-mercaptopurine, thereby opposing its transformation to 6-TGN. TPMT activity exhibits autosomal co-dominant polymorphism with about 11 % of the Caucasian population having intermediate activity due to heterozygosity at the TPMT locus. Approximately 1 in 300 individuals inherit homozygous TPMT deficiency. Eight mutations in the TPMT gene have now been reported which account for around 90 % of the cases with TPMT deficiency. A PCR strategy has been devised which allows the simultaneous detection of all eight known mutations using the LightCycler [56].

The TPMT phenotype of an individual can be determined by measurement of TPMT activity in erythrocytes. Most investigators have used a radioincorporation assay [57] for this purpose. A non-radioactive assay based on conversion of 6-thioguanine to 6-methylguanine which can then be quantified by HPLC has been described [58]. It has, however, been shown that some lots of 6-thioguanine are contaminated with an inhibitor of TPMT that will compromise the results obtained with this assay [59]. Individuals with TPMT deficiency are at high risk for thio-

purine-drug induced toxicity when these drugs are administered at the standard dosages [54, 60]. Identification of such individuals prior to initiation of drug treatment through measurement of TPMT phenotype is therefore necessary to successfully individualise therapy.

6-Thioguanine nucleotide concentrations

Several methods have been developed [61–64] for measurement of 6-thioguanine nucleotide concentrations in red blood cells (RBC). They generally involve hydrolysis to convert the nucleotides to their free base (6-TG), followed by extraction and HPLC separation. Comparative studies between these different methods are still lacking. A reciprocal relationship between erythrocyte TPMT activity and RBC 6-TGN concentrations has been documented in patients receiving azathioprine [65, 66]. Concentrations of RBC 6-TGN also correlated with myelosuppressive side effects in transplant recipients on treatment with azathioprine [67]. Excessively high, life-threatening 6-TGN concentrations were observed in patients with TPMT deficiency experiencing severe leucopenia [54, 60]. Monitoring of RBC 6-TGN can therefore be used to individualise therapy with azathioprine. In a prospective, randomised trial [68], renal allograft recipients were allocated to receive a low dose or a high dose of azathioprine. Doses in the latter group were adjusted to maintain RBC 6-TGN concentrations (measured twice weekly) between 100 and 200 pmol/8 $\times$ 10^8 RBCs. The cumulative incidence of first rejection episodes was reduced by 21 % in the high dose group. Based on the current data [55, 68], a putative therapeutic range for RBC 6-TGN concentrations in solid organ transplant recipients is 100–450 pmol/8 $\times$ 10^8 RBCs. A further prospective study, albeit not from the field of transplantation, evaluated the clinical utility of monitoring 6-TGN and TPMT genotyping in pediatric patients with inflammatory bowel disease receiving 6-mercaptopurine or azathioprine [69]. 6-TGN levels were significantly associated with therapeutic response independent of other potentially confounding factors. All eight patients who were heterozygous for a mutant TPMT allele achieved a clinical response, as opposed to 39/84 (46 %) patients who were homozygous for the wild type allele. RBC 6-TGN levels were significantly higher in the heterozygotes (median: 589 versus 256 pmol/8 $\times$ 10^8 RBCs). The authors suggest that the response to 6-mercaptopurine/azathioprine therapy was optimised at 6-TGN levels > 235 pmol/8 $\times$ 10^8 RBCs.

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