

Therapeutic Drug Monitoring of Everolimus: A Consensus Report

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Abstract: In 2014, the Immunosuppressive Drugs Scientific Committee of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology called a meeting of international experts to provide recommendations to guide therapeutic drug monitoring (TDM) of everolimus (EVR) and its optimal use in

clinical practice. EVR is a potent inhibitor of the mammalian target of rapamycin, approved for the prevention of organ transplant rejection and for the treatment of various types of cancer and tuberous sclerosis complex. EVR fulfills the prerequisites for TDM, having a narrow therapeutic range, high interindividual pharmacokinetic variability, and established drug exposure–response relation-

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ships. EVR trough concentrations (C_0) demonstrate a good relationship with overall exposure, providing a simple and reliable index for TDM. Whole-blood samples should be used for measurement of EVR C_0 , and sampling times should be standardized to occur within 1 hour before the next dose, which should be taken at the same time everyday and preferably without food. In transplantation settings, EVR should be generally targeted to a C_0 of 3–8 ng/mL when used in combination with other immunosuppressive drugs (calcineurin inhibitors and glucocorticoids); in calcineurin inhibitor-free regimens, the EVR target C_0 range should be 6–10 ng/mL. Further studies are required to determine the clinical utility of TDM in non-transplantation settings. The choice of analytical method and differences between methods should be carefully considered when determining EVR concentrations, and when comparing and interpreting clinical trial outcomes. At present, a fully validated liquid chromatography tandem mass spectrometry assay is the preferred method for determination of EVR C_0 , with a lower limit of quantification close to 1 ng/mL. Use of certified commercially available whole-blood calibrators to avoid calibration bias and participation in external proficiency-testing programs to allow continuous cross-validation and proof of analytical quality are highly recommended. Development of alternative assays to facilitate on-site measurement of EVR C_0 is encouraged.

Key Words: everolimus, mTOR inhibitor, therapeutic drug monitoring, transplantation, oncology

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INTRODUCTION

Therapeutic drug monitoring (TDM) is a well-established and recognized approach for guiding effective and safe immunosuppressive drug therapy in transplantation medicine. The immunosuppressant everolimus (EVR) is approved for the prevention of transplanted organ rejection and for the treatment of various types of cancer and tuberous sclerosis complex (TSC).¹ EVR is characterized by a narrow therapeutic window, rather high intraindividual and interindividual pharmacokinetic (PK) variability and established drug exposure–response relationships.² In the transplantation setting, overexposure to EVR may not only cause exacerbation of specific toxicities but also cause excessive immunosuppression likely to affect infection control, whereas very low levels of exposure may result in rejection. TDM was therefore recommended when EVR was first registered for organ transplantation about 10 years ago and is supported by substantial clinical evidence as demonstrated later in this document. In contrast, the potential value of TDM in managing EVR therapy in other clinical settings has not yet been fully established. When using published data to guide treatment regimens, correct translation of analytical methods and target concentrations to the specific clinical situation may be overlooked. In addition, the lack of between-method comparability and agreement on calibration complicates the interpretation of results and guidance on therapy.

In 2014, the Immunosuppressive Drugs Scientific Committee of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT)

called a meeting of international experts to discuss the most recent advances in EVR TDM. The aim of that meeting was to provide recommendations to guide its optimal use in clinical practice, taking into account drug characteristics, specific clinical situations, and methodological issues, based on in-depth literature research and expert discussions. Although consensus reports have been published for TDM of ciclosporin A (CsA), tacrolimus (TAC), sirolimus (SRL), and mycophenolic acid (MPA),^{3–10} this is the first to be developed to guide EVR TDM. It is intended for all professionals involved in the management of patients receiving therapy with EVR in transplantation and non-transplantation clinical settings, and aims to improve both standards of practice and patient care.

EVR FORMULATIONS

EVR has been available in the solid organ transplantation clinical setting since 2003. The drug was first registered (Certican; Novartis Pharma AG) in some European countries as an adjunctive immunosuppressive therapy in association with glucocorticoids (GCs) and low-dose CsA for the prevention of acute rejection in heart or kidney transplant recipients at the dose of 0.75 mg twice daily. Registration status and trade names may differ between countries. Although registered for use as a twice-daily administration regimen in transplantation (fitting with the coadministration of CsA), once-daily administration of EVR is conceivable in cases of poor compliance or coadministration with a once-daily formulation of TAC. The feasibility of this approach is supported by the relatively long half-life of the molecule, the approval of once-daily EVR formulations in other indications, and preliminary data obtained in kidney transplant,¹¹ and thoracic transplant recipients.¹²

Afinitor (Novartis Pharma AG) is a once-daily EVR formulation indicated in advanced hormone receptor-positive, human epidermal growth factor receptor 2 (HER2)-negative breast cancer, advanced kidney cancer, and advanced pancreatic neuroendocrine tumors. Votubia (Novartis Pharma AG) is another once-daily EVR formulation, more recently approved in the European Union for the treatment of (1) patients with TSC and subependymal giant cell astrocytoma (SEGA) or (2) adult patients with TSC and renal angiomyolipoma at risk of complications (based on factors such as tumor size, presence of aneurysm, or presence of multiple or bilateral tumors), but not requiring immediate surgery. In general, dosing of EVR for oncology indications is 10 mg once daily, with the exception of SEGA associated with TSC, for which it is 4.5 mg/m² once daily.

CHEMISTRY AND MECHANISM OF ACTION OF EVR

Chemistry

EVR is a 40-O-(2-hydroxyethyl) derivative of rapamycin,¹³ a macrolide antibiotic produced by *Streptomyces hygroscopicus*. Its molecular formula is C₅₃H₈₃NO₁₄ and its average molecular weight is 958.22.¹⁴ It is a white to faintly

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yellow powder, which is practically insoluble in water but soluble in organic solvents such as ethanol and methanol.

Mechanism of Action

EVR is an inhibitor of the mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine kinase that plays a central role in the regulation of many cellular functions, including metabolism, growth, proliferation, survival, and memory.¹⁵ EVR binds to the intracellular receptor FK-binding protein (FKBP)-12 in the cytoplasm of T cells to form a complex that binds with high affinity to a region in the mTOR protein C-terminus called FKBP 12-rapamycin binding (FRB), thereby inhibiting mTOR (Fig. 1). mTOR comprises 2 distinct kinase complexes: mTORC1 (the target for EVR) and mTORC2. The mTORC1 complex phosphorylates the p70 and p85 isoforms of the enzyme ribosomal protein S6 kinase beta-1 (S6K1), and the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). A further downstream target of p70S6K is the S6 ribosomal protein (rS6P). Inhibition of mTOR mainly causes dephosphorylation and inactivation of the 2 phosphorylated proteins P-p70S6K1 and P-4E-BP1, thereby inhibiting mTORC1-mediated signal transduction pathways. This modifies the cellular response of T cells to interleukin (IL)-2, in particular the production of ribosomal components necessary for protein synthesis and cell cycle progression. Consequently, mTOR inhibitors may also be called proliferation signal inhibitors.¹⁶ Another protein involved in the mTOR pathway is raptor, which forms a stoichiometric complex with mTOR and negatively regulates mTOR kinase activity.

GENERAL SAFETY

Safety, in terms of avoiding concentration-related adverse effects, is one of the major reasons for implementing TDM. Both mTOR inhibitors (EVR and SRL) are associated with several adverse effects,^{17,18} such as gastrointestinal disorders, hyperlipidemia, and interstitial pneumonitis,¹⁹ that may be sensitive to dose reduction and/or drug withdrawal, even if they do not seem to be directly concentration-related. Edema and mouth ulcers commonly occur and may also reflect overexposure. Impaired wound healing,²⁰ probably associated with the drugs' antiproliferative effects, is the reason for its delayed introduction in de novo situations with complex surgery. Hematotoxicity is usually moderate but represents the most significant complication supporting the need for TDM. The use of mTOR inhibitors in association with other hematotoxic drugs (MPA, ganciclovir) must be carefully monitored.

Renal outcomes are also important, and the precise contributions of calcineurin inhibitors (CNIs) and mTOR inhibitors to the development of nephrotoxicity remain a matter of debate: mTOR inhibitors have been shown to act in synergy with CNIs and increase their nephrotoxicity, in particular that of CsA. For example, the drug-drug interaction (DDI) of SRL and CsA leads to increased CsA concentrations in the blood and kidney, thereby aggravating renal dysfunction.²¹ Proteinuria has also been reported with mTOR inhibitors,²² resulting in the issuing of a specific warning.^{23,24} However, in practice, the combination of EVR and low-dose CNI results in similar renal function compared with MPA plus full-dose CNI.^{25,26} Providing the EVR dose can be maintained, the use of EVR with MPA in a CNI-free regimen results in a significantly

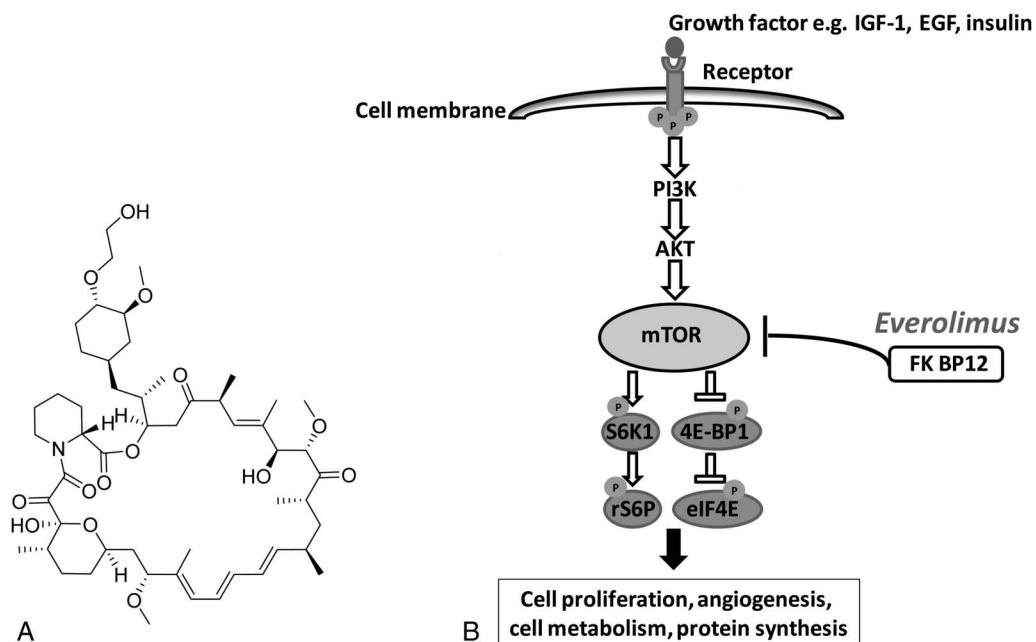


FIGURE 1. Everolimus. A, Molecular structure. B, Mechanism of action. EVR binds to FK-BP12 to block mTOR, resulting in inhibition of proliferation of B cells and T cells, angiogenesis, and cell metabolism. IGF-1, insulin-like growth factor 1; EGF, epidermal growth factor; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; FKBP 12, FK 506-binding protein of 12 KD; S6K1, ribosomal protein S6 kinase beta-1; rS6P, ribosomal S6 protein; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; eIF4E, eukaryotic translation initiation factor 4E.

better glomerular filtration rate (GFR), generally at the cost of a significantly higher incidence of (mostly low-grade) acute rejection.²⁷ The presence of proteinuria at the onset of treatment may compromise such an improvement.²⁸

PHARMACOKINETIC MONITORING

Pharmacokinetics

EVR is more hydrophilic than SRL; this confers greater solubility and stability and influences its PK characteristics. The hydrophilicity of EVR may have a role in its increased oral bioavailability, possibly through a reduced influence of drug efflux transporters^{29,30} and/or a lower clearance by cytochrome P450 (CYP)3A5.³¹ In blood, EVR is highly incorporated into erythrocytes (as also occurs with CNIs), and there is evidence that this binding is concentration dependent, justifying the use of whole blood rather than plasma for EVR quantification.³² A recent study in kidney transplant recipients demonstrated a strong correlation between EVR concentrations in whole blood and in peripheral blood mononuclear cells (PBMCs)—the immunosuppressive site of action of EVR.³³ EVR PK parameters should thus be assessed in whole blood.

EVR is orally active, with linear (dose-proportional) PK. Absorption is rapid, with peak concentration reached within 1.5–2 hours. The free fraction of EVR in plasma is approximately 0.26. There is a good correlation between predose or trough concentration (C_0) and area under the concentration–time curve (AUC) at steady state, and a positive, although weaker, correlation between C_0 and the total daily dose.² Although the bioavailability of EVR is greater than that of SRL, animal studies,³⁰ as well as the percentage of the drug dose found unchanged in the feces in humans,²⁴ suggest a bioavailability of less than 20%, even in the presence of CsA and GCs. EVR has a broad distribution in the body and is eliminated by substantial hepatic metabolism. The metabolic profile of EVR includes 11 metabolites and the primary route of elimination is biliary extraction as metabolites (98% versus 2% in the urine).² To the best of our knowledge, the pharmacological activity of the main metabolites of EVR has never been investigated,³⁴ contrary to that of a couple of minor metabolites, one of which was actually found to be active.^{35,36} EVR is a substrate of both the efflux pump known as the ATP-binding cassette subfamily B member 1 (ABCB1; P-glycoprotein) and the CYP metabolic enzyme (particularly CYP3A4, with CYP3A5 and CYP2C8 playing minor roles). Like CNIs, EVR is prone to substantial PK variability and numerous DDIs because of the involvement of ABCB1 and CYP3A4 in its PKs.²⁴ Although shorter than that of SRL, EVR has a long half-life of approximately 30 hours. Steady-state concentrations are, therefore, generally achieved within 4–7 days.² No data on circadian variation are available.

Population PK models of EVR have been developed. A model in heart transplant recipients reported an apparent clearance and distribution volume of 3.33 ± 0.20 L/h and 146 ± 33 L, respectively, and a significant influence of bilirubin concentration and CsA on EVR clearance.³⁷ In

kidney transplant recipients, using a 2-compartment structural model with first-order absorption with lag time, ideal body weight was found to be significantly related to the volume of distribution.³⁸ In neither study were other potential covariates found to be significant.

There is less intrapatent PK variability with EVR than with CNIs, but it remains high (45% for C_0 , 27% for the AUC in de novo kidney transplant recipients administered CsA), as does interindividual variability (55% for C_0 , 31% for the AUC).³⁹ Hepatic impairment has a major effect on EVR PK and dose reduction should be considered with careful monitoring.⁴⁰ Weight, age, and sex were not found to influence EVR exposure.³² Potential ethnic differences in EVR PK and their contribution to interindividual PK variability have not yet been fully determined, with inconsistent bioavailability data reported in African Americans.^{41,42}

Drug–Drug and Drug–Food Interactions

DDIs with EVR are very frequent and may involve both pharmacodynamics (PD) and PK. Both mTOR inhibitors have the same PK DDI profile as CNIs, dominated by interactions through ABCB1 and CYP3A. Most EVR PK DDIs involve inhibition or induction of metabolism by anti-infectives, such as macrolides and azoles (inhibitors) and rifamycins (inducers). DDIs are a major source of EVR PK variability and must be controlled by careful monitoring. Quantitatively, PK changes seen with EVR are higher than with TAC, but less significant and more easily manageable than those seen with SRL.⁴³ Compared with TAC, the management of EVR DDIs is easier because of its lower immunosuppressive potency and more favorable toxicity profile. The aims and endpoints of dose management have been described in 2 clinical case reports, describing metabolic induction by rifamycins⁴⁴ and inhibition by antifungal azole drugs.⁴⁵ Such case studies provide important additional information compared with the usual studies of the quantitative aspects of DDIs in healthy volunteers.

EVR exhibits DDIs with other immunosuppressants. GCs have a dual effect on EVR metabolism by CYP3A4, acting as strong inhibitors at acute high doses and as moderate inducers at chronic low doses.⁴⁶ CsA inhibits EVR metabolism by approximately 50%.^{47,48} In contrast, EVR exposure in kidney transplant recipients is not influenced by TAC concentration, and the EVR dose achieving equivalent exposure has been shown to be 1.5–2-fold higher with TAC than CsA.⁴⁶

EVR absorption is sensitive to fatty meals. In a single-dose study in healthy subjects, a high-fat meal delayed EVR t_{max} by a median of 1.25 hours and reduced C_{max} by 60% and AUC by 16%.⁴⁹ In a multiple-dose confirmatory study in kidney transplant recipients, a high-fat meal delayed t_{max} by a median of 1.75 hours and reduced C_{max} by 53% and AUC by 21%.⁴⁹ Thus, the different oral formulations of EVR should be administered consistently with or without food, as clearly explained in their respective prescribing information. Surprisingly, EVR C_0 showed no food effect, suggesting that although overall exposure is prone to a food effect, this does not translate into C_0 variations, the exposure index

mostly used for TDM. This represents a potential limitation of C_0 as a marker of exposure for TDM of EVR.

Compatibility of EVR PK Characteristics With the Prerequisites for TDM and TDM Strategy

The prerequisites for TDM in general have been widely described⁵⁰ and have been discussed specifically for EVR.⁴¹ They can be summarized as follows: drug choice appropriate to indication and patient subpopulation; convenient matrix and analytical method readily available; established exposure–response relationships; PD response not readily assessable; exposure–response occurs in the patient-specific subpopulation and indication; the drug has a narrow therapeutic range in the specific context; PK parameters are unpredictable because of variability or confounding factors; duration of therapy is sufficient to implement and benefit from TDM; and TDM makes a significant difference to clinical decision-making.

EVR fulfils most, if not all, of these criteria; in particular, a narrow therapeutic range, the risk of therapeutic failure, the risk of toxicity, and high PK variability that may result in unpredictable concentrations. Intrinsic, structure-related, chemical and pharmacological properties contribute to this large PK variability, but other important determinants exist, such as the influence of patient compliance, numerous EVR DDIs, and a food effect.

A strong recommendation for TDM of EVR exists in the transplantation setting, with a proposed target range of 3–8 ng/mL for EVR trough whole-blood concentration.²⁴ When EVR is used as monotherapy, eg, in case of malignancy indications, such as liver transplantation for hepatocarcinoma, higher therapeutic ranges are often targeted (6–10 ng/mL).^{51,52} The respective clinical evidence is described in detail below.

The most important development regarding the EVR target range is the evolution of the place of EVR in immunosuppressive regimens during the past decade, for example, in CNI minimization, treatment of malignancies in transplant recipients, or to avoid late graft dysfunction. Indeed, the real role for this class of drug is still being debated.^{53–55} Various factors associated with these roles influence the need for TDM. Alongside minimization of GCs and CNI, a current trend in immunosuppression for solid organ transplantation is the replacement of CsA by TAC. However, because of the potential for DDIs, the dose required to achieve a given EVR concentration target may differ according to the coadministered immunosuppressants. A comprehensive review on mTOR inhibitors and TAC in kidney transplantation was recently published, focusing on PK, exposure–response relationships, and clinical outcomes.⁵⁶ Long-term maintenance therapy with EVR is mostly limited by non–concentration-dependent intolerance. On an individual basis, a high C_0 may be well tolerated. However, long-term $C_0 > 8$ ng/mL are generally associated with high doses and therefore a risk of intolerance. Safety management generally involves EVR dose reduction or withdrawal.⁵⁷ Higher doses of EVR are used in non-transplantation settings, but there is currently no recommendation for EVR TDM in approved indications other than transplantation. The rationale for TDM in these settings requires further analysis of the available or emerging PK, safety, and clinical data. The

requirement for EVR TDM in evolving indications, such as hematology, will need to be evaluated in the future.

Recommendations: General Use of TDM for EVR

- EVR fulfils sufficient of the criteria for a TDM-guided therapy and therefore TDM is recommended for this drug, especially in the transplantation setting. EVR TDM is advised to limit the proportion of patients with subtherapeutic or suprathreshold exposure early after the initiation of EVR therapy and to detect underdosing or overdosing due to DDIs, dosing errors, or poor adherence.
- EVR PKs are linear, and trough concentrations (C_0) demonstrate a good relationship with overall exposure (AUC). Therefore, predose C_0 provides a simple and reliable index for TDM.
- Sampling should be standardized to occur within 1 hour before the next dose, which should be taken at the same time everyday and preferably without food. If the latter is not possible for practical or medical reasons, EVR should be dosed consistently with food to reduce fluctuations.
- The high uptake of EVR into erythrocytes results in the same recommendation as for CNIs, ie, to measure its concentration in whole blood. Ethylenediaminetetraacetic acid (EDTA) is the preferred anticoagulant because it minimizes problems with clotting and its use allows quantification of multiple immunosuppressive drugs in parallel.
- EVR steady-state concentrations should be monitored 4–6 days after administration of the first dose and after a change in dose, a change of coadministered CsA dose, or a change in cotherapy with other CYP3A4 or ABCB1 inhibitors/inducers. However, in some situations and under experienced guidance, earlier monitoring may be beneficial.
- EVR TDM is not usually an emergency, because of its long half-life, moderate safety concerns, and potential position as an adjunctive therapy. Although EVR TDM frequency is less than with CNIs because of the lower inpatient variability with EVR, the analytical turnaround time should ideally be the same as that of the associated CNI (except in cases of suspected DDI or if EVR is a major component of the immunosuppressive regimen when it should be the same).
- Efficacy in clinical trials was established using reference arms that may be different across countries and indications, for example, regarding induction therapy. Personalized target concentrations should follow the pattern of “an association, a target” rather than “a drug, a target” (taking into consideration the indication, posttransplantation time if applicable, background, clinical findings, and laboratory test results).

EVIDENCE-BASED TDM FOR EVR IN SPECIFIC CLINICAL SITUATIONS

General Considerations

Minimization or withdrawal of CNI exposure is expected to lower the incidence of, or at least delay, renal impairment and end-stage renal failure after long periods of maintenance therapy with these nephrotoxic drugs. With

respect to the introduction of EVR, there are currently 2 options: switching from a CNI to EVR at a predefined time after transplantation, or after the onset of renal impairment⁵⁸; or de novo introduction of EVR to preserve kidney function in patients with a high risk of renal failure.⁵⁹ In case of de novo use, attention must be paid to the risk of impaired wound healing associated with the antiproliferative properties of the drug, hence of the risk of overexposure. This is of particular importance in the context of liver and lung transplantation, in which the early introduction of GCs may confer the same risk. Therefore, the risk of insufficient immunosuppression must be countered using induction biotherapies and careful TDM of the remaining immunosuppressant drugs.

Owing to the antineoplastic properties of mTOR inhibitors, EVR may be preferentially used in the transplantation setting: (1) as part of the initial maintenance regimen to prevent the relapse of primary cancers, such as hepatocellular carcinoma (HCC) after liver transplantation, and (2) to replace CNIs in case of de novo cancer after transplantation (in this case, the recommended C_0 would be somewhat higher, at 6 ng/mL).⁶⁰ The rationale for using EVR in these 2 situations is also to avoid the risk of excessive immunosuppression associated with CNIs.

Kidney Transplantation

In kidney transplantation, two main EVR treatment strategies have been explored in clinical trials: CNI minimization and CNI withdrawal/elimination.⁶¹ With regard to the first strategy, combination therapy with EVR plus a reduced dose of a CNI (either CsA or TAC) has been studied mostly in de novo kidney transplant recipients and compared with MPA-based therapy (see **Table, Supplemental Digital Content 1**, <http://links.lww.com/TDM/A127>, which lists randomized controlled trials of EVR in combination with CNI). With regard to the second strategy, conversion of stable kidney-transplant recipients from CNI-based therapy to EVR-based therapy has been investigated at various time points, ranging from several weeks to several years after transplantation (see **Table, Supplemental Digital Content 2**, <http://links.lww.com/TDM/A128>, which lists randomized trials of conversion to a CNI-free EVR-based regimen). It is important to note that no clinical trials comparing fixed-dose versus concentration-controlled EVR therapy have been conducted. However, demonstration of an EVR concentration–effect relationship and identification of therapeutic and tolerated concentration ranges in the early registration trials provide a strong rationale for performing TDM of this drug. Because TDM was an integral part of most clinical trials performed during the last decade, an extensive body of literature exists to support TDM of EVR after kidney transplantation.

CNI Minimization

In a phase II trial⁶² and 2 phase III trials (RAD B201, RAD B251),^{63–65} several fixed doses of EVR were administered together with standard-dose CsA and GCs. In the 2 phase III trials, 2 different EVR dosing regimens were compared with a third approach in which patients also received standard-dose CsA (targeted to a C_0 of 150–400 ng/mL in weeks 1–4 and 100–300 ng/mL thereafter) and GCs with

mycophenolate mofetil (MMF) instead of EVR.^{63–65} Importantly, these phase III studies were all of a double-blind, double-dummy design, and TDM was not performed as part of routine clinical care. These trials demonstrated that EVR is as effective as MMF in preventing rejection, but that renal function was reduced when EVR was administered together with standard-dose CsA, most likely as a result of enhanced nephrotoxicity of the latter.^{63–65}

Prespecified post hoc analyses of PK data prospectively collected in these studies demonstrated an EVR concentration–effect relationship with regard to both efficacy (ie, freedom from acute rejection) and toxicity (thrombocytopenia, hypertriglyceridemia, and hypercholesterolemia).^{32,66–68} An EVR $C_0 \geq 3$ ng/mL [determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS)] was identified as the lower therapeutic concentration limit. The upper limit of the therapeutic concentration range could not be defined because few patients had a concentration >8 ng/mL. Simulation showed that TDM could optimize early EVR exposure (ie, increase the percentage of patients with a $C_0 \geq 3$ ng/mL).^{32,66,68} Interstitial pneumonitis, another serious adverse effect of mTOR inhibitors, does not seem to be directly related to measured EVR concentration.⁶⁹ However, pneumonitis was initially described at very high mTOR inhibitor concentrations and dose reductions were successful in alleviating symptoms, suggesting partial dose dependency. As a consequence, dose reductions are still recommended in less severe cases, whereas the drug should be withdrawn in severe cases.⁷⁰

The efficacy of EVR in combination with reduced-dose CsA and GCs was investigated in 2 subsequent phase IIIb trials (A2306, A2307).^{71,72} TDM was performed in both trials, targeting EVR to a $C_0 \geq 3$ ng/mL; no upper EVR concentration limit was specified. The difference between the 2 studies was the use of basiliximab induction therapy (only in A2307) and the targeted CsA exposure (lower in A2307). Both trials demonstrated that concentration-controlled EVR plus reduced-dose CsA provides effective protection against rejection, with good renal function.^{71,72} Analysis of the PK data collected in one of the studies (A2306) demonstrated that 14% of the patients randomized to the 1.5 mg/d EVR starting dose had early subtherapeutic EVR concentrations.⁷³ As a result of TDM, their EVR C_0 was uptitrated to a median concentration of 5.3 ng/mL, with an upper 90th percentile of 7.9 ng/mL. Early subtherapeutic concentrations were rare in the 3.0 mg/d EVR starting-dose group and their EVR C_0 distribution was skewed to the right. Many patients in the latter group subsequently underwent dose reductions because of adverse clinical events or laboratory abnormalities. The upper 90th percentile of the resulting EVR concentration range was 11.6 ng/mL in the 3.0 mg starting-dose group.⁷³ No reduction was seen in the incidence of rejection in patients with an EVR $C_0 > 8$ ng/mL; however, an increased incidence of thrombocytopenia and hypertriglyceridemia was observed at this EVR C_0 concentration. Based on these findings, an EVR C_0 therapeutic range of 3–8 ng/mL and a tolerated range of 3–12 ng/mL were proposed.⁷³

In a 24-month, 3-arm, open-label, controlled, phase IIIb clinical trial (A2309), de novo kidney transplant recipients were randomized to either EVR 1.5 or 3.0 mg/d (targeted to

a C_0 of 3–8 and 6–12 ng/mL, respectively) with reduced-exposure CsA, or to MPA plus standard-exposure CsA.^{74,75} At a 2-year follow-up, no differences were observed in the incidence of the composite endpoint “efficacy failure” between the 3 groups. The EVR 3–8 ng/mL target C_0 offered similar efficacy and renal function compared with the control group (MPA and full-dose CNI). Renal function was inferior in the EVR 6–12 ng/mL target group, in which patients experienced more adverse events (infections, posttransplantation diabetes mellitus and stomatitis). Post hoc analyses from this study also demonstrated that the risk of developing proteinuria (≥ 300 mg/g creatinine) and several other adverse effects was dose dependent.^{48,76} An EVR $C_0 > 8$ ng/mL was significantly associated with a 1.9-fold increased risk of proteinuria compared with a C_0 3–8 ng/mL ($P < 0.001$).⁷⁶ The 3–8 ng/mL concentration range was also associated with the lowest rate of wound-healing problems, peripheral edema, posttransplantation diabetes mellitus and dyslipidemia.⁴⁸ Primarily based on this trial, the US Food and Drug Administration granted EVR approval for the prevention of kidney allograft rejection in combination with reduced-dose CsA. Prescribing information includes a recommendation to monitor EVR concentrations and adjust the maintenance dose to achieve a C_0 within the 3–8 ng/mL target range (measured using an LC-MS/MS method).

In another trial in de novo kidney transplantation [Everolimus for renal cancer ensuing surgical therapy (EVEREST)], patients were randomized to receive standard-exposure EVR plus reduced-exposure CsA [with a 2-hour postdose (C_2) target of 350–500 ng/mL] or higher-exposure EVR with very low exposure CsA (C_2 target of 150–300 ng/mL).^{77,78} Patients in the latter group had a comparable low incidence of rejection, but did not have better renal function than those with the higher targeted CsA concentration range. The risk of rejection in the first 3 months after transplantation was significantly correlated with the presence of low EVR C_0 , but not with CsA exposure. Other studies of EVR plus reduced-dose CsA in de novo kidney transplant recipients have targeted a similar EVR C_0 3–8 ng/mL.⁷⁹

Although most studies have investigated EVR in combination with CsA, only limited data are available for EVR in combination with TAC.⁵⁶ In a 6-month open-label, randomized study (CRADUS09), EVR therapy was started within 24 hours after graft reperfusion to maintain EVR $C_0 > 3$ ng/mL, with a recommended maximum of 12 ng/mL. EVR was combined with either standard-exposure or reduced-exposure TAC (C_0 targeted to 8–11 and 4–7 ng/mL, respectively, during months 0–3) in de novo kidney transplant recipients.⁸⁰ In a 12-month open-label randomized study (ASSET), EVR was targeted to a C_0 of 3–8 ng/mL and combined for 3 months with low TAC (C_0 targeted to 4–7 ng/mL during months 0–3). At month 3, patients were randomized to either continue low TAC exposure or were targeted to very low TAC exposure (C_0 target 1.5–3 ng/mL).^{81,82} In both studies, patients received additional basiliximab induction therapy, with GCs, and no MPA control arm was included. EVR target concentration ranges were extrapolated from previous studies of EVR/CsA combination therapy. A post hoc analysis of the CRADUS09 study demonstrated that an EVR $C_0 \geq 3$ ng/mL

was associated with a significantly lower rate of acute rejection compared with a $C_0 < 3$ ng/mL, regardless of TAC target ranges ($P = 0.03$).⁵³

The importance of achieving adequate early posttransplantation EVR concentrations ($C_0 > 3$ ng/mL) was confirmed in a recent large phase III study in kidney allograft recipients (CRAD001AUS92).⁸³ Patients were randomized to EVR at 0.75 mg twice daily plus low-dose TAC or to MMF (2 g/d) plus standard-dose TAC. All patients received GCs and induction therapy as per local practice. In this study, noninferiority (10% margin) in the composite endpoint “efficacy failure rate” for EVR in combination with low TAC was not achieved, because of a higher rate of acute rejection. Despite higher rejection rates, there was a lower rate of graft loss and similar renal function in the EVR arm compared with the MMF arm.

CNI Withdrawal

The efficacy and safety of conversion to EVR from a CNI-based immunosuppressive regimen in stable kidney transplant recipients have been explored in several clinical trials. In the ZEUS study, EVR target concentrations were not based on previous data, but it was assumed that higher concentrations were necessary for adequate rejection prophylaxis compared with EVR-CNI combination therapy. An EVR target C_0 of 6–10 ng/mL was thus applied after CsA cessation.^{27,84,85} The ZEUS trial demonstrated that conversion from CsA to EVR may achieve better renal function compared with continued CsA therapy. However, compared with the patients continuing CsA, the EVR-treated CsA-free patients experienced more mild acute rejections, developed more circulating donor-specific anti-HLA antibodies, and more patients discontinued therapy because of adverse events.⁸⁶ Mean EVR C_0 during the trial was 6–7 ng/mL. Patients experiencing acute rejection in the first year had a mean EVR C_0 of 6.8 ± 2.6 ng/mL.²⁷ Mean EVR C_0 before withdrawal due to adverse events in the first year was 5.3 ± 2.2 ng/mL. Importantly, after 5 years, EVR was withdrawn in 37.4% of patients.^{27,84,85}

Comparable EVR C_0 targets were used in other conversion trials, including the Steroid or Cyclosporin Removal After Transplant using Everolimus (SOCRATES) study,⁸⁷ Certican Nordic Trial in Renal Transplantation (CENTRAL),^{88–90} APOLLO trial,^{91,92} and Assessment of Everolimus in Addition to CNI Reduction in the Maintenance of Renal Transplant Recipients (ASCERTAIN)⁹³ study (See **Table, Supplemental Digital Content 2**, <http://links.lww.com/TDM/A128>). From these trials, it seems that higher EVR concentrations may not provide better rejection prophylaxis, but may result in higher discontinuation rates. The risk of acute rejection after CNI withdrawal seems to depend mainly on the time after transplantation, being highest in these trials when conversion took place early after transplantation, but rarely occurring with maintenance regimens. In 1 conversion trial [Mycophenolate Sodium versus Everolimus or Cyclosporine with Allograft Nephropathy as Outcome (MECANO)], EVR was targeted to AUC_{0-12} [120 or 150 $\mu\text{g}\cdot\text{h}\cdot\text{L}^{-1}$ depending on whether EVR was measured with LC-MS/MS or fluorescence polarization immunoassay (FPIA), respectively].^{94,95} Switching from CNI-based immunosuppressive therapy to EVR at 6 months after

kidney transplantation was effective in preventing rejection, with acute rejection rates of 3% in the CsA group, 22% in the mycophenolate sodium (MPS) group and 0% in the EVR group ($P < 0.009$).⁹⁴ To date, no comprehensive (post hoc) PK investigations of the EVR concentration–effect and concentration–toxicity relationships in these CNI-elimination studies have been published. Further investigation is needed to obtain more insight into time dependency of EVR target concentrations and the relationship to EVR discontinuation.

Recommendations: EVR in Kidney Transplantation

- EVR should be targeted to a C_0 of 3–8 ng/mL, with a starting dose of 1.5 mg/d after de novo kidney transplantation when combined with reduced-dose CsA plus basiliximab and GCs.
- Limited evidence suggests that EVR should also be targeted to a C_0 of 3–8 ng/mL after de novo kidney transplantation when combined with reduced-dose TAC plus basiliximab and GCs.
- When EVR is given in combination with TAC in de novo kidney transplant recipients, a higher starting dose of 3 mg/d may be advisable to reach target concentrations early after transplantation.
- Targeting EVR to a $C_0 \geq 6$ ng/mL when eliminating CNIs in stable kidney transplant recipients seems reasonable, but lacks evidence from prospective dose-finding trials.
- Targeting EVR to a $C_0 > 10$ ng/mL in CNI-free regimens seems to offer no benefit in terms of reducing the risk of acute rejection and may increase the risk of developing drug-related adverse events.

Liver Transplantation

Minimizing the use of CNIs after liver transplantation reduces long-term complications, including nephrotoxicity.⁶¹ The safety and efficacy of SRL have not been fully established in liver transplant recipients.⁹⁶ Therefore, its use as primary immunosuppressant in de novo liver transplantation is not recommended. In addition, increased hepatic artery thrombosis has been observed within 30 days of transplantation in de novo liver transplant recipients treated with SRL and TAC/GCs compared with recipients treated with TAC/GCs.⁹⁶ However, SRL-induced hepatic artery thrombosis has not been reported since this initial study.⁹⁷

Early Introduction of EVR in Liver Transplantation

In a recent open-label study of EVR in de novo liver transplantation,⁹⁸ recipients were randomized on day 30 ± 5 to 1 of 3 treatment regimens: (1) EVR alone (C_0 3–8 ng/mL), (2) EVR with low-dose TAC (EVR C_0 3–8 ng/mL, TAC C_0 3–5 ng/mL), or (3) standard TAC therapy (C_0 6–10 ng/mL). Based on previous experience with SRL,⁹⁶ EVR was administered 30 days after liver transplantation to avoid wound-healing complications and hepatic artery thrombosis. Although treatment with EVR alone led to a higher rate of acute rejection, EVR with low-dose TAC had effects similar to those of standard TAC therapy. The reduction in adjusted estimated GFR (eGFR) 12 months after randomization was significantly lower in recipients treated with EVR and

low-dose TAC compared with standard TAC therapy ($P < 0.001$). The relative risk of severe infection was similar in these 2 treatment arms. Furthermore, the protective effect on eGFR of EVR with low-dose TAC compared with TAC monotherapy continued for 24 months after randomization.⁹⁹ These findings demonstrate the safety of starting EVR and low-dose TAC 30 days after surgery for the prevention of acute rejection after liver transplantation and show that this regimen leads to a significantly better renal function 24 months after transplantation compared with standard-exposure TAC.

Prevention of Chronic Rejection

Some patients develop severe rejection that cannot be controlled by TAC or high-dose GCs. Acute cellular rejection after liver transplantation can be treated with high-dose GC pulse therapy. However, apart from retransplantation, there is no established treatment of chronic (ductopenic) rejection. Prevention of B-cell maturation by mTOR inhibitors is anticipated to prevent chronic rejection after liver transplantation. The successful use of SRL¹⁰⁰ or EVR¹⁰¹ in combination with reduced-exposure CNI to treat severe chronic (ductopenic) rejection after liver transplantation has been reported in a limited number of cases from Japan (introductory EVR C_0 10–12 ng/mL; CsA C_0 100–200 ng/mL; TAC $C_0 < 5$ ng/mL; maintenance EVR C_0 5–8 ng/mL), but requires confirmatory studies.

Prevention of Recurrence of Hepatocellular Carcinoma After Liver Transplantation

Recurrence of HCC after surgery has been a severe problem in recipients of liver transplants from both deceased and living donors. The Milan criteria for transplantation were originally established to select patients with cirrhosis who have small HCC nodules and for whom a good outcome is anticipated, with low rates of recurrence.¹⁰² However, experience has accumulated of liver transplantation in patients with HCC who do not meet the Milan criteria. Recently, it was shown that the frequency of HCC recurrence in liver transplant patients receiving mTOR inhibitors was significantly lower than in those receiving CNIs (8.0% versus 13.8%, $P < 0.001$).¹⁰³ Despite the shorter follow-up for EVR-treated patients than those receiving SRL or CNIs (13, 30, and 43.2 months, respectively), the rate of HCC recurrence in EVR-treated recipients was significantly lower than in those treated with SRL and CNIs (4.1% versus 10.5% versus 13.8%, respectively; $P < 0.05$).

Recommendations: Liver Transplantation

- Early use of SRL after liver transplantation has been associated with an increased risk of hepatic artery thrombosis; therefore, it appears prudent to delay the introduction of EVR until 30 days after transplantation.
- EVR targeted to a C_0 of 3–8 ng/mL in combination with low-exposure TAC (target C_0 3–5 ng/mL) is as effective with regard to acute rejection risk as standard-exposure TAC (target C_0 6–10 ng/mL) and is associated with better renal function.

- Compared with CNI treatment, the administration of EVR is expected to reduce the recurrence of HCC after liver transplantation.

Heart and Lung Transplantation

EVR With CsA in De Novo Heart Transplant Recipients

In a 24-month, multicentre, randomized, double-blind, double-dummy, phase III study (B253), the efficacy, safety, and tolerability of 2 fixed doses of EVR were compared with azathioprine in de novo heart transplant recipients.¹⁶ Both EVR dose regimens were superior to azathioprine with respect to a composite endpoint {death, graft loss, retransplantation, loss to follow-up, biopsy-proven acute rejection [BPAR] of grade 3A [International Society for Heart and Lung Transplantation (ISHLT) 1990 grading] or rejection with hemodynamic compromise} and the incidence of repeated rejection episodes, and also the incidence of BPAR of grade $\geq 3A$. The severity and incidence of heart-allograft vasculopathy were also significantly lower ($P < 0.05$) in the patients receiving EVR than those receiving azathioprine.

In a post hoc analysis of this study, EVR $C_0 > 3$ ng/mL was significantly related to freedom from rejection ($P = 0.02$), and the 3 ng/mL cutoff therefore constituted the lower therapeutic concentration of EVR.¹⁰⁴ The upper limit of the therapeutic concentration range could not be defined because of the flat EVR concentration–safety parameter association (eg, for leukopenia, dyslipidemia, and renal insufficiency). For example, using a platelet count cutoff of $< 50 \times 10^9/L$ to define “clinically meaningful,” the incidence of thrombocytopenia was just 3%. Interestingly, EVR-related adverse events were manageable up to the highest C_0 observed in this population (22 ng/mL).¹⁰⁴ Further retrospective analysis of the exposure–effect data showed that EVR C_0 were stable in the first year posttransplantation and averaged 5.2 ± 3.8 and 9.4 ± 6.3 ng/mL in patients treated with EVR 1.5 and 3.0 mg/d, respectively. TDM simulation, based on 2 EVR dose adjustments and an initial starting dose of 1.5 mg/d, showed that the simulated BPAR rate was 21% with TDM (using a target range of 3–8 mg/mL) versus 26% in the group with fixed dosing.¹⁰⁵

In a single-center, observational study in Germany, EVR targeted to a C_0 of 3–8 ng/mL allowed a marked reduction of CsA concentration (58% from week 2 to month 12) in de novo heart transplant recipients, without significant loss of efficacy compared with MMF (mean dose 1.25–2.5 g/d) in combination with standard-dose CsA.¹⁰⁶

A randomized, open-label, noninferiority study (A2411) was conducted to examine whether renal toxicity was reduced with EVR plus reduced-exposure CsA compared with MMF (3 g/d) plus standard CsA.¹⁰⁷ Both study groups received GCs and antibody induction therapy according to local practice. At 12 months, the incidence of BPAR grade $\geq 3A$ and change in renal function (eGFR) in the concentration-controlled EVR group were similar to that in the MMF and standard-dose CsA group (22.8% versus 29.8% and 6.1 mL/min versus 4.3 mL/min, respectively).

The effect of reduced-dose CsA versus standard-dose CsA on renal function was investigated in a 6-month,

randomized, open-label study (A2403) in de novo heart transplant recipients treated with EVR and GCs.¹⁰⁸ Participating centers were permitted to use antithymocyte globulin (ATG) or IL-2 receptor antagonist induction consistently for all patients at that center. CsA was initiated at ≤ 12 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, except at centers using induction therapy, where local practice was followed. The dose was subsequently adjusted to maintain a predefined target CsA C_2 range (1000–1400 ng/mL) for all patients in the first 2 months. Predefined C_2 targets were lowered over the next 4 months, being higher in the standard-dose group than the reduced-dose group. EVR was initiated within 72 hours after transplantation at an initial dose of 0.75 mg twice daily, titrated after day 5 to achieve a C_0 in the range of 3–8 ng/mL. EVR with reduced-dose CsA resulted in similar efficacy as standard-dose CsA. At month 6, mean serum creatinine was 141.0 ± 53.1 $\mu\text{mol/L}$ in patients receiving standard-dose versus 130.1 ± 53.7 $\mu\text{mol/L}$ in patients receiving reduced-dose CsA ($P = 0.093$; primary endpoint). The incidences of BPAR grade $\geq 3A$, adverse events, and infections were similar between treatment groups. No renal function benefits were observed, possibly because of the inadequate adherence to reduced CsA exposure.

In an international, open-label, 24-month study (A2310), de novo heart transplant recipients were randomized to (1) standard-dose EVR with reduced-dose CsA, (2) high-dose EVR with reduced-dose CsA, or (3) MMF 3 g/d with standard-dose CsA.²⁵ All patients received GCs with or without induction, according to local practice. The combination of high-exposure EVR with CsA was associated with increased mortality, leading to cessation of recruitment to that study arm. These deaths occurred in patients with infected left ventricular assist devices pretransplantation in German centers and might have been caused by ATG induction leading to overimmunosuppression.¹⁰⁹ By month 24, the mortality rates were similar in the EVR and MMF groups (10.6% versus 9.2%, respectively), as were efficacy endpoints.

CNI-Free Regimens in De Novo Heart Transplant Recipients

SCHEDULE (The Scandinavian Heart Transplant Everolimus De Novo Study with Early Calcineurin Inhibitors Avoidance) was a 12-month, randomized, controlled, open-label trial in de novo adult heart transplant recipients.¹¹⁰ Patients were assigned within 5 days after transplantation to low-dose EVR with reduced-dose CsA ($n = 56$) or to standard-dose CsA ($n = 59$), with both MMF and GCs. All participants received induction therapy with ATG. EVR was initiated at a dose of 0.75 mg twice daily, adjusted to a target C_0 of 3–6 ng/mL during the first 7 weeks after transplantation and then 6–10 ng/mL after CsA withdrawal. CsA discontinuation took place at week 7 unless there was ongoing rejection at that time, in which case discontinuation could be postponed until week 11. After conversion to CNI-free treatment, both the incidence of BPAR overall and the incidence of treated rejection were significantly higher in the EVR group (both $P < 0.05$). However, this did not affect cardiac function at 12 months after transplantation. The mean whole-blood concentration of EVR from the time of randomization to month 12 was 7.0 ± 1.8 ng/mL in patients with grade 2R (ISHLT 2004

grading) rejections compared with 7.4 ± 2.3 ng/mL in those without rejection (not statistically significant). Measured GFR (mGFR) at 12 months after transplantation was significantly higher with EVR versus CsA (mean \pm SD: 79.8 ± 17.7 versus 61.5 ± 19.6 mL \cdot min $^{-1}$ \cdot 1.73 m $^{-2}$; $P < 0.001$), whereas the incidence of cardiac allograft vasculopathy (CAV) was lower in the EVR group (50.0% versus 64.6%, $P = 0.003$). Overall, there was a similar incidence of adverse events and serious adverse events in the 2 study groups, with a higher incidence of pneumonia and a substantially reduced risk of cytomegalovirus infection in the CNI-withdrawal arm.

The significant improvement in renal function in the CNI-free arm was confirmed in the follow-up analysis performed 36 months after transplant.¹¹¹ Despite the significantly higher incidence of BPAR without hemodynamic compromise in the CNI-free arm 12 months after transplant,¹¹⁰ there was no difference between the study groups regarding the incidence of BPAR after 36 months.¹¹¹

The results of an ongoing multicentre, randomized, controlled, open-label, 12-month study (MANDELA) including de novo heart transplant recipients in Germany are awaited.¹¹² After a 3-month CNI reduction phase, this study compares renal function (primary endpoint) and composite efficacy (at 12 months after randomization) in CNI-free patients treated with EVR (C_0 5–10 ng/mL) plus MPA and GCs, with that in patients receiving EVR (C_0 5–10 ng/mL) plus reduced-exposure CNI (TAC C_0 3–8 ng/mL or CsA C_0 50–150 ng/mL) and GCs.

Recommendations: EVR in De Novo Heart Transplantation

- The use of EVR in de novo heart transplant recipients requires TDM to achieve and maintain a recommended whole-blood target C_0 of 3–8 ng/mL in combination with reduced CsA dose. Use of EVR in combination with reduced-dose TAC may also be possible, but supporting evidence is limited. The CsA C_0 in patients receiving CNI-sparing regimens needs to be tightly controlled to achieve improved renal function.
- In a CNI-free regimen in combination with MPA and GCs, the EVR target C_0 range should be 5–10 ng/mL.
- Blood EVR concentrations >10 ng/mL are associated with an increased risk of adverse events and should be avoided.

De Novo Lung Transplantation

Experience with EVR in de novo lung transplantation is limited, possibly because of the negative outcome of 2 initial de novo lung transplantation studies with SRL. Both studies reported significant wound dehiscence and airway complications, leading to death in some patients.^{113,114} It is therefore recommended that mTOR inhibitor therapy is not started until the anastomosis and airways have healed,¹¹⁵ because of the drugs' inhibitory effects on growth factors and fibroblast proliferation.¹¹⁶ Consequently, all study protocols for the use of EVR in lung transplantation have avoided early administration of the drug.^{117–119}

A randomized, controlled study comparing azathioprine and conversion to EVR at month 3 in lung transplant recipients maintained on CsA and GCs (RAD001 B159)

demonstrated that the EVR regimen yielded a median steady-state blood EVR C_0 of 6.6 ng/mL (10th to 90th percentiles: 2.8 and 11.8 ng/mL, respectively).¹¹⁸ EVR concentrations were measured centrally, but investigators remained blinded to these values for the duration of the study, and no dose adjustments were made based on blood concentrations. The average dose and concentration of EVR remained stable between 12 and 24 months. CsA C_0 were slightly higher in EVR-treated patients during the first 2 months, but were then similar to those in azathioprine-treated patients for the remainder of the study. Approximately 95% of the patients in both treatment arms received GCs during the first year of the study. The composite endpoint of efficacy failure ($>15\%$ forced expiratory volume in 1 second (FEV₁) decline from baseline, graft loss, death, or loss to follow-up) occurred in 33.9% versus 21.8% of azathioprine-treated and EVR-treated patients, respectively, at 12 months ($P = 0.046$), but there was no difference in this composite endpoint at 24 months. The authors concluded that EVR demonstrated a slowing in loss of pulmonary function over time. Treatment discontinuations, particularly due to adverse events, serious adverse events, or high serum creatinine values, were more common with EVR compared with azathioprine.

CeMyLungs was a 3-year randomized, open-label, multicentre, investigator-driven superiority study comparing de novo enteric-coated MPS with delayed-onset EVR.¹²⁰ Before randomization, all patients received immunosuppression with CsA, MPS, and GCs. Patients were screened for eligibility once healing of the bronchial anastomosis had been confirmed by bronchoscopy between 1 and 3 months after transplantation. CsA doses were adjusted based on C_2 value and time after transplantation. Individual centers were allowed to use the assay of their choice to measure EVR concentrations when toxicity was suspected. EVR and MPS demonstrated equivalent efficacy in preventing the primary outcome of bronchiolitis obliterans syndrome at 3 years after lung transplantation, as well as secondary outcome parameters.

Results are awaited of the 4EVERLUNG study, an ongoing randomized, active-controlled, open-label, phase III study in de novo lung transplant recipients in Germany. This study compares a 12-month EVR-based quadruple immunosuppressive regimen with a center-specific CNI-based (either CsA or TAC) triple-drug immunosuppressive regimen.¹²¹

Personal experience (E.M.B.) has shown that it may be difficult to attain target EVR concentrations in lung transplant recipients with cystic fibrosis. Dose modifications are usually moderate, as the target concentration range is narrow. It is unusual for C_0 to be excessively out of range, except in cases of strong PK DDIs not prevented using TDM.

Recommendations: EVR in De Novo Lung Transplantation

- De novo EVR in lung transplantation should be avoided because of potential serious adverse effects, such as surgical complications and bronchial anastomosis. Therefore, introduction of EVR should be postponed until endoscopic confirmation of complete bronchial suture healing or until at least 3 months after transplantation.

- When EVR is administered in combination with a CNI, target EVR C_0 should be 3–8 ng/mL.
- Currently, there are insufficient clinical data to enable recommendations for appropriate therapeutic concentrations of EVR in CNI-free immunosuppressive regimens in lung transplant recipients.

Studies in Heart and Lung Transplant Maintenance

In a 12-month, open-label, multicentre, randomized, controlled study in Scandinavia [Nordic certican trial in heart and lung transplantation (NOCTET)], maintenance thoracic transplant recipients (190 heart transplants, 92 lung transplants) were randomized to continue their current CNI-based immunosuppression or to a quadruple regimen with EVR and a predefined CNI exposure reduction.¹²² EVR was initiated overnight with a target C_0 of 3–8 ng/mL. After an initial significant increase in infections in the EVR arm, reduction of EVR target C_0 to 3–6 ng/mL was recommended. On initiation of EVR, a parallel reduction of CNI dosage was performed to achieve a C_0 reduction of 30%–70% compared with baseline, with the target of achieving a CsA C_0 of 75 ng/mL or a TAC C_0 of 4 ng/mL. For patients in the EVR group who were receiving CsA and MMF, a 25%–50% reduction in MMF dose was recommended 1 week after the introduction of EVR, with further MMF dose reduction as required. In EVR-treated patients receiving TAC, MMF treatment was unchanged unless medically necessary. Concomitant medication with azathioprine, with or without GC therapy, was continued according to local practice.

Mean change in mGFR from baseline to month 12 was significantly in favor of EVR compared with controls [4.6 mL/min versus -0.5 mL/min ($P < 0.0001$) for heart and lung transplant recipients and 5.8 mL/min versus -0.1 mL/min ($P < 0.0001$) for heart transplant recipients only].¹²² At month 24, the improvement in mean mGFR remained significant in EVR-treated patients compared with controls in both subpopulations ($P < 0.001$).¹²³ The frequency of treated BPAR was similar between EVR and control group transplant recipients. A greater proportion of EVR-treated than control-group patients experienced adverse events, including serious adverse events, from baseline to month 12 ($P < 0.05$). However, there were no significant between-group differences in the rate of adverse events or serious adverse events (including pneumonia) during months 12–24. This study showed that concentration-controlled EVR therapy (C_0 3–8 ng/mL) with reduced CNI exposure had an acceptable safety profile in heart and lung transplant recipients, with a beneficial effect on renal function.

A subanalysis of the NOCTET study showed that EVR introduction and reduced CNI dose significantly improved renal function in maintenance heart and lung transplant recipients with preexisting moderate or severe renal failure (mGFR 30–59 or 20–29 mL·min⁻¹·1.73 m⁻², respectively). However, this beneficial effect was limited to patients undergoing conversion within 5 years of transplantation.¹²⁴

At 12 months after randomization in the NOCTET study, of the patients who had received a heart transplant >5.1 years previously, those treated with EVR had a significant morphological progression of CAV as measured by

virtual histology analysis of intravascular ultrasound data compared with the control group.¹²⁵ However, in the 5-year analysis, there was no difference in CAV progression between the study groups.¹²⁶

Recommendations: EVR in Heart and Lung Transplantation Maintenance

- Current data support the introduction of EVR in maintenance patients in the first 5 years after transplantation to improve renal function and to reduce the progression of CAV.

Oncology Indications

Angiogenesis is an important step in the proliferation of many tumors, and blockade of vascular endothelial growth factor may offer an opportunity to inhibit tumor growth. Since mTOR is essential for the activity of vascular endothelial growth factor, mTOR inhibitors could have an effect on tumor angiogenesis. Phase I studies confirmed the hypothesis that EVR might have an effect on renal cell carcinoma, a tumor type in which angiogenesis is known to play a crucial role in invasion and dissemination. In 92 patients with a range of different tumor types, EVR treatment was associated with partial responses in 4 patients, and 12 patients remained progression-free for at least 6 months, including 5 of 10 patients with renal cell carcinoma.¹²⁷ Furthermore, EVR was satisfactorily tolerated at doses up to 70 mg/wk and 10 mg/d. On a theoretical basis, dose selection for EVR might also be based on PD considerations, ie, the degree of inhibition of downstream mTOR pathways.¹²⁸ However, 10 mg/d seems to be the maximum tolerated dose. At this dose, EVR C_0 of up to 17.0 ng/mL have been observed, with large interindividual differences.¹²⁹

Efficacy was demonstrated in subsequent studies with EVR in renal cell carcinoma, and EVR was registered for this indication based on results of a large phase III study.¹³⁰ However, PK data were not available, so a concentration–effect relationship could not be demonstrated for either efficacy or toxicity. Only 1 study involving EVR in oncology has shown a concentration–effect relationship.¹³¹ In patients with metastatic renal cell carcinoma ($n = 42$), the median EVR C_0 was 14.1 ng/mL (range, 2.6–91.5 ng/mL). Fourteen (67%) versus 8 (38%) patients with median EVR C_0 above or below 14.1 ng/mL, respectively, were progression-free at 6 months ($P = 0.06$); median progression-free survival was 13.3 versus 3.9 months, respectively [hazard ratio (HR), 0.66; 95% CI, 0.33–1.31; $P = 0.23$], and median overall survival was 26.2 versus 9.9 months, respectively (HR, 0.62; 95% CI, 0.28–1.37; $P = 0.24$).

EVR is also approved for the treatment of breast cancer^{132–134} and neuroendocrine tumors.¹³⁵ Although there is a strong rationale for TDM of EVR in the treatment of solid-organ transplant recipients, this approach has not been widely studied in oncology settings and PK data were not systematically assessed for the potential benefit of EVR TDM in cancer patients. Although the relationship between EVR concentrations and efficacy is important, the relationship between EVR concentrations and toxicity may limit its long-term use. Determination of EVR concentrations at the onset of severe adverse events may enable a better understanding of pathophysiology, permitting dose reduction rather

than drug withdrawal in patients with high EVR concentrations.⁷⁰ This is of particular importance when EVR is used as combination therapy with the aim of improving long-term survival. It may be possible to perform retrospective analyses of data from previous studies. However, the ultimate goal is to include TDM of EVR in future studies to fully assess the value of this approach in cancer patients.

Recommendations: EVR in Oncology

- Further studies are required to determine the clinical utility of TDM for EVR in oncology settings.

Other Clinical Applications

EVR has also been used to treat several conditions other than the transplant and oncologic indications discussed above. In general, data on the PK of EVR and the potential benefit of TDM in the treatment of such diseases are very limited. Likewise, there is a lack of data pertaining to TDM of EVR in special clinical situations and populations. These include, but are not limited to, children (very young), pregnant women, critically ill patients, and the very old. It is the opinion of the authors that despite the lack of evidence, TDM may be justified in such special circumstances to get at least some indication of the exposure to EVR.

Pediatric Transplantation

Experience with EVR in pediatric transplantation is limited.¹³⁶ Published data are mostly uncontrolled and concern the use of EVR introduced shortly after transplantation, with reduced-dose CsA. TDM was performed as part of clinical care in some of these single-arm studies in kidney transplantation, targeting EVR to a $C_0 \geq 3$ ng/mL¹³⁷; 4–6 ng/mL in the first 6 months after transplantation followed by 3–5 ng/mL^{138,139}; and 5–10 ng/mL.¹⁴⁰ These target concentration ranges were extrapolated from data in adult patients. An ongoing, randomized, controlled clinical trial in pediatric de novo kidney transplant recipients (CRA-DLE; EudraCT 2010-024381-21)¹⁴¹ will compare standard-dose TAC plus MMF with reduced-dose TAC plus EVR and may provide more evidence to support the use of TDM for EVR in the pediatric population.¹⁴² Because of the paucity of data on the EVR concentration–effect relationship in this setting, no evidence-based recommendations can be made for TDM of EVR in children. Nonetheless, given the fact that the PKs of EVR in children are variable and related to age, weight, and body surface area,² and in the light of data obtained in adult transplant recipients, TDM of EVR in children is justified.

Tuberous Sclerosis Complex

TSC is a rare autosomal dominant genetic disease caused by mutations in the *TSC1* or *TSC2* tumor suppressor genes.¹⁴³ Both *TSC* gene products inhibit mTORC1, and it is believed that subsequent somatic mutation results in constitutive activation of mTORC1 in different organs. Overexpression of mTORC1, which is a critical regulator of cell growth and proliferation, leads to aberrant downstream signaling and growth of noncancerous hamartomas in the kidney (renal angiomyolipomas), brain (SEGA, cortical

tubers), heart (cardiac rhabdomyomas), liver, eyes, and skin (angiofibromas). Therapy with an oral mTOR inhibitor may target the constitutive mTOR overexpression in affected tissue and could be an effective treatment option for this genetic disorder.

After initial observations that mTOR inhibitors may cause regression in kidney and brain tumors, 2 randomized, double-blind, placebo-controlled phase III trials were initiated in patients with TSC. The Examining Everolimus in a Study of TSC-1 (EXIST-1) trial examined the effect of EVR (4.5 mg·m⁻²·d⁻¹; titrated to achieve blood C_0 of 5–15 ng/mL) on SEGA in patients aged up to 65 years [median age 9.5 years (range, 0.8–26.6 years)].^{144,145} TDM was considered an integral part of the study because participants included children using antiepileptic medication, known to interfere with EVR PK. The starting dose was chosen to be just less than the maximum tolerated dose in children with malignancies (5 mg·m⁻²·d⁻¹). After initial dose adjustment, the median EVR C_0 was maintained at 5–7 ng/mL during weeks 6–132. The EXIST-2 trial investigated a fixed dose of 10 mg EVR on renal angiomyolipoma in adult patients (median age, 31.0 years), stratified using enzyme-inducing antiepileptic drugs (EIAEDs).¹⁴⁶ Mean EVR C_0 was 7–10 ng/mL and exhibited large interindividual variability (56%–94%), with lower concentrations in patients using EIAEDs [5.10 (SD 3.02) ng/mL versus 10.41 (SD 9.47) ng/mL in patients not using EIAEDs].

In both studies, highly significant tumor reduction was seen in most patients, leading to approval of EVR in several countries for this indication. Despite lower concentrations, patients using EIAEDs had a similar response to therapy. There was only a weak indication that 2-fold higher EVR concentrations would result in slightly better efficacy (approximately 10%). In general, adverse events were consistent with those previously reported for patients using mTOR inhibitors. Long-term EVR therapy was safe and continued to be highly effective although adverse events required frequent dose reductions.^{145,147,148} Importantly, EVR toxicity did not seem to be related to TSC genetic background, which is known to be associated with altered mTOR expression. Even in children, EVR was generally well tolerated and adverse effects were manageable, mostly by dose reduction.

Based on these findings, EVR is recommended for first-line treatment of asymptomatic, growing renal angiomyolipoma associated with TSC and as an effective medical treatment option for growing but otherwise asymptomatic SEGA.¹⁴⁹ A large, randomized, placebo-controlled study is ongoing to determine the efficacy and safety of 2 EVR C_0 concentration ranges when used as adjunctive therapy in patients with TSC who have refractory partial-onset seizures [EXIST-3 (NCT01713946)].

Recommendations: EVR in TSC

- Firm evidence for the benefit of TDM of EVR in patients with TSC is lacking. As many patients with TSC require EIAEDs, TDM of EVR seems advisable at least for this population and PD findings suggest TDM might help avoid overexposure and improve safety.

- Further PK investigations are needed to better understand the minimum doses needed for efficacy and to explore the relationship between EVR concentrations and toxicity in this population.

Pulmonary Arterial Hypertension

EVR has also been used for the treatment of Pulmonary Arterial Hypertension (PAH), a disease in which proliferation of vascular cells plays an important role. In a single-center, open-label pilot study, 10 patients having progressive PAH despite the use of at least 2 vasodilating drugs were treated for 6 months with EVR, targeted to a C_0 of 5–8 ng/mL.¹⁵⁰ In 2 patients, EVR had to be stopped prematurely because of an adverse event (acute bronchitis and right cardiac decompensation). In the remaining 8 patients, a significant reduction in pulmonary vascular resistance was seen and a trend toward an improved 6-minute walking distance was observed. These preliminary findings may warrant a future randomized, controlled clinical trial of EVR for PAH.

Neurofibromatosis Type 2

Neurofibromatosis type 2 is a rare genetic disorder caused by inactivation of the *NF2* tumor suppressor gene and is characterized clinically by the development of multiple tumors, including bilateral vestibular schwannomas.¹⁵¹ Merlin, the product of the *NF2* gene, is a negative regulator of mTORC1 and loss of merlin activity results in activation of mTORC1 signaling in neurofibromatosis type 2-related tumors.¹⁵¹ In 2 phase II studies, the effect of EVR on progressive vestibular schwannomas in patients with neurofibromatosis type 2 was studied.^{152,153} In both studies, adult patients received EVR in a fixed dose of 10 mg per day and treatment was not guided by TDM. In the study by Karajannis et al,¹⁵² no benefit of EVR therapy was observed, whereas in the trial of Goutagny et al,¹⁵³ EVR seemed to stabilize tumor size in some patients. Interestingly, in the latter study,¹⁵³ EVR whole-blood predose concentrations were higher in stable patients than in progressive patients: median of 22.7 ng/mL (IQR 16.5–30.2) versus 10.6 ng/mL (IQR 9.7–15.5).

PHARMACOGENETIC MONITORING

Potential Pharmacogenes

EVR is metabolized through oxidation by cytochrome P450. CYP3A4 is a much more active catalyst than CYP3A5 (the recombinant CYP3A5/3A4 activity ratio is approximately 1:6)^{31,154} and CYP2C8 accounts for less than 10% of EVR's hepatic metabolism.³¹ As EVR is an ABCB1 substrate, this may limit its intestinal absorption as suggested from studies conducted in vitro and using knock-out mice.^{29,155,156} Hepatic extraction of EVR does not seem to be an active process because EVR has a very low affinity for the major uptake transporters expressed in hepatocytes.¹⁵⁷ However, canalicular excretion of the drug or its metabolites probably involves ABCB1. In the kidney, ABCB1 is not expected to play a significant role in EVR PK because renal elimination is a minor disposition pathway compared with biliary excretion.

The main proteins and corresponding genes involved in EVR PD are mTOR, also called FRAP (FKBP–rapamycin-associated protein) coded by the *FRAP1* (or *MTOR*) gene; FK-BP12 (*FKBP12*); p70s6K (*RPS6KB1*); and raptor (*RPTOR*).

Pharmacogenetic–Pharmacokinetic Relationship

CYP3A4

Data regarding the effect of CYP3A4 genetic variations on EVR PK (or PD) are still scarce. The *CYP3A4*1B* allele (rs2740574; c.-392G>A) showed no association with EVR dose-normalized concentrations in lung transplant recipients.¹⁵⁸ To the best of our knowledge, no other study has investigated the influence of this allele on EVR PK in solid organ transplantation. More generally, the functional consequence of this allele is very controversial. Although some in vitro studies suggested that it is associated with increased transcriptional activity,^{159,160} this was not found in another study¹⁶¹; the authors hypothesized that the transcriptional effect previously reported¹⁵⁹ was an artifact of the expression system used.¹⁶¹

In contrast, the *CYP3A4*22* (rs35599367; c.522-191C>T) variant has recently and more consistently been reported to be associated with decreased CYP3A4 activity in hepatocytes.¹⁶² To our knowledge, only 1 study has investigated its effect on EVR PK.¹⁶³ No significant influence on EVR PK was observed in relation to the variant, which was found in 9 of 97 patients; 8 were heterozygous carriers and 1 was a homozygous carrier of the *CYP3A4*22* allele, which is in accordance with the previously reported minor allele frequency (~5%).

CYP3A5

Six studies have been reported to date, showing no association between the common *CYP3A5*3* allele (rs776746; c.219-237G>A) and EVR blood concentrations, dose requirement, or PK parameters estimated using population PK approaches^{31,37,38,158,163–166} (Table 1). Three studies were conducted in stable kidney-transplant recipients not receiving CNIs,^{26,137,149} which ensured that enzyme inhibition did not influence the pharmacogenetic associations; 2 studies were in heart transplant recipients treated with CNIs.^{37,165} In 1 study, heart allograft rejection (4 episodes), new-onset type 2 diabetes (2 cases) and infections (8 cases) were not found to be related to CYP3A5 genotype.¹⁶⁵ In a study of lung transplant recipients treated with EVR alone (n = 6) or in combination with TAC (n = 57) or CsA (n = 2), comparable dose-normalized EVR steady-state C_0 were reported in CYP3A5 expressers (n = 11; *CYP3A5*1/*3* genotype) or nonexpressers (n = 54).¹⁵⁸ Further experimental studies using genotyped human liver microsomes demonstrated that the *CYP3A5*3* variant has no significant influence on EVR hepatic metabolism,³¹ possibly reflecting the fact that CYP3A4 is a better catalyst of EVR metabolism than CYP3A5. A study by Moes et al¹⁶³ suggested that neither *CYP3A5*3* nor its combination with *CYP3A4*22* had a significant influence on EVR PK.

TABLE 1. Studies of the Effects of the *CYP3A5*3* Allele on EVR PK

Type of Solid Organ Transplantation	No. Patients (Other Immunosuppressive Drugs Administered)	Main Results (<i>CYP3A5*1</i> Versus <i>CYP3A5*3</i> Carriers)	Reference
Kidney	n = 28 (MMF: n = 23 plus tapered GC regimen)	DN-C ₀ : 3.8 ± 1.8 versus 3.4 ± 2.0 ng·mL ⁻¹ ·mg ⁻¹ (<i>P</i> = 0.4417); DN-AUC _{0–12 h} : 61.1 ± 20.3 versus 69.6 ± 29.2 μg·h·L ⁻¹ ·mg ⁻¹ (<i>P</i> = 0.4417); n = 7 expressers	Picard et al ³¹
	n = 53 (prednisolone)	No influence on EVR PK (CL/F, V/F, first-order absorption rate constant) described using a 2-compartment model (in this study, TDM was performed to reach an AUC _{0–12 h} of 120 μg·h·L ⁻¹); n = 6 expressers	Moes et al ³⁸
	n = 97 (prednisolone) n = 184 patients receiving SRL or EVR (percent of patients receiving EVR not provided) (CNI: n = 105)	CL/F +12% (<i>P</i> = 0.15131); n = 15 expressers Significantly lower concentration/dose/kg ratio (at month 1 after treatment initiation); higher risk of proteinuria at month 6 for carriers of the <i>CYP3A4*1-CYP3A5*1</i> (AA-GA) haplotype; OR for moderate (>0.5 g/L) and significant (>1.5 g/L) proteinuria: 4.87 (95% CI, 1.48–16.35; <i>P</i> = 0.008) and 6.71 (95% CI, 2.08–21.63; <i>P</i> = 0.003)	Moes et al ¹⁶³ Bandur et al ¹⁶⁴
Heart	n = 30 (CsA and prednisolone)	Dose at month 1: 1.0 ± 0.5 versus 1.5 ± 0.8 mg (<i>P</i> > 0.05); C ₀ at month 1: 6.2 ± 2.7 versus 6.9 ± 2.9 ng/mL (<i>P</i> > 0.05); results also not significant at months 2, 12, and 36; n = 3 expressers	Kniepeiss et al ^{165,166}
	n = 59 (CsA, n = 42; TAC, n = 12; MMF, n = 4); GC, n = 59	No difference in apparent clearance (population PK analysis); n = 14 expressers	Lemaitre et al ³⁷
Lung	n = 65 (TAC, n = 57; CsA, n = 2; prednisone, n = 65)	Steady-state DN-C ₀ : 2.1 ± 1.1 versus 2.6 ± 1.7 ng·mL ⁻¹ ·mg ⁻¹ (<i>P</i> > 0.05); n = 11 expressers	Schoeppler et al ¹⁵⁸

CL/F, apparent oral clearance; DN, dose-normalized, OR, odds ratio; V/F, apparent volume of distribution.

A possible relationship between the *CYP3A4*1B-CYP3A5*3* haplotype and EVR-related (and SRL-related) adverse effects has been reported.¹⁶⁴ In kidney transplant recipients (n = 184) receiving either EVR or SRL, a significantly higher frequency of the *CYP3A4*1-CYP3A5*1* (AA-GA) haplotype was seen in patients with moderate (>0.5 g/L, ≤1.5 g/L) or significant (>1.5 g/L) proteinuria (*P* = 0.008 and *P* = 0.003, respectively). There were also significant differences in mTOR inhibitor C₀·dose⁻¹·kg⁻¹ ratios between the 2 haplotype groups. However, all patients were receiving CNIs, either de novo (44.5%) or as rescue therapy (55.5%), suggesting that DDIs may have been a confounding factor.

CYP2C8

*CYP2C8*3* is the most relevant variant allele of *CYP2C8* in whites. It denotes 2 highly linked variants, rs11572080 (c.416G>A; p.R139K) and rs10509681 (c.1196A>G; p.K399R), occurring at relatively high frequency in whites (11%–14%), but rarely in Asians and Africans.¹⁶⁷ Its effect on EVR PK has been studied in a range of transplantation settings.^{38,158,166} None of these studies found a significant relationship between EVR dosing or blood concentrations and this *CYP2C8* genotype, which is consistent with its minor role in EVR metabolism.³¹ The study in lung transplant recipients also failed to demonstrate any association with *CYP2C8*2* (rs11572103: c.805A>T; p.Ile199Phe) and *CYP2C8*4* alleles (rs1058930: c.792C>G; p.Ile264Met),¹⁵⁸ whereas the study in heart transplant recipients found no association between *CYP2C8*3* and EVR adverse effects.¹⁶⁶

ABCB1

Three pharmacogenetic studies also investigated the effects of *ABCB1* genetic polymorphisms on EVR PK in solid organ transplantation and none reported significant associations.^{37,38,158} No influence of *ABCB1* c.1236C>T, 2677G>A/T, 3435C>T or c.-129T>C single-nucleotide polymorphisms (SNPs) was seen on EVR apparent clearance, volume of distribution from the central compartment (V₁/F) or first-order absorption rate constant in a population PK study based on 783 C₀ blood concentrations from 53 kidney transplant recipients switched from a triple therapy (CsA, MMF, prednisolone) to a CNI-free dual therapy of EVR twice daily and prednisolone.³⁸ No effect of c.3435C>T *ABCB1* SNP on EVR PK was seen using a similar approach in heart transplant recipients.³⁷ Similarly, no effect of *ABCB1* haplotype c.1236-2677-3435 was seen on EVR steady-state dose-normalized C₀ in lung transplant recipients mostly cotreated with CNIs (87.7% receiving TAC and 3.1% CsA).¹⁵⁸

Data are lacking regarding the potential impact of *ABCB1* polymorphisms on EVR PD, in particular on the uptake of the drug by T cells.

Other Pharmacogenes

No associations were found in lung transplant recipients between EVR dose-normalized concentrations and 3 common variants in *NR1I2* (rs1523130, rs2472677, and rs763645), a gene coding for the nuclear pregnane X receptor (PXR) that regulates *CYP3A* and *ABCB1* gene expression.¹⁵⁸ Similarly, no influence of *NR1I2* rs2276706 and rs6785049 on EVR PK was reported in a population PK study in kidney transplant

recipients.³⁸ This is consistent with the absence of impact of *CYP3A* and *ABCB1* polymorphisms, suggesting that indirect modulation of EVR PK through PXR genetic variation is very unlikely.

Pharmacogenetic–Pharmacodynamic Relationship

Mutations of the gene encoding mTOR (especially in the FRB domain) or the proteins of the EVR signaling pathway (FK-BP12, p70S6K, raptor) might confer a resistance phenotype to the drug, as demonstrated in mammalian cell lines.¹⁶⁸ However, to our knowledge, no association study between such polymorphisms and EVR effects has been reported to date. A panel of genetic variations in mTOR ($n = 5$ tag SNPs), raptor ($n = 4$ SNPs selected based on the literature), and p70S6K ($n = 5$ tag SNPs) were investigated in 2 independent populations of kidney transplant recipients treated with SRL.¹⁶⁹ A significant association was found between a variant haplotype of the *MTOR* gene and decreased hemoglobin concentrations, but this might not have any clinical significance. None of the other genetic variations studied were associated with SRL adverse effects.

Recommendations: Genotyping

- There is insufficient evidence to recommend the prospective genotyping of *CYP3A5* and *CYP3A4* in solid organ transplant recipients for dose adjustment of EVR. However, combined *CYP3A5*3* and *CYP3A4*22* genotyping to identify patients with high or low *CYP3A* total activity might be proposed for the retrospective documentation of cases of unexpected EVR blood concentrations or adverse effects, combined with comprehensive exploration of potential drug–drug or food interactions.
- *ABCB1* genotyping cannot be recommended based on the available evidence.
- Further investigation of the pharmacogenetics of the mTOR pathway may be warranted, although the likelihood of potential clinical utility is low given the data previously generated for SRL.

PHARMACODYNAMIC MONITORING

PD monitoring aims to individualize drug therapy as a complement to TDM. It focuses on the drug's effects on target cells or target molecules. PD monitoring of immunosuppressive and anticancer drugs can be nonspecific (ie, investigating general effects on immune or tumor cells) or drug specific (ie, focusing on the molecular targets and precise pharmacological action of a particular medication). Limited data are available regarding PD monitoring of EVR. A variety of heterogeneous experimental approaches for both specific and nonspecific PD monitoring have been published. However, most assays are not standardized and have barely been validated. In addition, they are laborious and time-consuming and therefore not suitable for routine clinical use. Generally, single-center assay protocols have been used in small groups of patients and have not been cross-validated. To date, there is no evidence of an association between EVR PD markers and clinical outcome.

Nonspecific PD Monitoring of EVR

Approaches used for nonspecific PD monitoring of EVR include cell proliferation assays with lymphocytes or tumor cells; cytokine production in lymphocytes and T cells; intracellular production of ATP in CD4⁺ T cells; surface activation markers on T cells; and changes in the proportions of lymphocyte subsets.^{170–175} These are summarized below.

Proliferation inhibition has mainly been monitored using the thiazolyl blue tetrazolium bromide (MTT) assay. EVR has been shown to inhibit cell proliferation *in vitro* in a dose-dependent manner after stimulation of whole blood or isolated PBMCs. In kidney allograft recipients, *ex vivo* proliferation of PBMCs in response to stimulation with an anti-CD3 antibody was inhibited for up to 10 hours after a single EVR dose (0.75–1.5 mg).¹⁷⁰ Cell proliferation has also been used to assess the sensitivity of renal carcinoma cells to EVR. In a small study in 3 patients, tumor tissue was digested and isolated tumor cells were incubated *ex vivo* with different concentrations of EVR to assess the 50% inhibition of cell proliferation. The *ex vivo* sensitivity of renal carcinoma tumor cells was associated with clinical response.¹⁷⁵

Cytokine production has been assessed either by measurement of IL-10 concentrations in cell culture supernatants of stimulated PBMCs or by evaluating intracellular interferon gamma production in T cells using flow cytometry. Anti-CD3 antibody–induced IL-10 synthesis was reduced in PBMCs isolated from stable kidney transplant recipients after repeated EVR dosing compared with baseline values before initiation of EVR therapy and compared with levels in patients who received placebo rather than EVR in addition to CsA and prednisolone.¹⁷⁰ IL-10 production returned to baseline levels after cessation of EVR. In PBMCs, stimulated *ex vivo* for 12 hours using staphylococcal enterotoxin B, intracellular interferon gamma was more suppressed in stable liver allograft recipients administered EVR than those receiving CsA.¹⁷³

In a recent study,¹⁷⁴ the immunomodulatory effect of EVR in heart transplant recipients was evaluated using an FDA-approved “immune monitoring assay” (Immuknow; Cylex Inc, Columbia, MD) based on measurement of the increment in intracellular ATP after mitogenic stimulation of T cells (CD4⁺) with phytohemagglutinin. Results suggest that this biomarker may predict infections in such patients.

The cell surface T-cell activation marker CD26 (dipeptidyl peptidase IV) may also have potential as a biomarker. A pilot study found that its expression on CD3⁺ T cells was time-dependently inhibited by 30 nmol/L EVR in isolated PBMCs stimulated with phorbol-12-myristate-13-acetate for 72 hours.¹⁷¹

Liver transplant patients receiving EVR had increased percentages of total CD4⁺ T cells, naive CD4⁺ T cells, and naive CD8⁺ T cells, but lower percentages of CD8⁺ T cells, compared with patients receiving CsA.¹⁷³ mTOR inhibitors have been shown to be effective at inducing and maintaining regulatory T cells.¹⁷² They induce anergy in naive T cells, promote the expansion of regulatory T cells, and inhibit the maturation of dendritic cells, thus probably promoting immunologic tolerance. In the liver transplantation study, a higher proportion of regulatory T cells (CD3⁺, CD4⁺, CD25bright,

FoxP3⁺, CD127⁻) was seen in patients treated with EVR compared with CsA.¹⁷³ The frequency of regulatory T cells expressing the homing receptor CXCR3 was also higher with EVR.

To date, no study has investigated the association between nonspecific PD biomarkers of EVR treatment and clinical outcome. Drug selection or dose adaptation based on PD monitoring has not been used in either patients with cancer or transplant recipients administered EVR-based therapy.

Specific PD Monitoring of EVR

For drug-specific PD monitoring of EVR, measurements are feasible of mTOR activity, phosphorylation of its downstream targets 4E-BP1 (P-4E-BP1) and S6 kinase beta-1 (P-p70S6K1), p70S6K1 activity, and the subsequent phosphorylation of the ribosomal S6 protein (P-rS6P) (Table 2). Intracellular phosphorylated proteins can be detected by phosphoprotein-specific antibodies using Western blotting, enzyme-linked immunosorbent assay (ELISA) techniques (either singleplex or multiplex) after cell lysis and protein extraction, or by flow cytometry after cell permeabilization (phospho flow cytometry).¹⁷⁶ p70S6K1 activity can be measured by isotope-based assays using exogenously added ribosomal S6 and ³²P as substrates.^{177,178} In an observational cohort study, mTOR activity was measured using a GST-p70S6K fusion protein immobilized on an ELISA plate¹⁷⁹ and specifically phosphorylated by mTOR at position THR389. This phospho-epitope was then detected by an appropriate antibody in a sandwich assay.

Most of the studies using the assays mentioned above were performed with SRL. Few studies have been undertaken of specific PD monitoring of EVR. In oncology, studies have investigated dephosphorylation of P-4E-BP1 and P-p70S6K1, and also inhibition of p70S6K1 activity in response to EVR in tumor tissue and renal carcinoma cells.^{127,175,180} p70S6K1 activity in PBMCs has been used as a surrogate biomarker for the PD effect of EVR on tumor tissue and was included in dose-finding studies in cancer patients.¹²⁷ The largest investigation with EVR in transplantation used phospho flow cytometric quantification of p70S6K phosphorylation.¹⁸¹ The assay was first validated in vitro with SRL and mitogen-stimulated CD4⁺ T cells. Ex vivo PD investigations were then performed with stable kidney allograft recipients administered EVR-based or SRL-based therapy. Specific PD monitoring was combined with nonspecific monitoring of IL-2 production by T cells. In both EVR-treated and SRL-treated patients, there was a clear positive correlation

between IL-2-producing CD3⁺ T cells and the degree of p70S6K phosphorylation. However, no association between EVR C₀ and p70S6K1 dephosphorylation was observed.

Recommendations: PD Monitoring

- PD monitoring on a routine basis to guide EVR therapy cannot be recommended until more data on assay standardization, analytical cross-validation, and diagnostic validation in prospective clinical trials are available.

MEASUREMENT OF EVR CONCENTRATIONS

Sample Stability

EVR has been shown to be stable in EDTA-anticoagulated whole blood for up to 7 days when stored at 20°C or 30°C, and for 3 days at 37°C.^{182–184} A short-term stability study used the Innofluor Certican assay (Seradyn Inc, Indianapolis, IN) and an LC-MS/MS method to investigate samples taken from kidney transplant recipients and stored at 30°C in daylight for 3–7 days (EVR concentrations 1.7–17.6 ng/mL).¹⁸³ No statistically significant differences were found with either method in the EVR concentrations measured at day 3 or 7 compared with day 1. However, in hot geographical regions, samples received in central laboratories could be at higher temperatures (eg, temperatures in excess of 50°C) for several hours during transportation. In such cases, shipping on dry ice or with ice packs in insulated cold-boxes is recommended. No significant loss of EVR was reported when spiked whole-blood samples were stored at 4°C for 7 days or for at least 6 months at –20°C.¹⁸⁵ In addition, blood samples can be stored at –80°C without loss of EVR for up to 2 years.¹⁸⁶ Furthermore, EVR has been shown to be stable in blood samples for at least 3 freeze–thaw cycles.^{182,187–189}

EVR in dried blood spots from quality control (QC) samples collected on Protein Saver 903 sampling paper (Whatman GmbH) impregnated with a plasma-protein/ammonium acetate/formic acid solution was found to be stable for 3 days at 60°C and for 1 month at 4°C, 20°C, and 32°C.¹⁹⁰ Using nonimpregnated Whatman 31 ET CHR paper and whole blood spiked with EVR at concentrations of 3 ng/mL and 40 ng/mL, the EVR in the dried blood spots was stable for at least 7 days at 22°C and at least 2 days at 37°C.¹⁹¹

Recommendations: Sample Stability

- Transportation of EDTA-anticoagulated whole-blood specimens for EVR TDM from the site of collection to the laboratory without refrigeration is acceptable, providing the transportation time does not exceed 1 week at temperatures up to 30°C or 3 days at temperatures up to 37°C. Cooling of samples is advisable at higher temperatures. If multiparameter analysis including drugs with limited stability is intended from the same sample, long-term transportation and storage conditions should be adjusted appropriately. For prolonged storage times, specimens should be stored at –20°C or below.
- Laboratories performing TDM services are advised to be aware of sample stability issues and to include stability evaluation in their method-validation protocols. It is

TABLE 2. Specific EVR Targets Used as PD Biomarkers and Underlying Mechanisms

Marker	Mechanism
mTOR activity	Phosphorylates 4E-BP1 and S6K1
P-4E-BP1	4E-BP1 phosphorylated by mTOR
P-p70S6K1	S6K1 phosphorylated by mTOR
S6K1 activity	Phosphorylates ribosomal S6 protein
P-rS6P	Phosphorylated by S6K1

S6K1, ribosomal protein S6 kinase beta-1; P-p70S6K1, phospho-p70 S6 kinase beta-1; rS6P, ribosomal S 6 protein.

advisable to establish specific procedures for temperature control during transport and for storage and handling of samples, and to record the temperature throughout sample transport to detect system failures.

Analytical Methods for EVR Quantification

Chromatographic Procedures

Contemporary state-of-the-art chromatographic approaches for EVR TDM require mass spectrometry-based analyte detection. In contrast to other immunosuppressant drugs introduced before the routine availability of bioanalytical LC-MS/MS, most clinical trials for EVR have used this method rather than immunoassays.^{32,68,192–194} Consequently, a fully validated LC-MS/MS method is the more appropriate standard for EVR TDM.

Based on reports published in the late 1990s,^{182,195} LC-MS/MS applications for monitoring whole-blood EVR concentrations became widely available; combined multiplex assays enabling monitoring of CsA, TAC, SRL, and EVR quickly emerged.^{196,197} The use of UV detection (LC-UV) was explored as an alternative to LC-MS/MS.^{185,198} However, because of the rather low molecular extinction coefficient of the EVR triene chromophore, the general low selectivity of UV detection, even if higher wavelengths (such as 278 nm) are used and the very low target concentrations, this approach cannot be recommended.

State-of-the-art LC-MS/MS platforms for the measurement of immunosuppressant drugs, including EVR, mainly rely on one-dimensional or 2-dimensional chromatography (using a trap and an analytical column). Such methods are preceded by ZnSO₄ treatment of the whole-blood specimen and organic extraction followed by mass spectrometric detection in the “selected reaction monitoring” mode (also known as multiple reaction monitoring) using electrospray ionization. Alternatively, whole-blood samples supplied as dried blood spots can be used,^{191,199} but this approach has not yet entered routine clinical practice.

Analyte quantification is based on multilevel calibration with the pure analyte and the addition of an internal standard into the extraction procedure. Although in-house production of calibration materials is possible, use of commercial calibrators carrying the *in vitro* diagnostics Conformité Européenne (IVD-CE) mark is preferred to minimize calibration bias between centers. Since whole blood is the preferred matrix, both protein precipitation and erythrocyte lysis have to be performed. Organic solutions (methanol or acetonitrile) with 0.1–0.2 mol/L ZnSO₄ are frequently used; the use of pure organic solvent-based sample preparation protocols is now seen as outdated.^{200–202} To aid cell lysis, distilled water can be added before protein precipitation^{203–205} or samples can be frozen and thawed. Ten-minute incubation with ammonium bicarbonate has been proposed as an alternative.²⁰⁶

An appropriate internal standard must be added to the extraction solvent to allow for both fluctuation of extraction efficacy and mass spectrometer ion yield (ion suppression and ion enhancement). The optimal choice is a commercially available stable isotope-labeled derivative, for example

EVR-D₄ or ¹³C₂D₄. These have almost identical physicochemical properties as the analyte and chromatographically coelute in almost any assay set-up. However, attention should be given to possible labeled substance contamination with the non-labeled parent—a potential problem with all isotope-labeled internal standards. For instance, contamination of ¹³C₂D₄-EVR with SRL has been reported to be problematic in a combined EVR/SRL assays.²⁰⁶ Therefore, purity testing of all isotope-labeled internal standards before use is highly recommended. Currently, the SRL derivative 32-desmethoxyrapamycin (not commercially available)²⁰⁷ or the macrolide ascomycin (a structural homolog of TAC) is still used as internal standard in some laboratories. Although acceptable performance has been reported, particularly when using 32-desmethoxyrapamycin,^{207,208} isotope-labeled internal standards are preferable to the structural analogs that are not closely related to EVR.

In most published assays, EVR analysis in the mass spectrometer starts with *m/z* = 975.6 Da as the precursor ion; hence, the ammonium adduct is favored over the protonated species for analyte detection. The readout mass in the second ion selector is usually *m/z* = 908.5 Da. To allow accurate and reproducible chromatographic peak-area assessment, at least 8–10 data points must be recorded for each mass transition used. This can be challenging if all 4 immunosuppressant drugs have to be monitored in parallel and if ultrafast chromatography is used. Utmost care must be taken in the design and validation of an assay,²⁰⁹ so that ion-suppression effects are either completely avoided or evaluated and accounted for.²¹⁰

Some manufacturers (eg, Waters, Chromsystems, Recipe, AB Sciex) are introducing IVD-CE-marked assays.^{211,212} Use of such “kits” on existing internally qualified or even IVD-CE-certified LC-MS/MS platforms is perhaps preferable to in-house design and validation of a “laboratory-developed test” system. However, despite their ready availability, laboratories still need extended expertise to establish such assays by instrument qualification and performance evaluation, to maintain their daily performance, and to establish measures for timely LC-MS/MS troubleshooting and service.²⁰⁹

Although mostly restricted to tertiary-care centers, fully validated LC-MS/MS analysis is currently the preferred standard in EVR TDM. High-sensitivity LC-MS/MS procedures allow quantification limits at least down to 1 ng/mL and interassay coefficient of variation of <10%. However, results from the EVR International Proficiency Testing Scheme (Analytical Services International Ltd, www.bioanalytics.co.uk) and iC42 (Integrated Solutions in Clinical Research and Development, University of Colorado, Aurora, CO, www.zortracker.com) studies show broad interlaboratory variability with currently used (almost all developed in-house) procedures (Table 3),^{213,214} indicating a need for improved method standardization.

Recommendations: Chromatographic Methods

- A fully validated LC-MS/MS assay is the preferred method for the measurement of EVR concentrations. Chromatographic methods based on UV detection cannot be recommended.

TABLE 3. Representative Results for EVR From the International Proficiency Testing Program¹⁹⁷

	Challenge 71				Challenge 77			
	N	Patient Pool	Spike B	Spike C	N	Spike A	Spike B	Spike C
HPLC	125				142			
Mean		6	9.3	3.2		10	10.1	10
SD		0.5	0.6	0.5		1.1	1	1.2
CV (%)		8.3	6.5	15.6		11.0	9.9	12.0
Innofluor	9				4			
Mean		8.1	8.9	3.3		9.3	9.6	9.5
SD		1.7	1.1	0.5		0.5	0.5	0.2
CV (%)		21.0	12.4	15.2		5.4	5.2	2.1
QMS	40				44			
Mean		6.5	6.6	2		6.8	6.9	6.8
SD		0.7	0.8	0.5		0.9	1	0.9
CV (%)		10.8	12.1	25.0		13.2	14.5	13.2

CV, interlaboratory coefficient of variation; HPLC, high-performance liquid chromatography.

- For LC-MS/MS assays, the following tools might be helpful to achieve the required performance: (1) enhancement of cell lysis by: addition of distilled water before protein precipitation, freezing the samples, or incubation in the presence of ammonium bicarbonate; (2) ZnSO₄ treatment of the whole-blood specimen, followed by organic extraction; (3) 2-dimensional chromatography using a trap and an analytical column; (4) IVD-CE-labeled commercial calibrators instead of those produced in-house to avoid calibration bias between centers; and (5) use of the commercially available stable isotope-labeled derivatives of EVR as internal standards.
- Use of IVD-CE-marked LC-MS/MS assays or kits does not replace the need for careful verification of analytical performance within the laboratory in which they are used.
- Improvements in LC-MS/MS method standardization are needed.

Immunoassays for Measurement of EVR

Since the use of EVR as routine immunosuppressive therapy, commercial immunoassays have been used for TDM in transplantation centers that do not have adequate access to chromatographic techniques.

In conjunction with Novartis, Seradyn (Indianapolis, IN) developed the Innofluor Certican immunoassay (an FPIA) for monitoring EVR concentrations. With the introduction of the chemiluminescent microparticle immunoassay (CMIA; Abbott Diagnostics, Chicago, IL) and transition of the FPIA assays to the Architect clinical analyzer in 2008, Abbott announced that the TDx analyzer was to be discontinued, and support for the system was withdrawn by 2011.

A new CE-marked, 510(k)-cleared immunoassay—the Quantitative Microsphere System (QMS) EVR immunoassay—was subsequently introduced by ThermoFisher Scientific to monitor EVR in samples from kidney and liver transplant recipients. The method is a homogenous particle-enhanced turbidimetric assay based on competition between drug in the sample and drug coated onto a microparticle reagent. This immunoassay kit includes reagents, calibrators, and 3 QC

samples. The test can be established on different models of general chemistry analyzers using spectrophotometric detection, for example, CDx 90 (ThermoFisher), Hitachi 917 (Roche), Ortho Vitros 5, 1 FS Fusion, AU 640/680 (Olympus), DXc (Beckman), Indiko (ThermoFisher), Architect ci4100 (Abbott), JCA-BM6010 (DiaSys), and others.^{34,36,215–224} Like the Innofluor assay, the QMS assay is semiautomated, requiring a manual pretreatment procedure that is critical for the assay performance. The assay calibration range is 1.5–20.0 ng/mL. The method is relatively fast, has good reagent stability and satisfactory precision.^{217–219,223}

The assay revealed cross-reactivity of 59%–63% for 40-phosphatidylcholine–EVR, and <20% for 5 of the other metabolites, suggesting that drug concentrations would be overestimated in patient samples.²²⁵ This is particularly important because, so far, the issue of a contribution of any EVR metabolites to the pharmacological activity of the drug in vivo has not been elucidated conclusively. Based on the reported cross-reactivity of the EVR metabolites in the QMS assay, and on their concentrations relative to that of EVR, an average overestimation of approximately 40% can be expected.^{34,36} However, in practice, this is usually not the case, as explained below. Cross-reactivity with SRL was reported to be 46%,²¹⁷ so particular care is recommended shortly after switching from SRL. Detectable SRL concentrations in blood during the following week may compromise therapeutic decision-making because of the cross-reactivity of SRL and its metabolites in EVR immunoassays.²¹⁵

Early correlation data presented by the manufacturer showed only a slight positive bias between QMS performed on a Hitachi 917 instrument and a reference LC-MS/MS method.^{225,226} However, the possibility of the test being used on a variety of analyzers creates the need for careful evaluation of assay performance on specific platforms. Reported application data are contradictory. Data from 3 analytical centers (Australia, Poland, Spain) using CDx 90—the analyzer originally recommended by ThermoFisher for the QMS assay—show a significant negative bias of 18.7%, 32.9%, and 29.1%, respectively.^{216,218,220} Similarly, lower EVR

concentrations were reported recently using the QMS assay compared with LC-MS/MS, for a small sample ($n = 20$) with ThermoFisher Indiko (slope 0.880, intercept: 0.34), Beckman DXc (slope 0.832, intercept: 0.33), and AU 680 (slope 0.817, intercept: 0.39) analyzers.²²² Other groups reported no significantly different or even slightly higher results with QMS EVR applied on different biochemical analyzers (Hitachi 917, JCA-BM6010, Indiko, but also unspecified) and reference chromatographic methods.^{213,217,221,223,224,227,228} Results of a very recent study demonstrated similar long-term interlaboratory performance with the QMS assay or LC-MS/MS methods in US laboratories.²²⁹ These investigations used individual patient samples, pooled samples, and pooled samples enriched with either 46-OH-EVR or 39-O-desmethyl-EVR. In contrast, significant positive bias was reported in a broad spectrum of transplantation types for QMS applied on an Architect ci4100 analyzer compared with LC-MS/MS,²³⁰ suggesting that the 2 methods cannot be used interchangeably for EVR TDM.

Proficiency-testing programs have shown that results of the QMS immunoassay for patient samples are similar to those produced by chromatographic methods, but with approximately 30% lower recovery for samples spiked with EVR (Table 3). This is largely because the immunoassay calibration strategy is based on value-assigned calibrators and QC samples.^{222,223,227} As stated by the manufacturer, the immunoassay calibrators and controls were initially value assigned using a representative set of C_0 samples from kidney transplant recipients with traceability to LC-MS/MS values. The value-assigned concentrations of calibrators and controls were approximately 70% of their gravimetric concentrations. A second adjustment of the value-assigned concentrations of calibrators was performed at the end of 2011 and at the beginning of 2012 (ThermoFisher, personal communication, September 2014). This approach was designed to align the results in samples from patients with the “average” measurements obtained by LC-MS/MS methods. However, bias for individual patient samples may vary in either direction, depending on cross-reactivities with metabolites and other potential errors.²³¹ Another consequence of the manufacturer’s calibration strategy is that third-party control material containing spiked concentrations (also external QC samples) of EVR will not give the appropriate results when used in conjunction with the immunoassay, unless the values have been defined for this assay.²³¹ Similarly, external calibration materials are not appropriate for this assay.

Recommendations: EVR Immunoassays

- Studies comparing EVR concentrations measured with LC-MS/MS and the QMS immunoassay have reported inconsistent results when analyzing patient samples. This is most likely due to: (1) use of the assay on different types of analyzers; (2) the immunoassay calibration strategy based on value-assigned calibrators; and (3) variability in calibration from one reference chromatographic method to another. Further work is necessary to resolve this problem and, currently, use of the CDx 90 and Architect ci4100 analyzers cannot be recommended without performing a comparative study with patient samples at the analytical site. Use of the assay on other platforms should be approached with caution.

- Participation in an external QC program that includes the use of both spiked and pooled patient samples is highly recommended for sites using this immunoassay. It is also advisable to compare immunoassay measurements using a chromatographic reference method and real (nonpooled) samples when implementing the assay for patient samples.
- When using immunoassay, laboratories should inform the clinician that values obtained with different methods cannot be used interchangeably, because of differences in methods, method calibration, and cross-reactivity with metabolites.
- Laboratories using an immunoassay with cross-reactivity to SRL for TDM of EVR should alert their customers to the unreliability of EVR results during the first week after switching from SRL.

Immunoassays Validated for SRL

The very similar chemical structures of EVR and SRL result in a high probability of substantial cross-reactivity in immunoassays for either drug. The limited availability of assays suitable for EVR monitoring has prompted some laboratories to adopt commercial SRL immunoassays for the measurement of EVR. Some investigators suggest that the SRL microparticle enzyme immunoassay developed for the IMx analyzer (Abbott Park, IL) may be suitable as an alternative method for EVR monitoring, especially if chromatographic methods are not available.^{232,233} The SRL CMIA developed for the Architect analyzer (Abbott Diagnostics) has also been investigated for EVR monitoring.^{234–236} Good correlation was found between CMIA results and the FPIA InnoFluor Certican Assay, and also SRL microparticle enzyme immunoassay and LC-MS/MS. However, a correction factor was necessary with each set of data. Similar findings were seen with an antibody-conjugated magnetic immunoassay performed on the Dimension platform (Siemens Healthcare Diagnostics, Newark, DE).²³⁷ However, none of the SRL assays are formally released or validated for EVR measurement, and their use in clinical situations may be potentially dangerous for patients. Use of these uncertified methods to measure EVR may breach local regulatory guidelines.

Recommendations: Immunoassays Validated for SRL

- Use of SRL-dedicated immunoassays for EVR determination should be discouraged, since none are formally released or validated for EVR measurement.

Analytical Requirements

In general, immunoassays for immunosuppressive drugs suffer from a positive bias with respect to chromatographic assays, mainly because of cross-reactivity with metabolites of the target analyte. The proliferation of such immunoassays has often led to the use of target concentration ranges specific to particular assays. Attention has tended to focus more on assay precision than on absolute accuracy. Reproducibility of results is important because it is necessary to facilitate consistent dosing decisions over a period of time. The proliferation of reproducible, but different, results generated by a variety of assays is unhelpful and confusing in clinical settings.

Clinicians require methods that produce concentration measurements without bias, due either to cross-reactivity or calibration issues, so that patients can have their samples measured by any appropriate method, without any impact on dosing advice. Comparison of methods is hampered by the lack of certified reference materials and validated reference methods. Some progress has been made recently for TAC, for which a whole-blood certified reference material is now available.²³⁸ Two studies have shown that this reference material can be used to test the accuracy of assay methods and that standardizing all procedures used in LC-MS/MS methods can help to minimize between-method differences in accuracy.^{238,239}

The issue of accuracy has become even more important since the introduction of very low target concentration ranges for some immunosuppressive drugs. In some instances, the lower limits of the concentrations aspired to in-treatment protocols are close to or below the lower limits of quantification (LLOQ) for some of the analytical methods used. In 1 recent study, it was concluded that TAC concentrations should be targeted in the range 3–7 ng/mL.²⁴⁰ However, most patients had concentrations at or above the top of this range, at least in part because the analytical method used by most centers to measure the drug was never designed to measure TAC at such low concentrations. Subsequent discussions on the study stressed that attention should focus on the drug concentrations achieved, rather than those in the study protocol.²⁴¹ Of course, this assumes that the methods used are capable of measuring across the full target range and that all methods used have comparable accuracy.

Dose schedules for EVR are now following the trend established for TAC, with low doses being advocated in association with other immunosuppressive agents, in an attempt to reduce long-term toxicity.⁸¹ As a result, there is a need for EVR assays with LLOQ around 1 ng/mL and good reproducibility at this concentration.

To assure quality, consistency, and appropriate utility of results, analytical methods should be carefully validated according to the guidelines of appropriate international scientific societies and governmental agencies. For more information on analytical validation of methods for quantification of immunosuppressive drugs, readers are referred to a consensus report by Oellerich et al.⁴ Good standards of laboratory practices, as described in documents published by the College of American Pathologists (www.cap.org), the Clinical and Laboratory Standards Institute (www.clsi.org), and the International Organization for Standardization (www.iso.org), should also be followed when establishing an analytical service for EVR.

Recommendations: Analytical Requirements

- *Method specificity for the parent drug:* The therapeutic range intended by TDM is that of the parent drug. Therefore, analytical methods should be specific for EVR and this should be proven during method validation. If available, information on cross-reactivity with metabolites should be reported with a statement on clinical relevance. Currently, there is no evidence to support monitoring of EVR metabolite concentrations.

- *LLOQ:* An LLOQ close to 1 ng/mL should be achieved to ensure reliable assessment of low concentrations and to reveal inappropriate dosing or patient noncompliance issues. The method imprecision at the LLOQ should be <20%.
- *Measuring range:* Laboratories should characterize the working range of their method and, if dilution steps are required to allow quantification of high EVR concentrations, appropriate protocols should be developed and validated. In addition, a standard procedure for reporting concentrations outside the working range should be available.
- *Assay imprecision:* The aim should be a coefficient of variation of $\leq 10\%$ with respect to between-days imprecision. This is based on estimated variations that may result in suboptimal therapeutic decisions.
- *Assay inaccuracy:* Method accuracy should be established by comparison with a validated reference method. At the time of development of this consensus report, no exact-matching isotope-dilution mass spectrometry method was available. Therefore, fully validated LC-MS/MS-based procedures, for which assay performance is well documented, should be considered to be the reference.
- Further research is essential to achieve better standardization of EVR methods, including establishment of an appropriate reference method. Method comparison should be investigated by an unbiased regression procedure, for example, Deming regression or Passing–Bablok procedure.^{242,243} It should include a variety of pathological conditions (different transplantation types, time posttransplantation, ethnic backgrounds, age groups, etc.) and present a wide range of therapeutic values and values below and above the recommended therapeutic range. The following criteria are recommended for a method to be acceptable for selective determination of EVR, similar to other immunosuppressive drugs²⁴⁴: (1) linear regression slope within $\pm 10\%$ of the theoretical value of 1.0; (2) linear regression intercept not statistically different from zero; and (3) standard error of the estimate (S_{yx}) $\leq 10\%$ of the average of the therapeutic concentrations.

Method Calibration and Proficiency Testing

As mentioned above, for EVR measurement most centers currently use either a variety of chromatographic assays, based on mass spectrometric detection, or an immunoassay. Chromatographic assays are calibrated using calibrators prepared in-house or purchased from third-party sources. Immunoassays are calibrated by the manufacturer. In general, diagnostics manufacturers have striven to calibrate immunoassays as accurately as possible, with varying degrees of success. When samples are spiked with known concentrations of the drug of interest, results tend to be in broad agreement. More significant differences are usually seen between chromatographic and immunoassays because of cross-reactivity with metabolites. Users can check accuracy using third-party-prepared calibrators or controls, or by comparing their results with those given by external proficiency-testing material. As already mentioned for users of the QMS immunoassay, it is not possible to check calibrator accuracy by either of these methods if the samples are spiked with EVR. Since proficiency-testing programs have difficulty

obtaining samples from patients treated with EVR, samples spiked with the drug are often used. In addition, although external “pooled patient” proficiency-testing samples are very useful, they can also give an underestimate of errors that may occur in individual patients, as pooling blood tends to average down the magnitude of error that may occur in individual patients due to matrix effects. Therefore, confirmation of immunoassay measurements using a chromatographic reference method and real (nonpooled) samples is advised when starting application to patient samples and in particular situations (eg, on a monthly basis) when large individual deviations are suspected.

Recommendations: Method Calibration and Proficiency Testing

- Laboratories involved in TDM of EVR should participate in an external proficiency-testing program to allow continuous cross-validation and proof of analytical quality (eg, Analytical Services International Ltd, www.bioanalytics.co.uk or iC42, www.zortracker.com). In addition to spiked whole-blood samples, proficiency-testing samples should include samples without EVR and pooled samples from patients with different clinical indications for the drug, preferably including both transplantation and nontransplantation indications.
- In addition to participation in external proficiency testing, further actions are recommended to avoid calibration bias. These include the use of commercial whole-blood calibrators and QC materials (preferably from sources independent of the method manufacturers) and, periodically, the investigation of split specimens (as fresh material and after freezing) to control for calibration bias because of discrepancies between the matrices of the calibrators/controls (frozen) and the patient samples (fresh) if applicable. Development of a certified reference material would also be beneficial.

CONCLUSIONS

Therapeutic use of EVR has increased steadily since its development and now includes a broad spectrum of transplantation and nontransplantation indications. Because EVR has a narrow therapeutic index, TDM is mandatory in most clinical indications to avoid under-immunosuppression or over-immunosuppression, and to minimize the occurrence of adverse effects. The goal of this consensus report was to provide evidence-based recommendations for the optimal implementation of TDM of EVR in clinical practice. In particular, this report focuses on the best practice for TDM of EVR to allow treatment individualization. Recommendations are provided for therapeutic applications supported by a sufficient level of clinical evidence with TDM. Although there is much experience with TDM of EVR in de novo kidney transplantation and heart transplantation when EVR is combined with CsA and GCs, additional data are required to support the use of TDM of EVR with other cotherapies and in other transplantation types, and also in nontransplantation settings. Appropriately designed clinical trials have been initiated and their results are eagerly awaited. Further

prospective studies are encouraged, for example, to evaluate the potential benefit of TDM for EVR in situations requiring long-term therapy, such as breast cancer or TSC. From an analytical perspective, both the development of alternative assays to facilitate measurement of EVR on site and efforts to improve method standardization should be fostered.

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REFERENCES

1. Lebwahl D, Anak O, Sahnoud T, et al. Development of everolimus, a novel oral mTOR inhibitor, across a spectrum of diseases. *Ann N Y Acad Sci.* 2013;1291:14–32.
2. Kirchner GI, Meier-Wiedenbach I, Manns MP. Clinical pharmacokinetics of everolimus. *Clin Pharmacokinet.* 2004;43:83–95.
3. Jusko WJ, Thomson AW, Fung J, et al. Consensus document: therapeutic monitoring of tacrolimus (FK-506). *Ther Drug Monit.* 1995;17:606–614.
4. Oellerich M, Armstrong VW, Kahan B, et al. Lake Louise Consensus Conference on cyclosporin monitoring in organ transplantation: report of the consensus panel. *Ther Drug Monit.* 1995;17:642–654.
5. Shaw LM, Sollinger HW, Halloran P, et al. Mycophenolate mofetil: a report of the consensus panel. *Ther Drug Monit.* 1995;17:690–699.
6. Yatscoff RW, Boeckx R, Holt DW, et al. Consensus guidelines for therapeutic drug monitoring of rapamycin: report of the consensus panel. *Ther Drug Monit.* 1995;17:676–680.
7. Oellerich M, Armstrong VW, Schutz E, et al. Therapeutic drug monitoring of cyclosporine and tacrolimus. Update on Lake Louise Consensus Conference on cyclosporin and tacrolimus. *Clin Biochem.* 1998;31:309–316.
8. Morris RG. Cyclosporin therapeutic drug monitoring—an established service revisited. *Clin Biochem Rev.* 2003;24:33–46.
9. van Gelder T, Le Meur Y, Shaw LM, et al. Therapeutic drug monitoring of mycophenolate mofetil in transplantation. *Ther Drug Monit.* 2006;28:145–154.
10. Wallemaq P, Armstrong VW, Brunet M, et al. Opportunities to optimize tacrolimus therapy in solid organ transplantation: report of the European consensus conference. *Ther Drug Monit.* 2009;31:139–152.
11. Spagnoletti G, Favi E, Gargiulo A, et al. Once-a-day administration of everolimus is safe in de novo renal transplant recipients: 1-year results of a pilot study. *Transplant Proc.* 2011;43:1010–1012.
12. Guillemain R, Poinsignon V, Amrein C, et al. First pharmacokinetic evaluation of everolimus QD in thoracic transplant patients [Abstract P675]. *Transpl Int.* 2013;26(suppl 2):319–320.
13. Sedrani R, Cottens S, Kallen J, et al. Chemical modification of rapamycin: the discovery of SDZ RAD. *Transplant Proc.* 1998;30:2192–2194.
14. DrugBank. Everolimus (DB01590). 2013. Available at: <http://www.drugbank.ca/drugs/DB01590#properties>. Accessed February 12, 2015.
15. Wullschlegel S, Loewith R, Hall MN. TOR signaling in growth and metabolism. *Cell.* 2006;124:471–484.
16. Eisen HJ, Tuzcu EM, Dorent R, et al. Everolimus for the prevention of allograft rejection and vasculopathy in cardiac-transplant recipients. *N Engl J Med.* 2003;349:847–858.
17. Schaffer SA, Ross HJ. Everolimus: efficacy and safety in cardiac transplantation. *Expert Opin Drug Saf.* 2010;9:843–854.

18. Budde K, Neumayer HH, Lehne G, et al. Tolerability and steady-state pharmacokinetics of everolimus in maintenance renal transplant patients. *Nephrol Dial Transplant*. 2004;19:2606–2614.
19. Vandewiele B, Vandecasteele SJ, Vanwalleghem L, et al. Diffuse alveolar hemorrhage induced by everolimus. *Chest*. 2010;137:456–459.
20. Pascual J, Galeano C, Celemin D, et al. Uneventful thoracic healing with everolimus after aortic valve replacement. *Ann Thorac Surg*. 2007;84:271–272.
21. Podder H, Stepkowski SM, Napoli KL, et al. Pharmacokinetic interactions augment toxicities of sirolimus/cyclosporine combinations. *J Am Soc Nephrol*. 2001;12:1059–1071.
22. Torras J, Herrero-Fresneda I, Gulias O, et al. Rapamycin has dual opposing effects on proteinuric experimental nephropathies: is it a matter of podocyte damage? *Nephrol Dial Transplant*. 2009;24:3632–3640.
23. Food and Drug Administration. Drug safety labeling changes: Rapamune (sirolimus) oral solution and tablets. 2008. Available at: <http://www.fda.gov/Safety/MedWatch/SafetyInformation/Safety-RelatedDrugLabelingChanges/ucm106693.htm>. Accessed May 11, 2015.
24. Novartis Pharmaceuticals UK Ltd. Certican tablets: summary of product characteristics. Available at: <https://www.medicines.org.uk/emc/company/93/Novartis%20Pharmaceuticals%20UK%20Ltd>. Accessed December 13, 2015.
25. Eisen HJ, Kobashigawa J, Starling RC, et al. Everolimus versus mycophenolate mofetil in heart transplantation: a randomized, multicenter trial. *Am J Transplant*. 2013;13:1203–1216.
26. Oh CK, Huh KH, Ha J, et al. Safety and efficacy of the early introduction of everolimus with reduced-exposure cyclosporine in de novo kidney recipients. *Transplantation*. 2015;99:180–186.
27. Budde K, Becker T, Arns W, et al. Everolimus-based, calcineurin-inhibitor-free regimen in recipients of de-novo kidney transplants: an open-label, randomised, controlled trial. *Lancet*. 2011;377:837–847.
28. Schneer S, Kramer MR, Fox B, et al. Renal function preservation with the mTOR inhibitor, everolimus, after lung transplant. *Clin Transplant*. 2014;28:662–668.
29. Crowe A, Lemaire M. In vitro and in situ absorption of SDZ-RAD using a human intestinal cell line (Caco-2) and a single pass perfusion model in rats: comparison with rapamycin. *Pharm Res*. 1998;15:1666–1672.
30. Crowe A, Bruelisauer A, Duerr L, et al. Absorption and intestinal metabolism of SDZ-RAD and rapamycin in rats. *Drug Metab Dispos*. 1999;27:627–632.
31. Picard N, Rouguieg-Malki K, Kamar N, et al. CYP3A5 genotype does not influence everolimus in vitro metabolism and clinical pharmacokinetics in renal transplant recipients. *Transplantation*. 2011;91:652–656.
32. Kovarik JM, Kahan BD, Kaplan B, et al. Longitudinal assessment of everolimus in de novo renal transplant recipients over the first post-transplant year: pharmacokinetics, exposure-response relationships, and influence on cyclosporine. *Clin Pharmacol Ther*. 2001;69:48–56.
33. Robertsen I, Vethe NT, Midtvedt K, et al. Closer to the site of action; everolimus concentrations in peripheral blood mononuclear cells correlate well with whole blood concentrations. *Ther Drug Monit*. 2015;37:675–680.
34. Strom T, Haschke M, Zhang YL, et al. Identification of everolimus metabolite patterns in trough blood samples of kidney transplant patients. *Ther Drug Monit*. 2007;29:592–599.
35. Lhoëst GJ, Gougnard TY, Verbeeck RK, et al. Isolation from pig liver microsomes, identification by tandem mass spectrometry and in vitro immunosuppressive activity of an SDZ-RAD 17,18,19,20,21,22-tris-epoxide. *J Mass Spectrom*. 2000;35:454–460.
36. Zollinger M, Sayer C, Dannecker R, et al. The macrolide everolimus forms an unusual metabolite in animals and humans: identification of a phosphocholine ester. *Drug Metab Dispos*. 2008;36:1457–1460.
37. Lemaitre F, Bezian E, Goldwirt L, et al. Population pharmacokinetics of everolimus in cardiac recipients: comedications, ABCB1, and CYP3A5 polymorphisms. *Ther Drug Monit*. 2012;34:686–694.
38. Moes DJ, Press RR, den Hartigh J, et al. Population pharmacokinetics and pharmacogenetics of everolimus in renal transplant patients. *Clin Pharmacokinet*. 2012;51:467–480.
39. Kovarik JM, Kaplan B, Silva HT, et al. Pharmacokinetics of an everolimus-cyclosporine immunosuppressive regimen over the first 6 months after kidney transplantation. *Am J Transplant*. 2003;3:606–613.
40. Kovarik JM, Sabia HD, Figueiredo J, et al. Influence of hepatic impairment on everolimus pharmacokinetics: implications for dose adjustment. *Clin Pharmacol Ther*. 2001;70:425–430.
41. Mabasa VH, Ensom MH. The role of therapeutic monitoring of everolimus in solid organ transplantation. *Ther Drug Monit*. 2005;27:666–676.
42. Taber DJ, Belk L, Meadows H, et al. Racial comparisons of everolimus pharmacokinetics and pharmacodynamics in adult kidney transplant recipients. *Ther Drug Monit*. 2013;35:753–759.
43. Kovarik JM, Beyer D, Schmouder RL. Everolimus drug interactions: application of a classification system for clinical decision making. *BioPharm Drug Dispos*. 2006;27:421–426.
44. Lefevre S, Rebaudet S, Billaud EM, et al. Management of rifamycins-everolimus drug-drug interactions in a liver-transplant patient with pulmonary tuberculosis. *Transpl Int*. 2012;25:e120–e123.
45. Billaud EM, Antoine C, Berge M, et al. Management of metabolic cytochrome P450 3A4 drug-drug interaction between everolimus and azole antifungals in a renal transplant patient. *Clin Drug Investig*. 2009;29:481–486.
46. Christians U, Jacobsen W, Benet LZ, et al. Mechanisms of clinically relevant drug interactions associated with tacrolimus. *Clin Pharmacokinet*. 2002;41:813–851.
47. Brandhorst G, Tenderich G, Zittermann A, et al. Everolimus exposure in cardiac transplant recipients is influenced by concomitant calcineurin inhibitor. *Ther Drug Monit*. 2008;30:113–116.
48. Shihab FS, Cibrik D, Chan L, et al. Association of clinical events with everolimus exposure in kidney transplant patients receiving reduced cyclosporine. *Clin Transplant*. 2013;27:217–226.
49. Kovarik JM, Hartmann S, Figueiredo J, et al. Effect of food on everolimus absorption: quantification in healthy subjects and a confirmatory screening in patients with renal transplants. *Pharmacotherapy*. 2002;22:154–159.
50. Ensom MH, Davis GA, Cropp CD, et al. Clinical pharmacokinetics in the 21st century. Does the evidence support definitive outcomes? *Clin Pharmacokinet*. 1998;34:265–279.
51. De Simone P, Metselaar HJ, Fischer L, et al. Conversion from a calcineurin inhibitor to everolimus therapy in maintenance liver transplant recipients: a prospective, randomized, multicenter trial. *Liver Transplant*. 2009;15:1262–1269.
52. Saliba F, Dharancy S, Lorho R, et al. Conversion to everolimus in maintenance liver transplant patients: a multicenter, retrospective analysis. *Liver Transplant*. 2011;17:905–913.
53. Chan L, Hartmann E, Cibrik D, et al. Optimal everolimus concentration is associated with risk reduction for acute rejection in de novo renal transplant recipients. *Transplantation*. 2010;90:31–37.
54. Budde K. How to use mTOR inhibitors? the search goes on. *Am J Transplant*. 2011;11:1551–1552.
55. Naesens M. Switching from calcineurin inhibitors to mammalian target of rapamycin inhibitors—finally caught the right wave? *Transplantation*. 2011;92:728–730.
56. Shihab F, Christians U, Smith L, et al. Focus on mTOR inhibitors and tacrolimus in renal transplantation: pharmacokinetics, exposure-response relationships, and clinical outcomes. *Transpl Immunol*. 2014;31:22–32.
57. Manito N, Delgado JF, Crespo-Leiro MG, et al. Clinical recommendations for the use of everolimus in heart transplantation. *Transplant Rev (Orlando)*. 2010;24:129–142.
58. Flechner SM. Another step in defining the role of mTOR inhibitors in kidney transplantation. *Transpl Int*. 2010;23:1082–1083.
59. Ross H, Pflugfelder P, Haddad H, et al. Reduction of cyclosporine following the introduction of everolimus in maintenance heart transplant recipients: a pilot study. *Transpl Int*. 2010;23:31–37.
60. Monaco AP. The role of mTOR inhibitors in the management of post-transplant malignancy. *Transplantation*. 2009;87:157–163.
61. Peddi VR, Wiseman A, Chavin K, et al. Review of combination therapy with mTOR inhibitors and tacrolimus minimization after transplantation. *Transplant Rev (Orlando)*. 2013;27:97–107.
62. Kahan BD, Kaplan B, Lorber MI, et al. RAD in de novo renal transplantation: comparison of three doses on the incidence and severity of acute rejection. *Transplantation*. 2001;71:1400–1406.
63. Vitko S, Margreiter R, Weimar W, et al. Everolimus (Certican) 12-month safety and efficacy versus mycophenolate mofetil in de novo renal transplant recipients. *Transplantation*. 2004;78:1532–1540.
64. Lorber MI, Mulgaonkar S, Butt KM, et al. Everolimus versus mycophenolate mofetil in the prevention of rejection in de novo renal trans-

- plant recipients: a 3-year randomized, multicenter, phase III study. *Transplantation*. 2005;80:244–252.
65. Vitko S, Margreiter R, Weimar W, et al. Three-year efficacy and safety results from a study of everolimus versus mycophenolate mofetil in de novo renal transplant patients. *Am J Transplant*. 2005;5:2521–2530.
 66. Kovarik JM, Kaplan B, Tedesco Silva H, et al. Exposure-response relationships for everolimus in de novo kidney transplantation: defining a therapeutic range. *Transplantation*. 2002;73:920–925.
 67. Nashan B. Review of the proliferation inhibitor everolimus. *Expert Opin Investig Drugs*. 2002;11:1845–1857.
 68. Lorber MI, Ponticelli C, Whelchel J, et al. Therapeutic drug monitoring for everolimus in kidney transplantation using 12-month exposure, efficacy, and safety data. *Clin Transplant*. 2005;19:145–152.
 69. Baas MC, Struijk GH, Moes DJ, et al. Interstitial pneumonitis caused by everolimus: a case-cohort study in renal transplant recipients. *Transpl Int*. 2014;27:428–436.
 70. Duran I, Goebel PJ, Papazisis K, et al. Drug-induced pneumonitis in cancer patients treated with mTOR inhibitors: management and insights into possible mechanisms. *Expert Opin Drug Saf*. 2014;13:361–372.
 71. Vitko S, Tedesco H, Eris J, et al. Everolimus with optimized cyclosporine dosing in renal transplant recipients: 6-month safety and efficacy results of two randomized studies. *Am J Transplant*. 2004;4:626–635.
 72. Tedesco-Silva H Jr, Vitko S, Pascual J, et al. 12-month safety and efficacy of everolimus with reduced exposure cyclosporine in de novo renal transplant recipients. *Transpl Int*. 2007;20:27–36.
 73. Kovarik JM, Tedesco H, Pascual J, et al. Everolimus therapeutic concentration range defined from a prospective trial with reduced-exposure cyclosporine in de novo kidney transplantation. *Ther Drug Monit*. 2004;26:499–505.
 74. Tedesco Silva H Jr, Cibrik D, Johnston T, et al. Everolimus plus reduced-exposure CsA versus mycophenolic acid plus standard-exposure CsA in renal-transplant recipients. *Am J Transplant*. 2010;10:1401–1413.
 75. Cibrik D, Silva HT Jr, Vathsala A, et al. Randomized trial of everolimus-facilitated calcineurin inhibitor minimization over 24 months in renal transplantation. *Transplantation*. 2013;95:933–942.
 76. Wiseman AC, McCague K, Kim Y, et al. The effect of everolimus versus mycophenolate upon proteinuria following kidney transplant and relationship to graft outcomes. *Am J Transplant*. 2013;13:442–449.
 77. Salvadori M, Scolari MP, Bertoni E, et al. Everolimus with very low-exposure cyclosporine A in de novo kidney transplantation: a multicenter, randomized, controlled trial. *Transplantation*. 2009;88:1194–1202.
 78. Ponticelli C, Salvadori M, Scolari MP, et al. Everolimus and minimization of cyclosporine in renal transplantation: 24-month follow-up of the EVEREST study. *Transplantation*. 2011;91:e72–e73.
 79. Dantal J, Berthoux F, Moal MC, et al. Efficacy and safety of de novo or early everolimus with low cyclosporine in deceased-donor kidney transplant recipients at specified risk of delayed graft function: 12-month results of a randomized, multicenter trial. *Transpl Int*. 2010;23:1084–1093.
 80. Chan L, Greenstein S, Hardy MA, et al. Multicenter, randomized study of the use of everolimus with tacrolimus after renal transplantation demonstrates its effectiveness. *Transplantation*. 2008;85:821–826.
 81. Langer RM, Hené R, Vitko S, et al. Everolimus plus early tacrolimus minimization: a phase III, randomized, open-label, multicenter trial in renal transplantation. *Transpl Int*. 2012;25:592–602.
 82. Rostaing L, Christiaans MH, Kovarik JM, et al. The pharmacokinetics of everolimus in de novo kidney transplant patients receiving tacrolimus: an analysis from the randomized ASSET study. *Ann Transplant*. 2014;19:337–345.
 83. Qazi Y, Shaffer D, Kaplan B, et al. Efficacy and safety of everolimus with low-dose tacrolimus in de novo renal transplant recipients: 12-month randomized study [Abstract #713]. *Am J Transplant*. 2014;14 (suppl 3):80.
 84. Budde K, Lehner F, Sommerer C, et al. Conversion from cyclosporine to everolimus at 4.5 months posttransplant: 3-year results from the randomized ZEUS study. *Am J Transplant*. 2012;12:1528–1540.
 85. Budde K, Lehner F, Sommerer C, et al. Five-year outcomes in kidney transplant patients converted from cyclosporine to everolimus: the randomized ZEUS study. *Am J Transplant*. 2015;15:119–128.
 86. Liefeldt L, Brakemeier S, Glander P, et al. Donor-specific HLA antibodies in a cohort comparing everolimus with cyclosporine after kidney transplantation. *Am J Transplant*. 2012;12:1192–1198.
 87. Chadban SJ, Eris JM, Kanellis J, et al. A randomized, controlled trial of everolimus-based dual immunosuppression versus standard of care in de novo kidney transplant recipients. *Transpl Int*. 2014;27:302–311.
 88. Holdaas H, Bentdal O, Pfeiffer P, et al. Early, abrupt conversion of de novo renal transplant patients from cyclosporine to everolimus: results of a pilot study. *Clin Transplant*. 2008;22:366–371.
 89. Mjörnstedt L, Sørensen SS, von Zur Mühlen B, et al. Improved renal function after early conversion from a calcineurin inhibitor to everolimus: a randomized trial in kidney transplantation. *Am J Transplant*. 2012;12:2744–2753.
 90. Mjörnstedt L, Schwartz Sørensen S, von Zur Mühlen B, et al. Renal function three years after early conversion from a calcineurin inhibitor to everolimus: results from a randomized trial in kidney transplantation. *Transpl Int*. 2015;28:42–51.
 91. Budde K, Rath T, Sommerer C, et al. Renal, efficacy and safety outcomes following late conversion of kidney transplant patients from calcineurin inhibitor therapy to everolimus: the randomized APOLLO study. *Clin Nephrol*. 2015;83:11–21.
 92. Budde K, Sommerer C, Rath T, et al. Renal function to 5 years after late conversion of kidney transplant patients to everolimus: a randomized trial. *J Nephrol*. 2015;28:115–123.
 93. Holdaas H, Rostaing L, Serón D, et al. Conversion of long-term kidney transplant recipients from calcineurin inhibitor therapy to everolimus: a randomized, multicenter, 24-month study. *Transplantation*. 2011;92:410–418.
 94. Bemelman FJ, de Maar EF, Press RR, et al. Minimization of maintenance immunosuppression early after renal transplantation: an interim analysis. *Transplantation*. 2009;88:421–428.
 95. Moes DJ, Press RR, de Fijter JW, et al. Liquid chromatography-tandem mass spectrometry outperforms fluorescence polarization immunoassay in monitoring everolimus therapy in renal transplantation. *Ther Drug Monit*. 2010;32:413–419.
 96. Asrani SK, Wiesner RH, Trotter JF, et al. De novo sirolimus and reduced-dose tacrolimus versus standard-dose tacrolimus after liver transplantation: the 2000–2003 phase II prospective randomized trial. *Am J Transplant*. 2014;14:356–366.
 97. Trotter JF. Sirolimus in liver transplantation. *Transplant Proc*. 2003;35 (3 suppl):193S–200S.
 98. De Simone P, Nevens F, De Carlis L, et al. Everolimus with reduced tacrolimus improves renal function in de novo liver transplant recipients: a randomized controlled trial. *Am J Transplant*. 2012;12:3008–3020.
 99. Saliba F, De Simone P, Nevens F, et al. Renal function at two years in liver transplant patients receiving everolimus: results of a randomized, multicenter study. *Am J Transplant*. 2013;13:1734–1745.
 100. Shinke H, Hashi S, Kinoshita R, et al. Effectiveness of sirolimus in combination with cyclosporine against chronic rejection in a pediatric liver transplant patient. *Biol Pharm Bull*. 2013;36:1221–1225.
 101. Sato E, Hashi S, Taniguchi R, et al. Effectiveness of everolimus in combination with cyclosporine as treatment for chronic rejection in a pediatric patient undergoing liver transplantation. *Jpn J Ther Drug Monit*. 2014;31:1–5.
 102. Mazzaferro V, Regalia E, Doci R, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med*. 1996;334:693–699.
 103. Cholongitas E, Mamou C, Rodríguez-Castro KI, et al. Mammalian target of rapamycin inhibitors are associated with lower rates of hepatocellular carcinoma recurrence after liver transplantation: a systematic review. *Transpl Int*. 2014;27:1039–1049.
 104. Kovarik JM, Eisen H, Dorent R, et al. Everolimus in de novo cardiac transplantation: pharmacokinetics, therapeutic range, and influence on cyclosporine exposure. *J Heart Lung Transplant*. 2003;22:1117–1125.
 105. Starling RC, Hare JM, Hauptman P, et al. Therapeutic drug monitoring for everolimus in heart transplant recipients based on exposure-effect modeling. *Am J Transplant*. 2004;4:2126–2131.
 106. Lehmkühl HB, Mai D, Dandel M, et al. Observational study with everolimus (Certican) in combination with low-dose cyclosporine in de novo heart transplant recipients. *J Heart Lung Transplant*. 2007;26:700–704.

107. Lehmkuhl HB, Arizon J, Vigano M, et al. Everolimus with reduced cyclosporine versus MMF with standard cyclosporine in de novo heart transplant recipients. *Transplantation*. 2009;88:115–122.
108. Zuckermann A, Wang SS, Ross H, et al. Efficacy and safety of low-dose cyclosporine with everolimus and steroids in de novo heart transplant patients: a multicentre, randomized trial. *J Transplant*. 2011;2011:535983.
109. Hirt SW, Bara C, Barten MJ, et al. Everolimus in heart transplantation: an update. *J Transplant*. 2013;2013:683964.
110. Andreassen AK, Andersson B, Gustafsson F, et al. Everolimus initiation and early calcineurin inhibitor withdrawal in heart transplant recipients: a randomized trial. *Am J Transplant*. 2014;14:1828–1838.
111. Andreassen AK, Andersson B, Gustafsson F, et al. Three year follow up of the randomized SCHEDULE trial with everolimus initiation and early withdrawal of calcineurin inhibitor therapy in de novo heart transplant recipients—a multicenter, randomized Scandinavian trial. *J Heart Lung Transplant*. 2015;34(4 suppl):S129–S130.
112. Deuse T, Barten C, Bara C, et al. A multi-center, randomized, open-label, parallel group phase IV trial investigating the outcome on renal function, efficacy and safety of CNI-reduction or elimination with everolimus in de novo heart transplant recipients: the MANDELA study design [Abstract P107]. *Transpl Int*. 2014;27(suppl 3):S46.
113. King-Biggs MB, Dunitz JM, Park SJ, et al. Airway anastomotic dehiscence associated with use of sirolimus immediately after lung transplantation. *Transplantation*. 2003;75:1437–1443.
114. Groetzner J, Kur F, Spelsberg F, et al. Airway anastomosis complications in de novo lung transplantation with sirolimus-based immunosuppression. *J Heart Lung Transplant*. 2004;23:632–638.
115. de Pablo A, Santos F, Solé A, et al. Recommendations on the use of everolimus in lung transplantation. *Transplant Rev (Orlando)*. 2013;27:9–16.
116. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy. *Crit Rev Oncol Hematol*. 2005;56:23–46.
117. Snell GI, Levvey BJ, Zheng L, et al. Everolimus alters the bronchoalveolar lavage and endobronchial biopsy immunologic profile post-human lung transplantation. *Am J Transplant*. 2005;5:1446–1451.
118. Snell GI, Valentine VG, Vitulo P, et al. Everolimus versus azathioprine in maintenance lung transplant recipients: an international, randomized, double-blind clinical trial. *Am J Transplant*. 2006;6:169–177.
119. Parada MT, Alba A, Sepulveda C. Everolimus in lung transplantation in Chile. *Transplant Proc*. 2010;42:328–330.
120. Glanville AR, Abouyoun C, Klepetko W, et al. Three-year results of an investigator-driven multicenter, international, randomized open-label de novo trial to prevent BOS after lung transplantation. *J Heart Lung Transplant*. 2015;34:16–25.
121. Gottlieb J, Deuse T, Witt C, et al. Comparison of an innovative everolimus-containing quadruple immunosuppressive regimen versus a standard triple regimen in lung transplant recipients and its impact on renal function, safety and efficacy: the 4EVERLUNG study design. *J Heart Lung Transplant*. 2013;32(4 suppl):S171–S172.
122. Gullestad L, Iversen M, Mortensen SA, et al. Everolimus with reduced calcineurin inhibitor in thoracic transplant recipients with renal dysfunction: a multicenter, randomized trial. *Transplantation*. 2010;89:864–872.
123. Gullestad L, Mortensen SA, Eiskjaer H, et al. Two-year outcomes in thoracic transplant recipients after conversion to everolimus with reduced calcineurin inhibitor within a multicenter, open-label, randomized trial. *Transplantation*. 2010;90:1581–1589.
124. Arora S, Gude E, Sigurdardottir V, et al. Improvement in renal function after everolimus introduction and calcineurin inhibitor reduction in maintenance thoracic transplant recipients: the significance of baseline glomerular filtration rate. *J Heart Lung Transplant*. 2012;31:259–265.
125. Arora S, Erikstad I, Ueland T, et al. Virtual histology assessment of cardiac allograft vasculopathy following introduction of everolimus—results of a multicenter trial. *Am J Transplant*. 2012;12:2700–2709.
126. Arora S, Karason K, Eijsjær H, et al. The effect of everolimus initiation and calcineurin inhibitor reduction on allograft vasculopathy in maintenance heart transplant recipients: results of the NOCTET trial after 5 years. *J Heart Lung Transplant*. 2015;34(4 suppl):S32.
127. O'Donnell A, Faivre S, Burris HA III, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral mammalian target of rapamycin inhibitor everolimus in patients with advanced solid tumors. *J Clin Oncol*. 2008;26:1588–1595.
128. Sleijfer S, Wiemer E. Dose selection in phase I studies: why we should always go for the top. *J Clin Oncol*. 2008;26:1576–1578.
129. Taberero J, Rojo F, Calvo E, et al. Dose- and schedule-dependent inhibition of the mammalian target of rapamycin pathway with everolimus: a phase I tumor pharmacodynamic study in patients with advanced solid tumors. *J Clin Oncol*. 2008;26:1603–1610.
130. Motzer RJ, Escudier B, Oudard S, et al. Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. *Lancet*. 2008;372:449–456.
131. Thiery-Vuillemin A, Mouillet G, Nguyen Tan Hon T, et al. Impact of everolimus blood concentration on its anti-cancer activity in patients with metastatic renal cell carcinoma. *Cancer Chemother Pharmacol*. 2014;73:999–1007.
132. Baselga J, Campone M, Piccart M, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. *N Engl J Med*. 2012;366:520–529.
133. André F, O'Regan R, Ozguroglu M, et al. Everolimus for women with trastuzumab-resistant, HER2-positive, advanced breast cancer (BOLERO-3): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet Oncol*. 2014;15:580–591.
134. Piccart M, Hortobagyi GN, Campone M, et al. Everolimus plus exemestane for hormone-receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: overall survival results from BOLERO-2. *Ann Oncol*. 2014;25:2357–2362.
135. Pavel ME, Hainsworth JD, Baudin E, et al. Everolimus plus octreotide long-acting repeatable for the treatment of advanced neuroendocrine tumours associated with carcinoid syndrome (RADIANT-2): a randomised, placebo-controlled, phase 3 study. *Lancet*. 2011;378:2005–2012.
136. Ganschow R, Pape L, Sturm E, et al. Growing experience with mTOR inhibitors in pediatric solid organ transplantation. *Pediatr Transplant*. 2013;17:694–706.
137. Grushkin C, Mahan JD, Mange KC, et al. De novo therapy with everolimus and reduced-exposure cyclosporine following pediatric kidney transplantation: a prospective, multicenter, 12-month study. *Pediatr Transplant*. 2013;17:237–243.
138. Pape L, Offner G, Kreuzer M, et al. De novo therapy with everolimus, low-dose cyclosporine A, basiliximab and steroid elimination in pediatric kidney transplantation. *Am J Transplant*. 2010;10:2349–2354.
139. Pape L, Lehner F, Blume C, et al. Pediatric kidney transplantation followed by de novo therapy with everolimus, low-dose cyclosporine A, and steroid elimination: 3-year data. *Transplantation*. 2011;92:658–662.
140. Ferraresso M, Belingheri M, Ginevri F, et al. Three-yr safety and efficacy of everolimus and low-dose cyclosporine in de novo pediatric kidney transplant patients. *Pediatr Transplant*. 2014;18:350–356.
141. Ettenger R, Pape L, Tonshoff B, et al. Design and rationale of CRADLE: a randomized, phase III trial evaluating efficacy and safety of early everolimus introduction to reduce CNI exposure and to withdraw steroids in pediatric renal transplant recipients [Abstract #3806.163]. Presented at the Pediatric Academic Sciences Annual Meeting, May 5th, 2014. Vancouver, BC, 2014.
142. Tönshoff B. The use of everolimus in pediatric kidney transplantation. *Pediatr Transplant*. 2014;18:323–324.
143. Budde K, Gaedeke J. Tuberous sclerosis complex-associated angiomyolipomas: focus on mTOR inhibition. *Am J Kidney Dis*. 2012;59:276–283.
144. Franz DN, Belousova E, Sparagana S, et al. Efficacy and safety of everolimus for subependymal giant cell astrocytomas associated with tuberous sclerosis complex (EXIST-1): a multicentre, randomised, placebo-controlled phase 3 trial. *Lancet*. 2013;381:125–132.
145. Franz DN, Belousova E, Sparagana S, et al. Everolimus for subependymal giant cell astrocytoma in patients with tuberous sclerosis complex: 2-year open-label extension of the randomised EXIST-1 study. *Lancet Oncol*. 2014;15:1513–1520.
146. Bissler JJ, Kingswood JC, Radzikowska E, et al. Everolimus for angiomyolipoma associated with tuberous sclerosis complex or sporadic lymphangioliomyomatosis (EXIST-2): a multicentre, randomised, double-blind, placebo-controlled trial. *Lancet*. 2013;381:817–824.
147. Bissler JJ, Kingswood JC, Radzikowska E, et al. Everolimus for renal angiomyolipoma in patients with tuberous sclerosis complex or sporadic lymphangioliomyomatosis: extension of a randomized controlled trial. *Nephrol Dial Transplant*. 2015. In press. doi: 10.1093/ndt/gfv249.

148. Franz DN, Agricola K, Mays M, et al. Everolimus for subependymal giant cell astrocytoma: 5-year final analysis. *Ann Neurol*. 2015. In press. doi: 10.1002/ana.24523.
149. Krueger DA, Northrup H. Tuberous sclerosis complex surveillance and management: recommendations of the 2012 International Tuberous Sclerosis Complex Consensus Conference. *Pediatr Neurol*. 2013;49:255–265.
150. Seyfarth HJ, Hammerschmidt S, Halank M, et al. Everolimus in patients with severe pulmonary hypertension: a safety and efficacy pilot trial. *Pulm Circ*. 2013;3:632–638.
151. James MF, Han S, Polizzano C, et al. NF2/merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth. *Mol Cell Biol*. 2009;29:4250–4261.
152. Karajannis MA, Legault G, Hagiwara M, et al. Phase II study of everolimus in children and adults with neurofibromatosis type 2 and progressive vestibular schwannomas. *Neuro Oncol*. 2014;16:292–297.
153. Goutagny S, Raymond E, Esposito-Farese M, et al. Phase II study of mTORC1 inhibition by everolimus in neurofibromatosis type 2 patients with growing vestibular schwannomas. *J Neurooncol*. 2015;122:313–320.
154. Jacobsen W, Serkova N, Hausen B, et al. Comparison of the in vitro metabolism of the macrolide immunosuppressants sirolimus and RAD. *Transplant Proc*. 2001;33:514–515.
155. Chu C, Abbara C, Noël-Hudson MS, et al. Disposition of everolimus in mdr1a-/1b- mice and after a pre-treatment of lapatinib in Swiss mice. *Biochem Pharmacol*. 2009;77:1629–1634.
156. Lamoureux F, Picard N, Boussera B, et al. Sirolimus and everolimus intestinal absorption and interaction with calcineurin inhibitors: a differential effect between cyclosporine and tacrolimus. *Fundam Clin Pharmacol*. 2012;26:463–472.
157. Picard N, Levoir L, Lamoureux F, et al. Interaction of sirolimus and everolimus with hepatic and intestinal organic anion-transporting polypeptide transporters. *Xenobiotica*. 2011;41:752–757.
158. Schoeppler KE, Aquilante CL, Kiser TH, et al. The impact of genetic polymorphisms, diltiazem, and demographic variables on everolimus trough concentrations in lung transplant recipients. *Clin Transplant*. 2014;28:590–597.
159. Amirimani B, Walker AH, Weber BL, et al. Response: re: modification of clinical presentation of prostate tumors by a novel genetic variant in CYP3A4. *J Natl Cancer Inst*. 1999;91:1588–1590.
160. Amirimani B, Ning B, Deitz AC, et al. Increased transcriptional activity of the CYP3A4*1B promoter variant. *Environ Mol Mutagen*. 2003;42:299–305.
161. Spurdle AB, Goodwin B, Hodgson E, et al. The CYP3A4*1B polymorphism has no functional significance and is not associated with risk of breast or ovarian cancer. *Pharmacogenetics*. 2002;12:355–366.
162. Wang D, Guo Y, Wright SA, et al. Intrinsic polymorphism in CYP3A4 affects hepatic expression and response to statin drugs. *Pharmacogenomics J*. 2011;11:274–286.
163. Moes DJ, Swen JJ, den Hartigh J, et al. Effect of CYP3A4*22, CYP3A5*3, and CYP3A combined genotypes on cyclosporine, everolimus, and tacrolimus pharmacokinetics in renal transplantation. *CPT Pharmacometrics Syst Pharmacol*. 2014;3:e100.
164. Bandur S, Petrasko J, Hribova P, et al. Haplotype arrangement in CYP3A locus is associated with side effects of proliferative signal inhibitors in renal transplant recipients. *Transplantation*. 2011;91:e1–e2.
165. Knipeiss D, Renner W, Trummer O, et al. The role of CYP3A5 genotypes in dose requirements of tacrolimus and everolimus after heart transplantation. *Clin Transplant*. 2011;25:146–150.
166. Knipeiss D, Wagner D, Wasler A, et al. The role of CYP2C8 genotypes in dose requirement and levels of everolimus after heart transplantation. *Wien Klin Wochenschr*. 2013;125:393–395.
167. The Human Cytochrome P450 (CYP) Allele Nomenclature Committee. The human cytochrome P450 (CYP) allele nomenclature database. 2015. Available at: <http://www.cypalleles.ki.se/>. Accessed January 20, 2015.
168. Huang S, Bjornsti MA, Houghton PJ. Rapamycins: mechanism of action and cellular resistance. *Cancer Biol Ther*. 2003;2:222–232.
169. Woillard JB, Kamar N, Rousseau A, et al. Association of sirolimus adverse effects with m-TOR, p70S6K or Raptor polymorphisms in kidney transplant recipients. *Pharmacogenet Genomics*. 2012;22:725–732.
170. Böhrer T, Waiser J, Lichter S, et al. Pharmacodynamic effects of everolimus on anti-CD3 antibody-stimulated T-lymphocyte proliferation and interleukin-10 synthesis in stable kidney-transplant patients. *Cytokine*. 2008;42:306–311.
171. Shipkova M, Krizan E, Klett C, et al. Effect of immunosuppressants on CD26/dipeptidyl peptidase IV expression on CD3 cells in vitro [Abstract P530]. *Ther Drug Monit*. 2009;31:660.
172. San Segundo D, Fernández-Fresnedo G, Gago M, et al. Number of peripheral blood regulatory T cells and lymphocyte activation at 3 months after conversion to mTOR inhibitor therapy. *Transplant Proc*. 2010;42:2871–2873.
173. Roat E, De Biasi S, Bertonecchi L, et al. Immunological advantages of everolimus versus cyclosporin A in liver-transplanted recipients, as revealed by polychromatic flow cytometry. *Cytometry A*. 2012;81:303–311.
174. Ben Gal T, Israeli M, Yaari V, et al. Utility of immune monitoring in heart transplant recipients on everolimus-based immune suppression. *Clin Transplant*. 2014;28:428–433.
175. Grépin R, Ambrosetti D, Marsaud A, et al. The relevance of testing the efficacy of anti-angiogenesis treatments on cells derived from primary tumors: a new method for the personalized treatment of renal cell carcinoma. *PLoS One*. 2014;9:e89449.
176. Dieterlen MT, Bittner HB, Klein S, et al. Assay validation of phosphorylated S6 ribosomal protein for a pharmacodynamic monitoring of mTOR-inhibitors in peripheral human blood. *Cytometry B Clin Cytom*. 2012;82:151–157.
177. Lane HA, Thomas G. Purification and properties of mitogen-activated S6 kinase from rat liver and 3T3 cells. *Methods Enzymol*. 1991;200:268–291.
178. Gallant HL, Yatscoff RW. P70 S6 kinase assay: a pharmacodynamic monitoring strategy for rapamycin; assay development. *Transplant Proc*. 1996;28:3058–3061.
179. Leogrande D, Teutonico A, Ranieri E, et al. Monitoring biological action of rapamycin in renal transplantation. *Am J Kidney Dis*. 2007;50:314–325.
180. Boulay A, Zumstein-Mecker S, Stephan C, et al. Antitumor efficacy of intermittent treatment schedules with the rapamycin derivative RAD001 correlates with prolonged inactivation of ribosomal protein S6 kinase 1 in peripheral blood mononuclear cells. *Cancer Res*. 2004;64:252–261.
181. Hoernig A, Wilde B, Wang J, et al. Pharmacodynamic monitoring of mammalian target of rapamycin inhibition by phosphoflow cytometric determination of p70S6 kinase activity. *Transplantation*. 2015;99:210–219.
182. Segarra I, Brazelton TR, Guterman N, et al. Development of a high-performance liquid chromatographic-electrospray mass spectrometric assay for the specific and sensitive quantification of the novel immunosuppressive macrolide 40-O-(2-hydroxyethyl)rapamycin. *J Chromatogr B Biomed Sci Appl*. 1998;720:179–187.
183. Capone D, Gentile A, Polichetti G, et al. Stability of sirolimus and everolimus measured by immunoassay techniques in whole blood samples from kidney transplant patients. *Int J Immunopathol Pharmacol*. 2008;21:297–307.
184. Tszyszcznic W, Borowiec A, Pawlowska E, et al. Two rapid ultra performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) methods with common sample pretreatment for therapeutic drug monitoring of immunosuppressants compared to immunoassay. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2013;928:9–15.
185. Khoschsorur G. Simultaneous measurement of sirolimus and everolimus in whole blood by HPLC with ultraviolet detection. *Clin Chem*. 2005;51:1721–1724.
186. Salm P, Warnholtz C, Boyd J, et al. Evaluation of a fluorescent polarization immunoassay for whole blood everolimus determination using samples from renal transplant recipients. *Clin Biochem*. 2006;39:732–738.
187. Salm P, Taylor PJ, Lynch SV, et al. Quantification and stability of everolimus (SDZ RAD) in human blood by high-performance liquid chromatography-electrospray tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2002;772:283–290.
188. Baldelli S, Murgia S, Merlini S, et al. High-performance liquid chromatography with ultraviolet detection for therapeutic drug monitoring of everolimus. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2005;816:99–105.
189. Koster RA, Dijkers EC, Uges DR. Robust, high-throughput LC-MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus,

- everolimus, and sirolimus in whole blood. *Ther Drug Monit.* 2009;31:116–125.
190. van der Heijden J, de Beer Y, Hoogtanders K, et al. Therapeutic drug monitoring of everolimus using the dried blood spot method in combination with liquid chromatography-mass spectrometry. *J Pharm Biomed Anal.* 2009;50:664–670.
 191. Koster RA, Alffenaar JW, Greijdanus B, et al. Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. *Talanta.* 2013;115:47–54.
 192. Neumayer HH, Paradis K, Korn A, et al. Entry-into-human study with the novel immunosuppressant SDZ RAD in stable renal transplant recipients. *Br J Clin Pharmacol.* 1999;48:694–703.
 193. Dunn C, Croom KF. Everolimus: a review of its use in renal and cardiac transplantation. *Drugs.* 2006;66:547–570.
 194. Kingswood JC, Jozwiak S, Belousova ED, et al. The effect of everolimus on renal angiomyolipoma in patients with tuberous sclerosis complex being treated for subependymal giant cell astrocytoma: subgroup results from the randomized, placebo-controlled, phase 3 trial EXIST-1. *Nephrol Dial Transplant.* 2014;29:1203–1210.
 195. Vidal C, Kirchner GI, Wünsch G, et al. Automated simultaneous quantification of the immunosuppressants 40-O-(2-hydroxyethyl) rapamycin and cyclosporine in blood with electrospray-mass spectrometric detection. *Clin Chem.* 1998;44:1275–1282.
 196. Deters M, Kirchner G, Resch K, et al. Simultaneous quantification of sirolimus, everolimus, tacrolimus and cyclosporine by liquid chromatography-mass spectrometry (LC-MS). *Clin Chem Lab Med.* 2002;40:285–292.
 197. Streit F, Armstrong VW, Oellerich M. Rapid liquid chromatography-tandem mass spectrometry routine method for simultaneous determination of sirolimus, everolimus, tacrolimus, and cyclosporin A in whole blood. *Clin Chem.* 2002;48:955–958.
 198. Baldelli S, Zenoni S, Merlini S, et al. Simultaneous determination of everolimus and cyclosporine concentrations by HPLC with ultraviolet detection. *Clin Chim Acta.* 2006;364:354–358.
 199. den Burger JC, Wilhelm AJ, Chahbouni A, et al. Analysis of cyclosporin A, tacrolimus, sirolimus, and everolimus in dried blood spot samples using liquid chromatography tandem mass spectrometry. *Anal Bioanal Chem.* 2012;404:1803–1811.
 200. Taylor PJ. Therapeutic drug monitoring of immunosuppressant drugs by high-performance liquid chromatography-mass spectrometry. *Ther Drug Monit.* 2004;26:215–219.
 201. Korecka M, Shaw LM. Review of the newest HPLC methods with mass spectrometry detection for determination of immunosuppressive drugs in clinical practice. *Ann Transplant.* 2009;14:61–72.
 202. Adaway JE, Keevil BG. Therapeutic drug monitoring and LC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2012;883–884:33–49.
 203. Annesley TM, Clayton L. Simple extraction protocol for analysis of immunosuppressant drugs in whole blood. *Clin Chem.* 2004;50:1845–1848.
 204. Seger C, Tentschert K, Stoggl W, et al. A rapid HPLC-MS/MS method for the simultaneous quantification of cyclosporine A, tacrolimus, sirolimus and everolimus in human blood samples. *Nat Protoc.* 2009;4:526–534.
 205. Buchwald A, Winkler K, Epting T. Validation of an LC-MS/MS method to determine five immunosuppressants with deuterated internal standards including MPA. *BMC Clin Pharmacol.* 2012;12:2.
 206. Morgan PE, Brown NW, Tredger JM. A direct method for the measurement of everolimus and sirolimus in whole blood by LC-MS/MS using an isotopic everolimus internal standard. *Ther Drug Monit.* 2014;36:358–365.
 207. Heideloff C, Payto D, Wang S. Comparison of a stable isotope-labeled and an analog internal standard for the quantification of everolimus by a liquid chromatography-tandem mass spectrometry method. *Ther Drug Monit.* 2013;35:246–250.
 208. Valbuena H, Shipkova M, Kliesch SM, et al. Comparing the effect of isotopically labeled or structural analog internal standards on the performance of a LC-MS/MS method to determine cyclosporin A, everolimus, sirolimus and tacrolimus in whole blood. *Clin Chem Lab Med.* 2015. In press. doi: 10.1515/cclm-2015-0519.
 209. Vogeser M, Seger C. Pitfalls associated with the use of liquid chromatography-tandem mass spectrometry in the clinical laboratory. *Clin Chem.* 2010;56:1234–1244.
 210. Taylor PJ. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. *Clin Biochem.* 2005;38:328–334.
 211. Ji M, Kim S, Chung HJ, et al. Evaluation of the MassTrak Immunosuppressant XE Kit for the determination of everolimus and cyclosporin A in human whole blood employing isotopically labeled internal standards. *Clin Chem Lab Med.* 2011;49:2021–2027.
 212. Becker S, Thiery J, Ceglarek U. Evaluation of a novel commercial assay for the determination of cyclosporine A, tacrolimus, sirolimus, and everolimus by liquid chromatography-tandem mass spectrometric assay. *Ther Drug Monit.* 2013;35:129–132.
 213. Meyer EJ, Schniedewind B, Boucher R, et al. Cross-validation of everolimus LC-MS/MS and Thermo Fisher QMS therapeutic drug monitoring assays: the Zortracker Cross Validation and TDM Support Program [poster presentation]. Paper presented at: 13th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology; 2013; Salt Lake City, UT.
 214. Analytical Services International Ltd. Proficiency testing results. 2014. Available at: www.bioanalytics.co.uk/Results2014.php. Accessed May 27, 2015.
 215. Khoschsorur G, Fruehwirth F, Zelzer S, et al. Comparison of fluorescent polarization immunoassay (FPIA) versus HPLC to measure everolimus blood concentrations in clinical transplantation. *Clin Chim Acta.* 2007;380:217–221.
 216. Tran J, Ray J, Gao K, et al. Evaluation of the new ThermoFisher QMS everolimus assay [Abstract P70]. *Clin Biochem Rev.* 2010;31(4 suppl):S42–S43.
 217. Dasgupta A, Davis B, Chow L. Evaluation of QMS everolimus assay using Hitachi 917 Analyzer: comparison with liquid chromatography/mass spectrometry. *Ther Drug Monit.* 2011;33:149–154.
 218. Kunicki PK. Application of new QMS everolimus assay performed on CDx 90 analyzer (ThermoFisher) to therapeutic drug monitoring in transplant patients [Abstract P237]. *Ther Drug Monit.* 2011;33:542–543.
 219. Smith M, Plulik P. Evaluation of Thermo Scientific QMS everolimus immunoassay reagent [Abstract P235]. *Ther Drug Monit.* 2011;33:542.
 220. Zufía L, Ibáñez N, Aldaz A. Comparison of everolimus measured by different immunoassay methods [Abstract P236]. *Ther Drug Monit.* 2011;33:542.
 221. Christians U, Schniedewind B, Billaud E, et al. Cross validation of the Thermo Fisher QMS everolimus therapeutic drug monitoring assay run on four different immunoassay analyzer platforms with ten global LC-MS/MS laboratories [poster presentation]. Paper presented at: 13th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology; 2013; Salt Lake City, UT.
 222. Garrison K, Johnson-Davis KL, Wong SH. Everolimus QMS assay by using Thermo Fisher Indiko, Beckman DXc and AU680 analyzers and limited comparison to LC-MS/MS [oral presentation]. Paper presented at: 13th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology; 2013; Salt Lake City, UT.
 223. Yokobata K, Cheung C, Lee C, et al. QMS everolimus immunoassay—TDM for kidney, heart and liver transplant patients receiving everolimus therapy [poster presentation]. Paper presented at: 13th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology; 2013; Salt Lake City, UT.
 224. Shu I, Wright AM, Chandler WL, et al. Analytical performance of QMS everolimus assay on ortho Vitros 5,1 FS fusion analyzer: measuring everolimus trough levels for solid organ transplant recipients. *Ther Drug Monit.* 2014;36:264–268.
 225. Thermo Fisher Scientific Inc. *QMS Everolimus Immunoassay Package Insert; Revision 1*. Fremont, CA: Thermo Fisher Scientific, Inc; 2010.
 226. Thermo Fisher Scientific Inc. *QMS Everolimus Immunoassay Application Note 01/2010*. Fremont, CA: Thermo Fisher Scientific, Inc; 2010.
 227. Mori K, Kimura S, Matsui M, et al. Latex-enhanced turbidimetric immunoassay for everolimus in whole blood using the Nanopia TDM everolimus assay with the JCA-BM6010 automatic analyzer. *Ther Drug Monit.* 2014;36:677–680.
 228. Buthiau D, Bargnoux AS, Badiou S, et al. Evaluation of QMS everolimus assay using Indiko analyzer: comparison with an ultra-performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) method. *Ther Drug Monit.* 2015;37:275–278.
 229. Schniedewind B, Niederlechner S, Galinkin JL, et al. Long-term cross-validation of everolimus therapeutic drug monitoring assays: the Zortracker study. *Ther Drug Monit.* 2015;37:296–303.

230. Hoffer E, Kurnik D, Efrati E, et al. Comparison of everolimus QMS immunoassay on Architect ci4100 and liquid chromatography/mass spectrometry: lack of agreement in organ transplanted patients. *Ther Drug Monit.* 2015;37:214–219.
231. Thermo Fisher Scientific Inc. *QMS Everolimus Immunoassay Package Insert*. Fremont, CA: Thermo Fisher Scientific, Inc; 2014.
232. Bouzas L, Tutor JC. Determination of everolimus in whole blood using the Abbott IMx sirolimus microparticle enzyme immunoassay. *Clin Biochem.* 2007;40:132–136.
233. Pieri M, Miraglia N, Gentile A, et al. Quantification of sirolimus and everolimus by immunoassay techniques: test specificity and cross-reactivity evaluation. *Int J Immunopathol Pharmacol.* 2008; 21:585–594.
234. Coentrao L, Carvalho C, Sampaio S, et al. Relationship between everolimus blood concentration assessed using the Innofluor Certican Fluorescence Polarization Immunoassay and the Architect i System sirolimus chemiluminescent microparticle immunoassay. *Transplant Proc.* 2010;42:1867–1869.
235. Dasgupta A, Moreno V, Balark S, et al. Rapid estimation of whole blood everolimus concentrations using architect sirolimus immunoassay and mathematical equations: comparison with everolimus values determined by liquid chromatography/mass spectrometry. *J Clin Lab Anal.* 2011;25:207–211.
236. Hermida-Cadahia EF, Tutor JC. Determination of everolimus in blood samples from kidney and liver transplant recipients using the sirolimus chemiluminescence magnetic microparticle immunoassay (CMIA) on the Architect-i1000(R) system. *Scand J Clin Lab Invest.* 2012;72:180–183.
237. Bouzas L, Tutor JC. Determination of blood everolimus concentrations in kidney and liver transplant recipients using the sirolimus antibody conjugated magnetic immunoassay (ACMIA). *Clin Lab.* 2011;57:403–406.
238. Levine DM, Maine GT, Armbruster DA, et al. The need for standardization of tacrolimus assays. *Clin Chem.* 2011;57:1739–1747.
239. Annesley TM, McKeown DA, Holt DW, et al. Standardization of LC-MS for therapeutic drug monitoring of tacrolimus. *Clin Chem.* 2013;59: 1630–1637.
240. Ekberg H, Tedesco-Silva H, Demirbas A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med.* 2007;357: 2562–2575.
241. Ekberg H, Mamelok RD, Pearson TC, et al. The challenge of achieving target drug concentrations in clinical trials: experience from the Symphony study. *Transplantation.* 2009;87:1360–1366.
242. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, Part I. *J Clin Chem Clin Biochem.* 1983;21:709–720.
243. Linnet K. Evaluation of regression procedures for methods comparison studies. *Clin Chem.* 1993;39:424–432.
244. Shaw LM, Annesley TM, Kaplan B, et al. Analytic requirements for immunosuppressive drugs in clinical trials. *Ther Drug Monit.* 1995;17: 577–583.