Title: **Everolimus Personalized Therapy: Second Version of Consensus Document by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology**

Authors

ABSTRACT

The Immunosuppressive Drugs Scientific Committee of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) has established the second version of the consensus document to guide therapeutic drug monitoring (TDM) of everolimus (EVR) and its optimal use in clinical practice following 5 years after the first version of publication in 2016. Replacing the first document from 2016, the current version provides an update focused on new developments that arose during the last 5 years. EVR is the second generation of the mammalian target of rapamycin (mTOR) inhibitoers, approved for the prevention of rejection after organ transplantation, and for the treatment of various types of cancers and tuberous sclerosis complex. In clinical use, TDM is strongly recommended for optimal dosage adjustment of EVR, especially in the patients receiving organ transplantation, because of the narrow therapeutic window, wide inter-individual variation in pharmacokinetics, and various adverse reactions. Whole blood sampling is routinely used in the general TDM, but several alternative sampling strategies have been developed which may be used properly by each clinical situation and each status of patient. The target therapeutic window of EVR is recommended between 3 and 8 ng/mL in kidney, liver and heart transplantation with the sampling in whole blood ideally within 1 hour before the next dosage (through concentration) in combination with calcineurin inhibitors. In the case of the introduction of EVR into cancer treatment, relatively higher trough concentration (> 10 ng/mL) would be associated with better progression free survival. Although various types of methodologies in measurement of whole blood concentration of EVR are available, the differences among methods should be carefully considered when determining EVR concentrations as well as evaluating blood concentration measurements. Participation in external proficiency-testing programmes to allow continuous cross-validation and proof of analytical quality is highly recommended.

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

1. INTRODUCTION

Therapeutic drug monitoring (TDM)-based adequate immunosuppressive therapy following organ transplantation has been well acknowledged as a cornerstone to rescue the patients with end-stage organ failure [1-4]. Two calcineurin inhibitors cyclosporine (CsA) and tacrolimus (Tac) were introduced around 1980 and 1990, respectively, and both agents were established as key players to prevent acute cellular rejection taking into account HLA compatibility [5 6,7]. However, various adverse reactions with or without drug-drug interactions with pivotal CYP3A inhibitors were raised as problems in organ transplant patients such as infections by over-immunosuppression, kidney injury, hyperlipidemia, hyperkalemia and so on [8]. More recently, mycophenolate and everolimus had been combined with the calcineurin inhibitors to reduce the exposure of each drug and, therefore, reduce the risk of adverse drug reactions related to high exposure [9-11].

The second generation of mammalian target of rapamycin (mTOR) inhibitors everolimus (EVR) is approved ~~as~~ for the prevention of transplanted organ rejection and for the treatment of various types of cancer and tuberous sclerosis complex (TSC). Immunosuppressive therapy with EVR should be in consideration of its narrow therapeutic window, large intra- and inter-individual pharmacokinetic (PK) variability, and well-established drug exposure-response relationships. [4]. In patients receiving organ transplantation, overexposure to EVR exacerbates certain adverse reactions as well as causes immunological compromise due to over-immunosuppression. In contrast, insufficient exposure to EVR increases the risk of rejection of the transplanted organ. Therefore, TDM had been recommended simultaneously when EVR was registered for suppression of rejection after organ transplantation, and TDM-based EVR dose adjustment is supported by substantial clinical evidence, as described below. In addition to the organ transplant area, the effectiveness of TDM-based EVR therapy as an anticancer strategy is growing to be established through numerous clinical trials. Accurate and precise measurement of EVR concentration in whole blood is a matter of course for the smooth implementation of EVR TDM, but the analytical methods, their characteristics, and the target concentration for each method are often overlooked. Furthermore, the lack of agreement on comparability and calibration between analytical methods strongly affects the interpretation of results and the drug treatment itself.

In 2016, the Immunosuppressive Drugs Scientific Committee of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) published the first edition of consensus document for TDM of EVR. The aim of the present document is to update information to guide EVR TDM. This document is intended for all professionals involved in the management of patients receiving pharmacotherapy with EVR in a variety of clinical settings, including the organ transplants and oncology, and aimed to improve both standards of practice and patient care.

2. PHARMACEUTICS

2.1 Chemistry

Everolimus (EVR) is a white to faintly yellow powder practically insoluble in water [<0.01% in water, 0.1 N hydrochloric acid, and citrate buffer (pH 2.0 – 10.0)]. EVR is soluble in organic solvents such as ethanol and methanol. EVR has a molecular weight of 958.25 g/mol and a molecular formula of $C_{53}H_{83}NO_{14}$ [12]. EVR is chemically similar to the original mTOR inhibitor, sirolimus (SRL) and is the 40-*O*-(2-hydroxyethyl) derivative~~, which means there is an additional hydroxyethyl chain in position 40~~. This renders the molecule more hydrophilic and with different pharmacokinetic and pharmacodynamic properties. EVR is a signal transduction inhibitor targeting the mammalian (later adjusted to the 'mechanistic') target of rapamycin (mTOR) [12].

2.2 Formulations

EVR has been marketed by Novartis as Afinitor®, Votubia®, Certican® and Zortress® [12]. It is available in different formulations and dosages (0.25 mg, 0.5 mg, 0.75 mg~~-~~, and 1 mg) as oral tablets, and tablets for suspension. The first generic was introduced by Teva Europe, then subsequently in Australia, the US and Canada. More recently several other generics have been approved in the US (Everolimus Hikma Pharms®, Everolimus Par Pharm® and Everolimus BIOCON®) and in Canada (Everolimus-PMS®).

Preparations for topical use of EVR are in development. The preparation of EVR micelles or nanosuspension for ocular administration and expected to be a new dosage form for corneal transplantation immunological rejection or other ocular disease. Especially for corneal transplantation, due to the presence of blood-eye barrier, if a systemic administration is used, a large dose of oral administration is required to be effective in the eye, which will undoubtedly increase the risk of side effects such as thrombocytopenia and dyslipidemia which can occur in about 30% of the patients that take the drug. In contrast to systemic administration, the topical preparation for the eye has the advantages of small dosage and mild side effects and may prove advantageous in corneal transplantation [13].

3. PHARMACOLOGY

3.1 Mechanism Of Action

Following entry into the cytoplasm, EVR binds noncovalently to FK506 binding protein-12 (FKBP12) (Figure 1) [14,15]. FKBP12 is a 12 kDa cytosolic protein that is abundantly expressed in all tissues and functions as a *cis/trans* peptidyl prolyl isomerase (PPIase) that catalyzes interconversion between prolyl *cis/trans* conformations [15-17]. The EVR-FKBP12 complex specifically binds with high affinity to a region in targets mTOR protein near the C-terminus called FKBP12-rapamycin binding (FRB), thereby inhibiting its kinase function [14,15].

3.2 Immunosuppressive Agent

EVR functions as an immunosuppressant by blocking the response of T cell activation by cytokines, primarily interleukin-2 (IL-2), by interfering with some of the signals resulting from IL-2 binding to its receptor and ultimately blocking cell cycle progression from G1 to S phase [18-21]. The interleukin-2 receptor (IL-2R) is a heterotrimeric protein, in which signaling is initiated as IL-2 binds to the receptor at the cell surface. This leads to signal transduction through the tyrosine kinases Jak1 and Jak3, resulting in activation of the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) [22]. PI3K phosphorylates the inositol ring of the phosphatidylinositol 4,5-bisphosphate (PIP2) in the membrane to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3) [23]. This enables the recruitment and activation of several proteins, including phosphoinositide-dependent protein kinase-1 (PDKD1) and the serine/threonine kinase protein kinase B (PKB), also known as AKT [18,23]. AKT phosphorylates tuberous sclerosis complex (TSC-2), which is a subunit of TSC a GTPase-activating protein (GAP) for Ras homolog enriched in brain (RHEB) [18,24-26], Phosphorylation of TSC inhibits its GAP activity, thereby reducing the GTPase activity of RHEB. This increases the fraction of RHEB in the GTP-liganded state, and RHEB in the GTP-liganded configuration, activates mTORC1 [18]. Insulin-activated AKT phosphorylates mTORC1-associated proline-rich AKT Substrate 40 (PRAS40), causing it to dissociate from RAPTOR, serving to activate mTORC1 signaling independent of TSC1/2 [26,27].

There are at least two functionally distinct molecular complexes in which mTOR is a functional catalytic unit, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [15,28]. The mTORC1 is comprised of mTOR, regulatory-associated protein of mTOR (RAPTOR) and mammalian lethal with Sec13 protein 8 (mLST8) plus other species and condition dependent proteins [26]. The mTORC2 is comprised of mTOR, a rapamycin-insensitive companion of mTOR (RICTOR), and several other proteins including Rac1 and mammalian stress-activated protein kinase interacting protein 1 and protein observed with RICTOR (PROTOR) [26].

Activated mTORC1 mediates downstream effects through the phosphorylation of multiple substrates [29,30]. The activation of mTORC1 phosphorylates and induces the activity of the Ribosomal protein S6 kinase beta-1 (S6K1, p70S6 kinase, p70S6K) [31] which exists in a complex with Eukaryotic initiation factor 3 (eiF3), which in turn phosphorylates ribosomal protein S6 (rpS6) to promote ribosome biogenesis. At the same time, mTORC1 phosphorylates and inactivates the translational repressor 4EBP1 [31-34]. The translation of 7-methyl guanosine (m7G)- capped mRNAs requires the eIF4F complex, which is composed of eIF4E, eIF4A, and eIF4G [35,36]. The eIF4F complex binds to the m7G cap of mRNAs and facilitates their association with the ribosome. Hypophosphorylated 4EBP1 binds to the mRNA cap recognition protein eIF4E, preventing the formation of the eIF4F complex and thereby blocking translation [37]. The phosphorylation of 4EBP1 by mTORC1 blocks its ability to bind to eIF4E, resulting in an increased translation of capped mRNAs [38] which in turn, is required for the entry of cells into S phase of the cell cycle [15,18,23,39-42]. The phosphorylation of unc-51 like autophagy activating kinase 1 (ULK1) reduces autophagic activity; mTOR activity prevents ULK1 activation by disrupting its interaction with AMP-activated protein kinase (AMPK), a key promoter of autophagy [18]. The mTORC1

mediated phosphorylation of Hypoxia-inducible factor-1 (HIF-1) has a role in multiple physiological responses to hypoxia, such as erythropoiesis and glycolysis, which quickly counteract oxygen deficiency, as well as promoting angiogenesis and cell proliferation [18,43,44].

EVR modifies the T-cell responses by associating with the intracellular protein FKBP12. The FKBP12-EVR complex binds directly to mTOR1 and inhibits its functions [18]. Inhibition of mTOR primarily causes dephosphorylation and inactivation through the interactions with S6K1and 4EBP1 leading to G1 cell cycle arrest [45]. mTORC2 remains relatively unaffected by mTORC1 inhibitors such as EVR, and can respond by upregulating AKT activity [18]

3.3 Anticancer Agent

Over-activation of the PI3K/AKT/mTOR signaling axis is one of the most activated pathways in human cancers [26]. The detailed knowledge of the molecular targets of rapalogs (rapamycin analog) made possible the use of these drugs in the treatment of cancer. EVR is one among the rapalogs, which have been evaluated as monotherapy and in combination therapies in the treatment of human cancer. The first of such indications to be approved for EVR treatment was advanced renal cell carcinoma in patients whose disease progressed on or after treatment with vascular endothelial growth factor (VEGF)-targeted therapy [46]. This demonstrated the benefits of a combination therapy including drugs for specific targets relevant for such cancers.

Constitutively activated mTOR functions in supplying carcinoma cells with oxygen and nutrients by increasing the translation of HIF1 and supporting angiogenesis [47]. Studies in prostate cancer cell lines have shown that EVR down-regulates HIF-1α expression [48]. mTOR inhibitors have been shown to block HIF-1α function and have antiangiogenic effects, suggesting another possible mechanism for EVR anticancer activity [44,49].

~~In 2012, the phase 3 study BOLERO-2 demonstrated that EVR in combination with exemestane could improve the median progression-free survival compared to exemestane alone in advanced hormone receptor-positive and HER2-negative breast cancer [50]. Although still not curative, this combination of an aromatase inhibitor and an mTOR inhibitor also illustrates the benefits of targeted therapy since they are better tolerated, have fewer contraindications, and improve quality of life compared to traditional chemotherapy [51].~~

~~Other studies in the following~~Along the years~~,~~ ~~have led to more~~several approvals by the FDA and EMA have been obtained by EVR, ~~so currently, EVR is also~~which is now an option in combination therapies for advanced renal cell carcinoma, pancreatic neuroendocrine tumors and progressive, nonfunctional gastrointestinal and lung neuroendocrine tumors. ~~It should be noted that for the cancers mentioned here, the indications are restricted to the second or third line of treatment and for subgroups~~. In

addition, there are several hundreds of ongoing studies investigating EVR and other drugs that inhibit the mTOR complexes for cancer treatments [52].

One more example where EVR is specifically targeting the (noncancerous) disease, is in patients with the tuberous sclerosis complex. This disease is caused by mutations in the above-mentioned TSC1 and 2 -genes, leading to constitutive activation of the mTOR pathway. This dysregulation of mTOR is regulated by EVR, which has proven effective for seizure control in patients with the disease [53].

3.4 Antiviral Activity

The immunosuppressive regimen following solid organ transplantation exposes graft recipients to an increased risk of opportunistic viral, bacterial, and fungal infections [54,55]. Interestingly, patients receiving mTORi-based immunosuppressive therapy have a lower incidence and severity of the most common opportunistic viral pathogens seen in transplant recipients (i.e., cytomegalovirus [CMV] and BK polyomavirus) [56-58].

The antiviral properties of mTORi have been attributed to a variety of mechanisms [59]. The activity of mTORi can improve memory T-cells quality, functionality, and efficacy in response to viral stimuli [60,61]. Moreover, the inhibition of mTOR can block downstream effects related to virus replication and growth [62,63]. There is experimental evidence in vitro and in vivo as well as clinical evidence for the antiviral properties of EVR against CMV, Epstein-Barr virus (EBV), human herpesvirus 8 (HHV8), hepatitis B virus (HBV), human immunodeficiency virus (HIV) and human papillomavirus (HPV) [56].

The protective effect in EVR-treated patients against CMV reactivation and disease is supported by its ability to boost CMV-specific CD8+ T-cell response [61,64]. Moreover, in vitro, EVR treatment delayed and suppressed viral DNA synthesis and release from human embryonic lung (HEL) cell culture supernatant [65]. Human CMV (HCMV), like multiple other viruses that rely on m7G-cap-dependent mRNA translation, requires mTOR complexes, mainly mTORC1 activation, for efficient virus replication [38,62,66]. Therefore, HCMV strives to maintain mTOR kinase activation as detected by phosphorylation of 4EBP1 and S6K with either RAPTOR or RICTOR depletion in infected cells. Experimental depletion of RAPTOR or RICTOR also showed differing effects on the accumulation of viral proteins. HCMV produced an alteration of substrate specificity between both mTOR complexes in infected cells [66-69]. The mTOR signaling translational control of viral proteins kinase is involved in the production of all classes of HCMV proteins and mainly at the early stage of the infection [66].

4. MEASUREMENT OF EVR

4.1 Sample Handling

4.1.1 Primary Sample Matrix: Whole Blood

The appropriate collection and handling of samples intended for determination of EVR concentrations was addressed in the previous version of this Consensus Document [4]. Due to high EVR incorporation and concentration dependent binding into the erythrocytes, whole blood is the recommended specimen for EVR quantification, preferably ethylenediaminetetraacetic acid (EDTA) as the anti-coagulant. This is furthermore supported by the demonstration of a strong correlation between EVR concentrations in whole blood and in peripheral blood mononuclear cells (PBMCs) – the immunosuppressive site of action of EVR [70].

EVR is stable for 3 days at temperatures up to 37°C, 1 week at temperatures up to 30°C, but for prolonged storage times, specimens should be stored at –20°C or below [4].

4.1.2 Alternative Sampling Matrices

For the use of alternative matrices to facilitate measurement of everolimus (EVR) concentrations in limited samples volumes, an adequate sensitivity, selectivity, and robustness are a requirement. The interest in these matrices is steadily growing and, respectively, there are currently many studies reporting on the analytical performance of methods developed to be applied in clinical practice. The focus now is on dried blood spots (DBS), volumetric absorptive microsampling (VAMS), oral fluid (OF), and peripheral blood mononuclear cells (PBMCs).

4.1.2.1 Dried Blood Spots (DBS) And Volumetric Absorptive Microsampling

The use of DBS is a minimal-invasive, cost and time saving sampling method, which allows ~~can be an alternative to the traditional blood sampling for TDM of EVR. The procedure is both cost and time saving, and it also allows~~ for multiple sampling ~~within a dose interval~~, simplifying the determination of the area under the curve (AUC) [71,72] and can be performed by patients, at-home. ~~This method offers the advantage to be implemented at home by the patients and small amounts of blood is needed (usually 10-20ml). However, adequate patient training might be critical for optimal sample collection [73]. In addition, some important analytical issues should be carefully evaluated [74]. These include for example the requirement of a relatively large disc, the controversial and intricate desorption approaches, and the lack of feasible extraction strategies. Most recently~~ Le J. et al. described a new LC-MS/MS method including incubation of the DBS in water before adding acetonitrile and zinc sulfate as protein-precipitators that lead to high dissociating efficiency and improvement of analytical results. Effective enrichment and purification of the analytes were achieved by using cold-induced phase separation techniques [75]. Deprez and Stove implemented a method that combined a fully automated DBS extraction with an online coupled LC-MS/MS [76].

Factors such as the influence of blood spot volume, hematocrit, drying time and stability of the analyte are also critical for validation of DBS methods [74,77]. Among other immunosuppressive drugs EVR and sirolimus were reported to be more susceptible to influences by heat degradation, hematocrit and drying time [77]. Knapen et al. and Deprez et al. ~~successfully validated an EVR DBS sampling method over a range of 3–75 µg/L and an analysis time of 3.5 min in cancer patients [78]. Although accuracy and precision were satisfactory at hematocrit values ≥0.25 L/L, this was not true at 0.20 L/L hematocrit and EVR concentrations of ≥20 µg/L. This problem could be solved by preparing a full set of calibration standards at 0.20 L/L [79]. Similarly, Deprez and Stove~~ observed a hematocrit-dependent relative recovery in their DBS method, ~~with~~ the latter showing lower hematocrit values yielding higher relative recoveries (and vice versa) and a difference of >15% at hematocrit of 0.18 and 0.60 [76].

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Results from the study of Veenhof H. et al in transplant patients showed that their DBS sampling method for sirolimus and EVR cannot replace whole blood sampling. However, according to the authors, if the clinical setting is compatible with less strict limits for clinical relevance, this DBS method could be suitable for application [80]. On the other hand, Willemsen et al. demonstrated the clinical validity of DBS sampling for analysis of EVR concentrations in patients with cancer as a practical alternative for whole blood concentration determinations [81]. The group of Bressán et al. demonstrated the feasibility of the clinical application of a dried matrix on paper discs method for the simultaneous quantification of four immunosuppressants (tacrolimus, sirolimus, EVR and cyclosporin) and reported about interchangeability with whole blood sampling [82].

~~New generation sampling procedures such as VAMS have been introduced as an effort to mitigate some of the bias encountered with classical DBS methods, particularly the hematocrit effect. The VAMS technique allows the collection of a fixed volume of blood sample (approximately 10–30 µL) directly from a finger prick or pool of blood [83].~~

Currently available results from the evaluation of new generation VAMS based EVR methods, which is expected to reduce some analytical biases, are still controversial. Whereas in general acceptable precision and bias to results from conventional venous whole blood sampling were reported, problems with the microsampling due to variability in the fill volume of the tip caused by differences in sample viscosity and filling time were noticed [84,85][Lee, J in press]. Using the Mitra® (Neoteryx LLC, Torrance, USA) devices hematocrit-, blood sampling time-, and concentration-related recovery effects, as far as observed, were within the requirements of the purpose of the analytics in some evaluations [85,86]. However, Verheijen et al. reported about considerable biases from −20 to 31% in their study and method over a 30–50% hematocrit range [87].

In conclusion, although DBS and VAMS represent a promising alternative to conventional venous whole blood sampling, further evidence with a higher number of samples and broader patient populations is required to identify and overcome possible pitfalls. Moreover, results from a proficiency testing pilot for immunosuppressant microsampling assays demonstrated that these methods show higher interlaboratory variation compared to whole blood methods. As far as this variation can

influence clinical decision making, the authors are calling for harmonization and standardization of the analytics as well as for regular availability of proficiency testing for laboratories involved in patient care [Veenhof H et al., ahead of print].

4.1.2.2 Oral Fluid

Oral fluid (OF) has been considered an alternative and non-invasive method to venipuncture blood sampling [71,72]. Recently published multidrug LC-MS/MS method for the determination of five immunosuppressants in oral fluid was validated also for the determination of EVR concentrations [88].

In addition, Molenaar-Kuijsten reported the results of a small study with cancer patients treated with EVR for which they measured EVR concentrations in saliva by LC-MS/MS to investigate the possibility to use it for prediction of stomatitis. Although the salivary drug concentration tended to be higher in patients with stomatitis, the relationship was not significant [89]. Moreover, these EVR concentrations were poorly correlated with those in whole blood. The high variability of EVR PK in saliva (interindividual variability of 67.7%) was considered the most critical reason to not conclude the feasibility of this method in patients with stomatitis. It could be related to several factors such as salivary gland function, water and electrolyte balance, protein binding and the pH differences. Based on current knowledge, there is no evidence in support of the use of OF as an alternative matrix to monitor the therapy with EVR.

4.1.2.3 Intracellular Concentration Monitoring

The intracellular concentrations appear more closely related to drug efficacy than blood concentrations [90]. Intracellular quantification of EVR was described PBMC by different groups [70,91,92] and supposed to be able to improve prediction of rejection in transplantation [93]. However, significant variability of results was reported in published studies underlining the critical importance of proper validation of preanalytical and analytical steps for intracellular concentration assays.

Currently, there are two articles published on EVR monitoring in PBMCs by LC-MS/MS, with controversial results about the correlation between intra-PBMCs and whole blood concentrations [70,91]. Whereas Robertsen et al. [70] showed a high correlation between EVR whole blood- and PBMCs concentrations revealing a weak role of ABCB1-mediated efflux from PBMCs, this was not the case with the study of Roullet-Renoleau et al. [91]. More recently, Pensi et al. developed and validated a LC-MS/MS method coupled with automated online solid-phase extraction (SPE) for the simultaneous tacrolimus and EVR quantification in PBMCs to study their potential pharmacokinetic interactions [92]. The authors concluded that the method is suitable to be used in the clinical setting to investigate drug exposure at the active site [92]. The potential additive value of using this alternative sample material compared to the established whole blood remains to be demonstrated.

~~4.1.2.4 Sample Stability In Alternative Matrices~~

~~Stability data reported for EVR monitoring in whole blood have been largely confirmed also for analysis based on the use of dried blood [4]. According to the study of Verheijen et al. VAMS-EVR samples were stable for nearly a year (362 days) at room temperatures [87]. However, as far as the evidence available with the application of alternative sample matrices (dried blood, oral fluid, or tissue material) is in general limited and potentially dependent on the sample collection materials used (e.g. type of paper or tubes) laboratories working with those matrices are advised to be aware of sample stability issues and to include stability evaluation in their method validation protocols.~~

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4.2 Analytical Methods

Because the most fundamental clinical trials for the establishment of EVR TDM applied liquid chromatography with mass spectrometric detection (LC-MS/MS) for the measurement of drug concentrations this analytical technology was decisive for the development of therapeutic targets in clinical practice. Accordingly, fully validated LC-MS/MS methods that fulfill recommended analytical acceptance criteria are the preferred standard for EVR TDM [4,94]. Analytical tools that may support achievement of clinically required performance are deeply discussed in the latest IATDMCT consensus by Shipkova et al [4].

Chromatographic methods based on UV detection do not provide the required quality for this narrow therapeutic index drug and are not recommended. The "Chromatographic Procedures" described in the first version of the consensus [4] remain valid.

In recent years, various concepts and technical options have been explored in the ~~ISD~~ immunosuppressive drugs analysis to both improve analytical performance parameters (range, precision, accuracy) and to facilitate the practical aspects of using the LC-MS/MS technique. One of the ways to improve the method may be the use of a new type of column (e.g. small particle solid-core packing), which significantly shortens the run-time [95]. Replacing commonly used protein precipitation with a semi-automated solid-phase extraction (SPE) on a 96 well HLB microelution plate resulted in a chromatographically pure sample and a faint matrix effect. The advantage of the method is a wide range (0.1-50 ng/mL) with a low lower limit of quantification (LLOQ) [96]. A new sample preparation technique for the analysis of 4 immunosuppressants including EVR was proposed by Kvamsøe et al [97]. Salting Out-Assisted Liquid-Liquid Extraction (SALLE) is a specialized type of LLE in which water-miscible solvents are used and phase separation is achieved by adding salt first. In the presented procedure, the authors used 100 µL of a blood sample, added 100 µL of 5M NaCl and 400 µL of acetonitrile-methanol (87.5:12.5 v/v). With an almost complete extraction yield, the very low LLOQ level of 0.06 ng/mL was obtained for EVR. In the case of SALLE, it is possible to apply the sample directly without evaporating it; an additional benefit is the low matrix effects, but the analyst must take into account the need for a mass analysis of sodium [M + Na]+ adducts [97]. ~~Another procedure proposed in the analysis of immunosuppressive drugs (including for EVR) was QuEChERS (quick, easy, cheap, rugged, effective and safe) based on SPE and matrix solid phase dispersion techniques [98]. The idea is based on extraction (here: ethyl acetate), adding inorganic salt (here: anhydrous magnesium~~

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~~sulfate) and separating the organic layer with a sorbent (here: ethylenediamine-N-propyl silane). However, the appropriate range of the method for EVR (1-100 ng/mL) was obtained with a large (500 µL) sample volume. The disadvantage of the method is also the significant matrix effect and the long run time (ca. 12 min for EVR), which creates the need for further improvements.~~

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Interest in automating the various steps of routine LC-MS/MS analysis is growing steadily. Analysts use 96 well microplates and pipetting robots [96,97], but the real step forward seems to be the introduction to the routine TDM of the assay available on a fully automated LC-MS/MS based clinical analyzer. Hörber et al. [99] evaluated the performance over 6 months in the 24/7 routine laboratory of Thermo Scientific™ Cascadion™ SM Immunosuppressant Panel. The main practical advantages reported were: "no manual sample handling" and the possibility of using technical staff without MS experience, after only 5 days of training. Time will show whether such MS analyzers with applications will be able to replace both classic LC-MS/MS methods and immunochemical methods, opening a new era in laboratory medicine, including for TDM of immunosuppressive drugs.

Bruns et al. [100] presented a quantitative method for the determination of 4 immunosuppressants (including EVR) using equipment dedicated to the qualitative (identification) High Resolution MS (HRMS) analysis. The UPLC-TOF-MS was used, which compared to the classic LC-MS/MS provided equal quantitative results and similar operating parameters with the same sample preparation procedure. The advantage of using such equipment is the simpler development of the analytical method plus the possibility of obtaining data on metabolites. The results of this publication show a new application that could be a promising alternative for some laboratories.

Finally, the proposed reference method LC-MS/MS (combined with SPE) for the determination of immunosuppressive drugs (including EVR) in blood should be noted. This methodology published in 2020 by Taibon et al. [101] presents a refined analytical procedure which, due to attention to detail, is characterized by very good accuracy and precision, and therefore high reliability of measurements in wide concentration ranges (0.25-50 ng/mL for EVR). In the current absence of established reference materials for EVR, the methodology can act as a reference procedure for other laboratories.

Whereas at the time of the development of the first Consensus Document only one immunoassay was available to clinical laboratories to quantify EVR (the Quantitative Microsphere System (QMS)® EVR immunoassay, Thermo Fisher Scientific), two further immunoassays were introduced more recently. These include the Roche Elecsys® everolimus electrochemiluminescence immunoassay (ECLIA) and the Siemens Healthcare Diagnostics everolimus affinity chrome-mediated immunoassay (ACMIA) for the Dimension Chemistry Systems. In contrast to the QMS® EVR that can be run on multiple open clinical chemistry platforms like AU 640/680/5800 (Beckman), DxC (Beckman), Indiko (Thermo Fisher), Architect ci4100 (Abbott) etc., the other two assays can be applied only in combination with the immunochemical modules provided by their manufacturer. Whereas the QMS® and the ECLIA are semi-automated procedures requiring manual sample pretreatment before analysis, with the ACMIA the full process is automated. ~~Table1 summarizes performance characteristics of the different~~

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~~analytical methods.~~ Although the immunoassays almost comply with analytical requirements such as quantification limits close to 1 ng/mL (except QMS® ; 1.5 ng/mL), inter-assay coefficient of variation of <10% and dynamic measurement ranges satisfactory for performing a predose-concentration based TDM, they suffer from considerable cross-reactivity to both active and non-active metabolites of EVR that in addition vary between the immunoassays. This leads to inconsistent (biased) results both against the LC-MS/MS methods and between the three immunoassays [4,94,102-107]. Importantly, the manufactures follow different calibration strategies to deal with the cross-reactivity issue. The QMS® applies value-assigned concentrations of calibrators using a factor set at approximately 70% of their gravimetric concentrations with the goal to compensate for the 'average' bias against concentrations obtained by LC-MS/MS [4]. The ECLIA and ACMIA do not use correction factors in calibration to align their results to LC-MS/MS results but ECLIA was found to overestimate EVR concentrations in transplant recipients (relative bias of 36.5% and 26.5% at 3 and 8 ng/mL EVR, respectively [102]). Comparing ECLIA results to QMS® revealed a mean positive bias of 32.6% and 37.9% in two single center studies [103,106]. ACMIA manufacturer reported good correlation with LC-MS/MS (slope of 1.06 (n=295, correlation coefficient r=0.965)). However, a recently published customer study that compared ACMIA to QMS® with samples from kidney, liver and lung transplanted patients by the same statistical method observed high constant (intercept of 0.679 ng/mL) and proportional bias (slope of 1.326) between the two assays [107]. Correspondingly there was a 39.9% mean positive bias of ACMIA vs. QMS®.

~~The QMS® applies value-assigned concentrations of calibrators using a factor set at approximately 70% of their gravimetric concentrations with the goal to compensate for the 'average' bias against concentrations obtained by LC-MS/MS [4]. In addition to not considering inter-patient variability of the bias around the 'average' as caused by individual differences in cross-reactivates with metabolites and other potential errors, two further problems of the concept have been reported more recently. First, underestimation of EVR concentrations in samples from heart transplant recipients (mean 15.5% lower concentrations) probably related to insufficiency of the QMS® value assignment that was developed by a training set of samples from liver and kidney transplant patients only [103] and second, assay inconsistency over time. Six years results of the Zotracker-EVR study showed a significant shift in QMS® results during the observation period, after having initially results similar to that of LC-MS/MS a significant positive bias emerged [104].~~

~~The ECLIA and ACMIA do not use correction factors in calibration to align their results to LC-MS/MS results. Correspondingly, the ECLIA was found to overestimate EVR concentrations in transplant recipients. An estimated average relative bias of 36.5% and 26.5% were reported at the medical decisions points of 3 and 8 ng/mL EVR, respectively, as evaluated by Bland-Altman plot in a multicenter study [102]. This is in agreement with the bias observed in two additional analytical trials [103,105]. Comparing ECLIA results to QMS® revealed a mean positive bias of 32.6% and 37.9% in two single center studies [103,106].~~

~~ACMIA is the most recently launched immunoassay and respectively there is almost lacking practical experience regarding its performance. According to the manufacturer's information in the package~~

~~insert comparison of ACMIA results with a LC-MS/MS method used as the reference showed a Deming regression equation with an intercept of 0.1 ng/mL and a slope of 1.06 (n=295, correlation coefficient r=0.965). The respective values for the comparison ACMIA vs. QMS® (n=125) were reported to be 0.44 ng/mL (intercept), 1.09 (slope) and a correlation coefficient of 0.954 (Dimension Everolimus (EVRO) Assay, 11417409_En Rec. 01, 2021-05). However, a recently published customer study that compared ACMIA to QMS® with samples from kidney, liver and lung transplanted patients by the same statistical method observed higher constant (intercept of 0.679 ng/mL) and proportional bias (slope of 1.326) between the two assays [107]. Correspondingly there was a 39.9% mean positive bias of ACMIA vs. QMS®. These controversial results highlight the need of further independent studies to collect more evidence regarding the analytical performance of the ACMIA.~~ Table1 summarizes performance characteristics of the different analytical methods.

In conclusion, based on the currently available knowledge, EVR concentrations generated with different analytical assays should not be used interchangeably. Laboratory reports should include information on the assay used. Switching between assays needs careful assessment of expected deviations and their therapeutic relevance with samples representative for the local patient population. Re-baselining of individual patients is recommended to ensure proper patient handling.

4.3 Analytical Method Standardization

In addition to differences of assay design and actual measurement procedure, inconsistency in method calibration by using different EVR calibration materials, which are not related to a proper reference, strongly contributes to disagreement of interlaboratory results [108]. ~~The concept of metrological traceability guarantees the worldwide comparability of analytical results and has been firmly established in laboratory medicine for decades. Especially for small organic molecules such as substrates and metabolites, but also for drugs which are structurally very simple and unambiguously characterizable, the goal must be to use metrological traceability as the basis for standardization [109]. Harmonization, on the other hand, should be reserved for molecules that are not uniquely characterizable, i.e., where the measurand represents an analyte group rather than a single analyte [110]. Therefore, EVR, due to its synthetic origin, is a molecule for which standardization should follow metrological traceability.~~

~~The basic prerequisite for traceability is the definition of the measurand, i.e., when a uniquely identifiable analyte is measured in a specific matrix and when SI units are used for the reporting of the result. Therefore, methods which are calibrated for a specific analyte, but which also cross-react with metabolites and affect the measurement cannot meet the requirements of this definition. This is currently the case for most assays available on the market for the measurement of immunosuppressants, especially if ligand binding assay formats are employed [111]. Metrological traceability itself rests on three pillars: 1) access to reference materials in matrices that are metrologically traceable to pure substances and SI units; 2) availability of reference methods and 3) establishment of an ISO 15195 accredited measurement service.~~

Commenté [LF6]: Definitions. I propose to delete these parts.

Currently, for EVR a single reference method is described [101], which can also be used for standardization of cyclosporine A, tacrolimus, and sirolimus. The ISO 15193 compliant method is listed in the JCTLM (Joint Committee for Traceability in Laboratory Medicine) database and has an expanded uncertainty (k = 2) of less than 2.7% for EVR concentrations greater than 1 ng/ml. Unfortunately, no reference materials for EVR certified by metrological institutions such as the Joint Research Centre of the European Commission (JRC) (https://crm.jrc.ec.europa.eu/) or the National Institute for Standardization (NIST) (https://www.nist.gov/srm) are available. Although there are commercial suppliers offering reference materials only those intended to be used for quantitative analysis in biological materials can be applied for the clinical diagnostics. Only ISO34 certification ensures full metrological traceability of a pure compound or solutions prepared from it in terms of "higher metrological order". Regarding the purity assessment method used in reference material characterization, it can be noted that quantitative NMR spectroscopy (qNMR) is increasingly taking on a pioneering role and is beginning to replace the mass balance approach [112].

Currently, it is not possible to have metrological traceability, at least a complete documentation of the standardization should be provided. This includes the description of the reference materials used (origin, purity, certificates, etc.), the measuring equipment, the monitoring methods used (reference methods), an estimation of the measurement uncertainty [113,114] as well as a risk analysis regarding missing elements with respect to analytical accuracy. This description of the measuring equipment is flanked by analytical data of a validation, together these parts result in a test report, which is the operational basis of the measuring equipment. This applies above all to lab developed tests, but also to industrially manufactured analytical offerings.

4.4 Recommendation

Analytical recommendations provided in the first version of this document remain unchanged [4].
Additional recommendations include:

Concentrations obtained with LC-MS/MS, QMS, ECLIA and ACMIA should not be used interchangeably. Identification of the analytical assay used by the laboratory should be provided to the customer. Careful assessment of expected deviations and their therapeutic relevance with samples representative for the local patient population is required before switching of assays. Re-baselining individual patients is a helpful tool to ensure proper clinical handling. Continuous participation in an external QC program that includes the use of both spiked and pooled patient samples is highly recommended.

5. PHARMACOKINETIC MONITORING

5.1 Pharmacokinetics

Average pharmacokinetic parameters for EVR in sub-populations are summarized in Table 2. This has been quoted in several recent reviews on the use of EVR, but it should be noted that much of the PK data on transplant recipients originates from the early studies (Table 2). Regarding the use of EVR in cancer treatment, data from newer studies are included. The suggested target ranges for therapeutic

drug monitoring of EVR are to a large degree based on observational studies, which in combination with a few prospective studies provide the rationale for the suggested levels. Since the previous version of the EVR consensus document, the recommended TDM target levels have been discussed in several comprehensive reviews and meta-analyses for solid organ transplantation as well as for cancer treatment; some of these are quoted in the paragraph on TDM strategy below.

In addition to the EVR PK data in Table 2, some further characteristics should be mentioned. The bioavailability is consistently reported as 16%, but this figure originates from a study in rats [XXXXXXXXX] and similar data for humans have not been published. The relative bioavailability of the dispersible tablet formulation is however well documented to around 90% relative to the standard tablet formulation [115]. The PK of everolimus appears to be similar in patients with and without cystic fibrosis [116]. Everolimus is a substrate for the drug transporter P-glycoprotein 1 [(permeability glycoprotein, P-gp) also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) or cluster of differentiation 243 (CD243)] and for metabolism via CYP3A4. Accordingly, hepatic dysfunction and CYP3A4 inhibition may increase systemic exposure while the opposite will occur if combined with drugs that induce CYP3A4. Drug-drug interactions are described in the following paragraphs.

5.2 Drug-drug and Drug-food interactions

Drug–Drug interactions (DDI) and Drug–Food Interactions 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.

DDIs are a major source of EVR PK variability and must be identified early and controlled by careful monitoring. Quantitatively, the PK changes seen with EVR are higher than with TAC, but less significant and more easily manageable than those seen with SRL [117]. Compared to TAC, the treatment of DDI of EVR is simpler mainly due to its lower immunosuppressive potency and more favorable safety profile.

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Commenté [LF7]: Repetition.

A recent study on the ~~pharmacodynamic~~ PD ~~drug-drug interaction~~DDI in PBMC of healthy volunteers has raised additional evidence about the antagonist effect between EVR and TAC at higher concentrations. The interaction is seen at immunophilin FKBP12 saturation levels in lymphocytes. Although the interaction is not additive, the ~~pharmacodynamic~~ PD interaction of TAC with EVR enhanced its inhibition of T cell proliferation in vitro. However, in both drug therapeutic ranges, the antagonist effect is not observed [118].

CsA inhibits EVR metabolism by approximately 50% [119,120]. EVR exposure in kidney transplant recipients is not influenced by TAC concentration [121], and the EVR dose achieving equivalent exposure has been

shown to be 1.5 – 2-fold higher with TAC than CsA [122]. EVR exhibits DDI with other immunosuppressants. ~~GCs~~ Glucocorticoids 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 [122].

~~As discussed, most EVR PK DDIs involve either inhibition or induction of metabolism through the CYP3A pathway Therefore drug dosing should be adjusted based on strength and "direction" of the effect.~~ Case examples of dose adjustments in EVR treated patients involving enzyme induction by rifamycins [123] and inhibition by antifungal azole drugs [124] also provide important additional information compared with the usual studies on the quantitative aspects of DDI in healthy volunteers. Regarding treatment with anti-tuberculous concomitant treatment and immunosuppressive drugs, recent investigation proposes the use of rifabutin instead of rifampicin because of fewer potential interactions. However, more studies should help to support these results [125].

A recent review on CYP3A interactions proposes guidelines for patients on anticancer drugs where ~~everolimus~~ EVR is included [126,127]. DDI further explored clinically relevant interactions of lapatinib/everolimus with fluconazole and verapamil. The high incidence of DDI in cancer treatment is mainly due to a weak screening for DDI. Pharmacists could be actively involved in the identification of these interactions [127]. Another study in oncology highlighted the added effects among tyrosine kinase inhibitors and ~~everolimus~~EVR, in renal cell carcinoma and CNS tumors. ~~Everolimus~~ EVR increases the concentration of dasatinib at the tumor level. The combination inhibits both mTOR and RTK/MAPK signaling. Furthermore, inhibition of P-gp~~P~~ by ~~everolimus~~ EVR prevents the efflux of dasatinib from its action site [128].

Given the increased recreational use of marijuana among the population due to changes in legislation [129], it is important to raise awareness of the potential PK interaction of cannabidiol. DDI varies depending on the THC/CBD ratio, dose, and route of administration. The potential interaction with mTor inhibitors is enabled through the blockage of the CYP3A4/5 enzymes [129,130]. ~~On the other hand, CBD will be an inhibitor of UDP-glucuronosyltransferase 1A9 and 2B7 [129].~~

5.3 TDM Strategy

There is a significant body of evidence to recommend TDM of everolimus in organ transplant recipients~~. Observational studies and prospective clinical trials in kidney and liver as well as heart transplants have consolidated a therapeutic range of the everolimus whole blood trough concentration (C0) 3-8 µg/L, when combined with low dose calcineurin inhibitor. These results were summarized and discussed by van Gelder & al in a 2017 paper which includes references to the original studies~~ [10].

Following the introduction of everolimus into cancer treatment protocols, the potential for improved treatment using TDM even on this indication has been investigated. Several observational studies demonstrated an exposure - effect relationship. Studies including patients with various cancers where everolimus was used, found reduction of tumor size or progression free survival (PSF) significantly

better in patients with everolimus C0 above 11.9 [131], 14.1 [132] and 15.7 µg/L [133] (the latter was a meta-analysis of 5 phase 2-3 studies). One more study of 44 evaluable patients with breast cancer did not find a statistically significant relation between C0 and PSF [134]. In a review of these studies Falkowski and Woillard concluded that a lower limit for the therapeutic range of everolimus C0 could be set at 12 ng/mL [135]. So far, no prospective study to evaluate whether this level is the most appropriate recommendation.

In the BOLERO-2 trial where everolimus was combined with exemestane to treat breast cancer, everolimus was discontinued in 19% of the patients due to toxicity. The most frequent adverse events were stomatitis, infections, rash, pneumonitis, and hyperglycemia [136]. There are observations indicating a correlation between the incidence and severity of adverse events and the exposure to everolimus, both in terms of AUC and Cmax [135,137]. One prospective, randomized cross-over trial investigated the pharmacokinetics of dosing everolimus 5 mg twice daily compared to the standard 10 mg once daily, since this might pave the way for a more tolerable regimen. With the 5 mg twice daily regimen, Cmax was reduced from the average 61.5 µg/L (CV% 29,6) on 10 mg once daily to 40.3 µg/L (CV% 46.6) while maintaining the AUC (435 vs 436 µg·h/L respectively). The C0 concentrations were 9.6 µg/L on the once-daily dose vs 13.7 µg/L on the twice daily [138]. Observational studies have suggested that although one may experience stomatitis, pneumonitis, rash and other adverse events already at low levels, the risk gradually increases with C0 and that higher grade toxicity will be more frequent with trough concentrations at 15 to 26 µg/L [131,133,134]. Based on these data an upper level for the therapeutic range for everolimus in cancer treatment has been suggested around 20 µg/L [135].

Commenté [LF8]: I would delete this as it is in the Cancer TDM part

Regarding the use of everolimus on the indication tuberous sclerosis in children, TDM is required and dosing should be adjusted to attain a blood concentration 5-15 µg/L [12]. This range was the target in an open-label study of children with SEGA, although in this study the actually obtained concentrations were 4.2 µg/L (range, 2.0 to 11.0) at 3 months and 5.0 µg/L (range, 1.6 to 15.3) at 6 months [139].

5.4 Pharmacometrics and Model-informed precision dosing / Pharmacometrics

5.4.1 Population PK Modeling Of EVR And Pharmacometric Approaches

A population PK model describes how a drug behaves in patients and can be used to offer guidance for proposing dosage regimens to achieve and maintain the therapeutic goal for each individual patient. The first everolimus population PK model was developed based on data collected during the early clinical trials, when the drug was evaluated as a prophylactic agent against acute rejection episodes after kidney transplantation. Kovarik et al. (2001) [140] used clinical trial data from 673 patients consisting of an approximate equal mix of pre-dose troughs and 4 sample PK profiles collected over an eight-hour post-dose interval. The structural model that best describes the data was a one-compartment model with first-order input, linear clearance, and dose dependent bioavailability. Since then, there have been multiple everolimus population PK models published covering different patient populations and therapeutic usages for different clinical indications. Table 3 provides an overview of the published literature.

In addition to everolimus population PK models in renal transplant recipients [141-144], other populations include heart transplant recipients [145], cancer such as patients with thyroid cancer [137], renal cell carcinoma [146], and breast cancer patients [143], and most recently tuberous sclerosis complex patients [147]. Depending on the richness of the collected concentration data, models have been parametrized as one- or two-compartment models, with a semi-physiological liver model with transit compartment absorption as the most recent iteration [143,148]. Most studies also included covariate analyses identifying a size parameter (BSA, BMI, or allometrically scaled weight) and hematocrit as the most frequently retained parameters in the final model. Other covariates identified have included pharmacogenetic variants such as CYP3A5*1, ABCB1 haplotype, PPARA*42, PPARA*48, and POR*28 [137,149]. However, although several studies identified significant association (or trends) between genetic variants and everolimus clearance, it is unclear whether these studies were adequately powered to fully quantify such relationships (Table 3).

More recently, some more complex EVR pharmacometrics models have been developed and described (Table 3). The first was a semi-mechanistic everolimus model for cancer patients described by van Erp [148] and which was subsequently used as a basis for the analysis of PK study data in other type of patient (renal transplant patients, other types of cancer) [144]. These authors demonstrated that the population PK model accurately and precisely predicts future everolimus exposure based on previous PK assessments. In addition, this study showed the potential added value of hematocrit-normalized whole-blood concentration measurements, as a promising strategy to further optimize everolimus therapy in patients with extreme hematocrit values. Another pharmacometrics approach, using a nonparametric modeling platform and in combination with a Bayesian estimator, was reported by the Limoges group in 2018 [149]. This approach was developed to simultaneously predict everolimus exposure in whole blood and peripheral blood mononuclear cells (PBMCs) and provides a basis for the exploration of the relationship between intracellular drug exposure and therapeutic and adverse effects. Lastly, a promising new approach using machine learning (ML) algorithms to estimate everolimus exposure built from data extracted from the Immunosuppressants Bayesian Dose Adjustment (ISBA) program was reported by the same group [150]. These ML model was trained on both simulated data from a previously published POPPK model and experimental data from the ISBA program to predict everolimus exposure (AUC0-12h) based on a limited sampling strategy (pre-dose, ~1 h, and ~2 h whole blood concentrations). The ML algorithm slightly outperformed their current Bayesian estimator in terms of better AUC predictive performance based on prediction error expressed as root mean squared error. However, despite these findings and the increasing application of ML using big data sets, it is likely that the introduction of ML in precision dosing will not lead to the disappearance of population PK, quantitative systems pharmacology, and other pharmacometrics methods [151]. Given that a major advantage of pharmacometrics models is that the biological plausibility of the models is considered, the addition of artificial intelligence and ML methodologies should be viewed as a welcome addition to the model-informed precision dosing (MIPD) armamentarium.

~~5.4.2 Model-Informed Precision Dosing And Available Services~~

~~Many studies report the benefit of pharmacokinetically guided dosing and model-informed precision dosing (MIPD) as an attractive approach to further improve the efficacy, safety, and clinical outcomes [152]. To date, however few studies have documented utility and improved clinical outcomes of the MIPD approach, so there is an important need for further investigation in larger prospective studies [153,154].~~

Commenté [LF9]: Nothing much to say there.

~~5.4.3 Model-informed precision dosing (MIPD) of EVR.~~

~~The published EVR population PK models as summarized in Table 3 form an attractive basis for integration into MIPD algorithms and decision support platforms. Several studies have validated sparse sampling strategies and have shown that when used in conjunction with a Bayesian estimator using maximum a posteriori Bayesian (MAPB) estimation, the generation of everolimus precision dosing regimens to attain the predefined therapeutic target(s) is quite feasible.~~

Commenté [LF10]: Same. My opinion is all as been said in the 5.4.1

~~Table 4 summarizes the stepwise process of the model-informed Bayesian estimation process to generate precision dosing regimens. An example of such Bayesian adaptive control has been published for the mTOR inhibitor sirolimus [155,156]. In a similar fashion everolimus dose individualization can be achieved by using (a) a population model, defined as the summary of everolimus PK parameter estimates (e.g. absorption rate, oral clearance, and volume of distribution), covariates (such as body weight) and their respective standard deviations (or coefficients of variation) to define the parameter distribution within to select the initial dosing regimen for that particular patient based on chosen goals; and (b) entering results of timed concentration measurements as feedback information to update the parameter estimates to go from 'population average' to 'individual' estimates; (c) a maximum a posteriori probability Bayesian fitting algorithm that is part of clinical precision dosing software; (d) calculation of the subsequent individualized dosage regimen to precisely reach the target.~~

~~There are several programs who have published on their MIPD strategy for everolimus therapy. The Immunosuppressant Bayesian Dose Adjustment (ISBA) program at Limoges University Hospital, France has developed and validated multiple Bayesian estimators and limited optimal sampling designs for immunosuppressive drugs across different indications including everolimus [145,149,157]. Other examples of the application of MIPD have been described by groups at Leiden University Medical Center, and Radboud University Medical Center in the Netherlands [143,144,148].~~

Commenté [LF11]: These are general information –and even team commercial information…

6. CLINICAL PHARMACOLOGY

6.1 Kidney Transplantation

6.1.1 The therapeutic window for EVR after kidney transplantation

In the 2016 version of the EVR consensus document the therapeutic window for patients after kidney transplantation was defined as 3-8 ng/mL [4]. The lower concentration limit was based on indications of

reduced efficacy below 3 ng/mL, largely based on an analysis in almost 700 kidney transplant patients simultaneously treated with cyclosporine [158]. Freedom from acute rejection was significantly related to pre-dose concentrations of EVR, with an incidence of 68% at 1.0–3.4 ng/ml, 81–86% at 3.5–7.7 ng/ml, and only a small further improvement of efficacy to 91% at 7.8–15.0 ng/ml (P =0.03). The importance of achieving everolimus pre-dose concentrations above 3 ng/ml was underscored by a Cox proportional hazards analysis that indicated a significantly higher risk of acute rejection of nearly 3-fold when levels were less than 3 ng/mL compared with levels above this cut-off value. The incidence of thrombocytopenia increased with higher exposure to EVR. A subsequent study showed that maintaining EVR pre-dose concentrations in the range 3 to 8 ng/mL in the first posttransplant year with reduced-exposure cyclosporine is associated with good efficacy and safety profiles [159].

In the multicenter US 92 study, where everolimus was combined with low exposure tacrolimus, showed a high incidence of acute rejection in patients with below-the-target EVR concentrations at days 3 (62.8%), 7 (55.8%), and 14 (33.5%) [160]. The incidence of treated BPAR was 64.7% among patients with EVR concentrations below 3 ng/mL and strikingly lower at 14% among those patients with EVR concentration above 3 ng/mL [161].

6.1.2 Development of Donor Specific Antibodies

While the studies mentioned above focused on acute (cellular) rejection episodes within the first 6 or 12 months after transplantation, in the last ten years several studies have been published with focus on development of de novo Donor Specific Antibodies (dnDSA). One of the first to publish on this topic was Liefeldt. He randomized 127 patients to continue on cyclosporine or switch to everolimus therapy, 3 months after kidney transplantation [162]. Seven out of 65 (10.8%) patients on cyclosporine developed dnDSA after a median of 991 days, while 14/61 patients (23.0%) randomized to everolimus developed dnDSA after 551 days (log-rank: p = 0.048).

In the ELEVATE study 715 de novo kidney transplant recipients were randomized at 10-14 weeks to convert to everolimus (n = 359) or remain on standard calcineurin inhibitor (CNI) therapy (n = 356; 231 tacrolimus; 125 cyclosporine), all with mycophenolic acid and steroids [163]. When dnDSA was assessed at months 12 and 24 in patients with no DSA at time of transplant, Class II DSA was more frequent in the everolimus cohort at month 12 (18.4% [30/163] vs. 11.1% [22/199] in the CNI group), but showed less difference at month 24 (13.1% [17/130] vs. 10.7% [18/169]). However, no data were reported on the association between the everolimus concentrations and development of dnDSA. It remains unclear whether or not everolimus exposure in the upper end of the therapeutic window of 3 to 8 ng/mL, or above 8 ng/mL, will lead to less DSA development.

6.1.3 Concentration-controlled studies:

The TRANSFORM study needs to be mentioned, as it was the largest RCT in the transplant field, so far. In a multicenter noninferiority trial, 2037 de novo kidney transplant recipients were randomized to receive, in combination with induction therapy and corticosteroids, everolimus with reduced-

exposure CNI (everolimus arm) or mycophenolic acid (MPA) with standard-exposure CNI (MPA arm) [164]. The everolimus dose was subsequently adjusted to target an everolimus trough concentration of 3–8 ng/mL throughout the study. Treated biopsy-proven acute rejection, graft loss, or death at post-transplant month 12 occurred in 14.9% and 12.5% of patients treated with everolimus and MPA, respectively (difference 2.3%; 95% confidence interval, 21.7% to 6.4%). De novo DSA incidence at 12 months and antibody-mediated rejection rate did not differ between arms.

In the ATHENA study 655 de novo kidney transplant patients were randomized to 1 of 3 treatment arms—EVR/TAC, EVR/CsA, or MPA/TAC—with similar tacrolimus exposure in the EVR/TAC and MPA/TAC groups [165]. Again, everolimus target concentrations were 3 – 8 ng/mL, in both EVR groups. The primary endpoint of ATHENA was eGFR at 12 months, and although non-inferiority was aimed for, eGFR was numerically inferior in both everolimus groups compared with MPA/TAC [166]. A concern was the high patient withdrawal rate in ATHENA, with only 52% of randomized patients making it to month 12 on trial, on study drug, and with an eGFR recorded [167]. Unfortunately, analysis in a concentration-effect relationship for everolimus on the incidence of adverse events has not been published.

In the pediatric era, Ahlenstiel-Grunow and colleagues conducted a randomized controlled trial comparing a group of pediatric kidney transplant recipients (n=31) whom immunosuppression was guided using the viral load of virus-specific T cells in addition to therapeutic drug monitoring (TDM) to a group of patients (n=33) with treatment adjusted only with TDM [168]. From month-1, patients were treated with cyclosporine with a target of 50-100 ng/mL and then 30-75 ng/mL from month-6 and everolimus with a target of 3-6 ng/mL and then 2-5 ng/mL form month-6. The primary outcome was eGFR at month-24 and was not different between the groups. Coupling a pharmacodynamics monitoring approach to TDM allows decreasing the dosage (0.8±0.3 vs 1.2±0.5 mg/m2, p=0.004) and trough levels of everolimus (3.5±0.7 vs 4.5±0.8 ng/mL, p<0.001). There were also numerically less patients with ≥1 biopsy-proven acute rejections in the intervention arm. While this was a small study, it underlines the potential interest of guiding everolimus exposure using a pharmacodynamics test.

6.1.4 Immune response after vaccination.

As a substudy to the OPTIMIZE study [169] Boer et al published data on the response after COVID-19 mRNA vaccinations [170]. They showed that an immunosuppressive regimen without MMF/MPA, a lower CNI dose, and usage of everolimus were associated with a higher humoral response rate against COVID-19 after vaccination in elderly patients after transplantation without any treatment against rejection. This confirmed data from an earlier study that reported low seroconversion rates in patients on MMF/MPA maintenance treatment. Recent data showed that within the group of MMF/MPA treated patients the MPA-AUC is predictive of the likelihood of seroconversion [Meziyerh et al, submitted to CPT]. To the best of our knowledge such data relating everolimus concentrations with seroconversion rates are not available.

6.2 Liver Transplantation

As clinical management of liver transplant patients improved with time, the goal in this kind of transplantation has shifted from avoiding immunological complications (i.e graft rejection) to reducing the burden of pathological and treatment complications. Renal failure, new onset diabetes mellitus and hypertension now individually account for a 2-fold increase in patients' mortality and are the primary focus to take a step-in liver transplant patients' management [171]. The role of immunosuppressive drugs, and particularly calcineurin inhibitors (CNI), in the onset of such adverse events is recognized, and therefore there is a need for defining therapeutic strategies aiming at addressing these adverse drug reactions (ADR).

Everolimus is labeled in some countries as part of the immunosuppressive regimen indicated to prevent graft rejection in liver transplantation and may be a key player in decreasing ADR rate in liver transplant patients. Another advantage of everolimus-based treatment is its ability to sharply decrease the incidence of cytomegalovirus infections compared to mycophenolic acid-based treatment [172]. The drug has been evaluated in clinical strategies to decrease CNI exposure or to withdraw CNI from patients' immunosuppressive regimen.

6.2.1 Everolimus as an agent to decrease CNI exposure

In an attempt to reduce immunosuppressive drug regimens' long term toxicity, and particularly nephrotoxicity, everolimus can be added to the patient's treatment allowing decreasing CNI dose and most importantly CNI whole blood exposure. Dose ranging studies have shown that the efficacy of everolimus is related to the whole blood trough concentrations (Cmin) with patients having Cmin below 3 ng/mL being at higher risk for three-year graft rejection (50% versus 14% in patients with Cmin between 3 and 6 ng/mL) [173]. Most of the recent randomized control trials comparing immunosuppressive drug regimen composed of everolimus with a target concentration of 3 to 8 ng/mL and a CNI with a reduced exposure (tacrolimus Cmin between 3 and 5 ng/mL) reported a better renal function in the experimental arm at month 12 [174], month 24 [175] and later [176,177]. It is noteworthy that, when tacrolimus minimization below 5 ng/mL is not obtained, the gain in preventing renal function deterioration is weaker [178]. Everolimus should be introduced from post-operative day 30 to avoid delayed wound healing events. A real life cohort data also confirms the benefit of the treatment on preventing renal function deterioration when everolimus initiation is performed early [179] while there is minimal benefit from late introduction of the drug (e.g after 6 months) [180]. It still has to be determined whether a de novo minimized tacrolimus scheme (i.e with a tacrolimus target Cmin < 6 ng/mL from day-1) compares favorably to everolimus with reduced tacrolimus as this first regimen might decrease the toxic pressure on kidney [181]. Beside wound healing events, starting everolimus before day 30 can be associated with a possible lower efficacy with numerically more events of graft rejection, especially subclinical rejection [182]. The safety profile of everolimus in clinical trials shows more proteinuria, hematological disturbance and hypercholesterolemia. Long-term use displays more statin treatment but no more cardiac events in the patients treated with everolimus [175]. Globally, the treatment discontinuation rate in recent randomized clinical trials is high with everolimus and higher than in CNI/corticosteroids or CNI/mycophenolic acid arms with rates ranging from 18 to 37% according to study protocols and follow-up duration [174,183].

6.2.2 Everolimus as an agent to withdraw CNI from immunosuppressive treatment

Another option for the use of everolimus in liver transplantation is to use the drug as a substitute for a CNI agent. As the safety profile of CNI is expected to be less favorable than everolimus' one, this strategy may lead to a decrease in the rate of CNI specific ADR such as chronic renal failure, diabetes and hypertension. A large clinical trial has been conducted in de novo liver transplant patients comparing 3 arms: everolimus with tacrolimus discontinuation, everolimus with reduced tacrolimus and a control tacrolimus arm [184]. In this study, everolimus was dosed to maintain a Cmin concentration of 3-8 ng/mL from day 5 to the end of month 3 and then a target increase to 6-10 ng/mL was proposed. Tacrolimus was then progressively eliminated by the end of month 4. Unfortunately, the 'everolimus with tacrolimus elimination arm' was prematurely terminated due to an excess in graft rejection. Still, recent data obtained from randomized control trials and a prospective cohort show that tacrolimus elimination can be achieved in a selected population of liver transplant recipients by initially targeting everolimus Cmin of 8-12 ng/mL (or at least 6-10 ng/mL) then 6-10 ng/ml from month 6 and finally 6-8 ng/mL from year 1 [182,183,185]. However, if such a strategy can lead to reduced kidney function deterioration, it also exposes patients to a probable less efficient immunosuppressive treatment as suggested by the higher, although not always statistically significant, rate of biopsy proven acute rejection in these studies. A large drop out is also expected when everolimus monotherapy is applied with around 20% and 50% of patients discontinuing their treatment after 6 months and 2 years, respectively [182,183].

6.2.3 EVR as an agent to prevent of Chronic Rejection

Some patients develop severe rejection that cannot be controlled by TAC or high-dose glucose steroids (GCs). Acute cellular rejection after liver transplantation can be treated with high-dose GC pulse therapy. However, apart from re-transplantation, 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 SRL [186] or EVR [187] 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 C0 10–12 ng/mL; CsA C0 100–200 ng/mL; TAC C0 5 ng/mL; maintenance EVR C0 5–8 ng/mL), however, more evidence supporting the introduction of EVR to treat antibody-mediated rejection is needed in future studies [188]. Furthermore, a meta-analysis showed an increase of acute rejection episodes after mTOR initiation with CNI withdrawal during the first six months after the procedure. Trials which include MPA plus EVR and EVR in monotherapy have shown less BPAR incidence [10) Nashan PMID: 28230639].

Commenté [LLJP12]: Mismatch

6.2.4 Prevention of Recurrence of Hepatocellular Carcinoma After Liver Transplantation

HCC is the main cause of liver cancer in the world [189] and the fourth etiology of cancer-related death [Estimated age-standardized mortality rates (World) in 2020, worldwide, both sexes, all ages (excl. NMSC) [190].

Recurrence of HCC after liver transplantation in cirrhotic patients (10 - 20%) [191,192] has been a serious problem in deceased and living donors. HCC recurrent recipients have a lower median 1-year survival after diagnosis and 67% exhibit extrahepatic cancer [192]. CNIs are carcinogenic, and sustained higher levels of CNIs correlate with a higher rate of HCC recurrence leading to a poor prognosis [191,193,194]. Milan transplant criteria were originally established to select patients with cirrhosis with small HCC nodules for whom a good outcome is expected, with lower rates of recurrence [195]. As many transplant centers have the possibility of enlisting patients beyond Milan criteria, they show a worse prognosis compared to HCC-free recipients [178,196]. mTOR signaling (cell growth, metabolism, proliferation, and apoptosis inhibition) is concerned in several key stages of the neoplastic process (development, progression, and spreading) [194,197]. Furthermore, upregulation of the mTOR pathway is a feature in different types of cancer, including HCC [178,194]. A recent meta-analysis revealed that HCC patients treated with mTOR inhibitors show a lower rate of recurrence compared to the CNI arm [178]. Aligned with the study by Cholongitas et al. the recurrence of HCC was significantly lower compared to CNI (8.0% vs 13.8%, P , 0.001) [196].

Despite the shorter follow-up for patients treated with EVR than for those receiving SRL or CNI (13, 30, and 43.2 months, respectively), the rate of HCC recurrence in recipients treated with EVR was significantly lower than in those treated with SRL and CNI (4.1% vs 10.5% vs 13.8%, respectively; P, 0.05).

A recent meta-analysis reported an increase of HCC recurrence free survival 1 and 3 years after transplantation in patients taking mTOR inhibitors compared to standard CNI therapy (Risk-Ratio 1.09, 95% CI 1.01 1.18 and 1.1, 95% CI: 1.01-1.21 respectively) Furthermore, the recurrence rate was lower in the mTOR inhibitor group (RR: 0.67, 95% CI: 0.56-0.82) [198]. On the other hand, Kang et al [199] found that in the EVR cohort, patients exhibited an increased number of tumors and microvascular invasion compared to the EVR-free arm. However, in patients with microvascular invasion and who exceeded the Milan criteria, the time to recurrence was similar between the studied groups, while the patients in the EVR arm had significantly longer overall survival than in the non-EVR arm. In addition, there is increasing evidence that EVR restricts HCC progression and recurrence [191,200]. Another important finding is that when EVR Cmin are maintained higher than or equal to 6 ng/ml from month 6 to 12, lower HCC recurrence rates are achieved [201]. Nitta et al. [192] observed that patients treated with a synergistic combination of sorafenib plus EVR with through levels above 5 ng/ml may have prolonged survival rates. This approach blocks the activation of the PI3K/Akt and Ras-MAPK signaling pathways, whether activated yields to shorter survival [197]. Emphasizing one of the challenges of the combination therapy is the management of adverse effects and comorbidities [192]. The current recommendation to avoid HCC recurrence in liver transplant recipients is to reduce immunosuppression to the lowest effective dose to prevent rejection of the graft [202].

Currently, evidence on the use of EVR to prevent recurrence of HCC is still limited due to the small number of studies and patients [203]. Several clinical trials are ongoing [194] so we expect the results could support better clinical decisions.

6.2.5 Recommendations: Liver Transplantation

- In liver transplant recipients Cmin, everolimus, targeted at 3 to 8 ng/mL, can be used to reduce tacrolimus Cmin to 3-5 ng/mL from post-operative day 30. It allows prevention of renal function deterioration. Late introduction of the drug (since 6 months after transplantation and ahead) has little effect.

- In case of need of complete elimination of CNI from the immunosuppressive treatment, an everolimus Cmin target of 8-12 ng/mL (or at least 6-10 ng/mL) until month 6, then 6-10 ng/ml from month 6 and finally 6-8 ng/mL from year 1, should be targeted but this strategy has been shown to be less efficient in preventing graft rejection.

- Compared to CNI treatment, EVR administration with relatively higher trough concentration [LF1] is expected to reduce the recurrence of HCC after liver transplantation due to its ability to inhibit cellular growth, proliferation, angiogenesis, and survival [204].

6.3 Heart and Lung Transplantation

6.3.1 Heart Transplantation: EVR with CNI

Little has been published since the last consensus document. EVR has shown its superiority over azathioprine on ~~In a 24-month, multicentre, randomized, phase III study (B253), both fixed doses of EVR were Both EVR dose regimens were superior to azathioprine with respect to a~~ 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 < 3A [205] and. ~~The severity and incidence of cardiac allograft vasculopathy (CAV) were also significantly lower (P < 0.05) in the patients receiving EVR than those receiving azathioprine.~~

The EVR therapeutic target in combination with CNI was set to 3-8 ng/mL based on post hoc analysis and TDM simulation of PK results of the B2253 study, which observed freedom from rejection with EVR C0 > 3 ng/mL (P = 0.02) and a lower incidence with this target range compared to a EVR fixed dose regimen [206,207]. Whereas 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) [206]. This therapeutic range allows decreasing CsA concentration in de novo heart transplant recipients, without significant loss of efficacy

~~In a single-center, observational study in Germany, EVR targeted to a C0 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~~ [208] while ensuring similar efficacy and change in renal function when compared to MMF~~.~~

~~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.~~[209]~~. Both study groups received glucosteroids (GCs) and antibody induction therapy according to local practice. At 12 months, the incidence of BPAR grade ≥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 a 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.~~[210]~~. CsA was initiated at ≤12 mg·kg-1·d-1, except at centers using induction therapy, where local practice was followed. The CsA C2 target range for all patients was 1000–1400 ng/mL in the first 2 months. Predefined CsA C2 targets were lowered over the next 4 months, being higher in the standard-dose group than the reduced-dose group. Post-transplant EVR was initiated 1.5 mg/ daiy) to achieve a C0 in the range of 3–8 ng/mL after day 5. EVR with reduced-dose CsA resulted in similar efficacy as standard-dose CsA, whereas the renal function (primary endpoint) was significantly different between the study groups at month 6 (mean serum creatinine 141.0 ± 53.1 mmol/L standard-dose CsA group versus 130.1 ± 53.7 mmol/L reduced-dose CsA group). No renal function benefits were observed, possibly because of the inadequate adherence to reduced CsA exposure using a target C2 range in the study group.~~.

~~In the largest EVR study so far, the 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.~~[211]~~. 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 pre-transplantation in German centers and might have been caused by ATG induction leading to overimmunosuppression. At month 24, the mortality rates were similar in the EVR and MMF groups (10.6% versus 9.2%, respectively), as were efficacy endpoints.~~

The large multicentre, randomized, controlled NOCTET (Nordic certican trial in heart and lung transplantation) study in Scandinavia explored the benefit of a quadruple regimen with EVR and a predefined CNI exposure reduction on renal function in maintenance thoracic transplant recipients (190 heart transplants) [212]. The quadruple regimen consisted of EVR (target C0 to 3–6 ng/mL), CsA (C0 of 75 ng/mL) or TAC (C0 of 4 ng/mL), MMF (1000mg / day) or azathioprine, with or without GC therapy according to local practice.

Mean change in mGFR from baseline to month 12 was significantly in favor of the recipients receiving quadruple immunosuppression compared with controls receiving standard triple immunosuppression without EVR (5.8 mL/min versus 20.1 mL/min, P<0.0001) and ~~. In the follow-up period the favorable trend of the quadruple therapy on renal function~~ continued at month 24 [212] up to month 60~~. with a mGFR improvement from 52.8 ± 14.5 to 54.2 ± 19.2 mL/min in the ERL group, the improvement in mean mGFR remained significant in EVR-treated patients compared with controls in both subpopulations (P <0.001).~~ Heart and lung transplant recipients with preexisting moderate or severe renal failure (mGFR 30–59 or 20–29 mL/min2/1.73 m2, respectively) benefited particularly from the experimental arm.

The frequency of treated BPAR was similar between quadruple 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~~ [212].

~~A sub-analysis 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/min2/1.73 m2, respectively). However, this beneficial effect was limited to patients undergoing conversion within 5 years of transplantation~~ [213]. Overall, this study showed that a quadruple regimen with EVR (C0 3–6 ng/mL) with reduced CNI exposure has a beneficial effect on renal function in the long-term in heart transplant recipients and an acceptable safety profile.

~~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~~ [213]~~.~~

Commenté [LF13]: Not a single study after 2013 in that part. We absolutely need to reduce it.
Plus I missed: Anthony et al., JACC Heart Fail

6.3.2 Heart Transplantation: EVR in CNI-Free Regimens

Over the last 10 years the focus in EVR trials was to evaluate the potential of EVR in renal sparing regimens mainly CNI-free regimens, because CNIs are the most important contributors for end-stage renal disease (ESRD) in the long-term [214].

The first multicenter, randomized controlled trial explored the potential of a CNI-free regimen using EVR to protect renal function in 60 patients after heart transplantation in Germany [215]. More than one year after transplantation patients (mean time, 5.4 ± 3.2 years) were converted from a CNI regimen to a CNI-free regimen with EVR (target C0 4-8 ng/mL) plus MMF (1000 mg twice daily) and prednisone (5 mg/day). EVR (start fixed dose of 1.5 mg/day) target blood C0 levels ranged from 4 to

8 ng/mL, when CNI stopped two weeks after EVR initiation. Six months after conversion to the CNI-free EVR regimen creatinine clearance significantly improved compared to the control group with CNI (42.2 ± 21.6 vs 61.8 ± 23.4 ml/[min · 1.73 m2], p= 0.018)). Of interest, most CNI-related adverse events (e.g. arterial hypertension) resolved in the CNI-free group without losing the efficacy.

~~A couple of years later an observational single-center study in Scandinavia confirmed the benefit of a conversion from a CNI-based to a CNI-free EVR-based immunosuppression to improve renal function in maintenance patients with ESRD including dialysis [216].~~

Commenté [LF14]: Old study, the historical information has been provided by the previous paragraph.

As a consequence of these results the randomized, controlled, open label SCHEDULE (The Scandinavian Heart Transplant Everolimus De Novo Study with Early Calcineurin Inhibitors Avoidance) trial in de novo adult heart transplant recipients was designed [217]. 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 C0 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) but without hemodynamic compromise and stable 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) was significantly higher with EVR versus CsA (mean ~~6~~ SD: 79.8 ± 17.7 versus 61.5 ± 19.6 mL/min21/1.73 m2; P<0.001) at 12 months after transplantation. Additionally, the incidence of CAV was lower in the EVR group (50.0% versus 64.6%, P = 0.003).

Follow-up analyses at the study timepoints 36 months and 60 to 72 months post-transplant confirmed the significant improvement in renal function in the CNI-free arm [218,219]. Whereas, there was no difference between the study groups regarding the incidence of BPAR at both study time points. Overall, there was a similar incidence of adverse events and serious adverse events in the study groups, with a higher incidence of pneumonia and a substantially reduced risk of cytomegalovirus infection in the CNI-withdrawal arm. Additionally, the incidence of CAV was reduced in the EVR treated patients compared to CsA treated patients up to 7 years after HTx [219].

In Germany, the MANDELA study (NCT00862979) was the first multicentre, randomized, controlled, open-label, study comparing two EVR-facilitated CNI reduction strategies (reduction vs withdrawal) [214]. In addition, MANDELA investigated the drug combination of TAC plus EVR in a randomized study

for the first time. After randomization at month 6 post-transplant, a 2-month CNI reduction (control group) or CNI withdrawal (study group) phase followed. The study compares renal function (primary endpoint) and composite efficacy at month 18 post-transplant in CNI-free patients treated with EVR (C0 5–10 ng/mL) plus MPA and GCs, with that in patients receiving EVR (C0 5–10 ng/mL) plus reduced-exposure CNI (TAC C0 3–8 ng/mL or CsA C0 50–150 ng/mL) and GCs. Renal function was improved in the first weeks after CNI reduction or withdrawal in both study groups compared to baseline with continuation up to study end. However, renal function was significantly better in the CNI-free compared to the CNI reduction group at the study end.

Although the incidence of BPAR was higher in the EVR CNI-free group compared to the control group, none of the rejections led to hemodynamic compromise. Interestingly, 40% of the BPAR episodes occurred in patients with a EVR C0 target level below <5 ng/mL. This indicates that the incidence of BPAR in a EVR CNI-free regimen could potentially be reduced with more careful regulation of EVR blood concentrations. As known from earlier EVR studies the risk of CMV infection reduced in both groups but with a pronounced effect in the CNI-free group compared to the CNI-reduced group.

6.3.3 Recommendations: EVR use with and without CNI after heart transplantation.

- The use of EVR in de novo heart transplant recipients requires TDM to achieve and maintain a recommended whole-blood target C0 of 3–8 ng/mL in combination with reduced CNI dosages.
- The CNI C0 in patients receiving CNI-sparing regimens needs to be tightly controlled to achieve improved renal function.
- In a quadruple regimen with CNI, other cell cycle inhibitors and steroids EVR C0 target levels should be 3-6 ng/mL to lower the risk of infections.
- In a CNI-free regimen in combination with MPA and GCs, the EVR target C0 range should be 5–10 ng/mL, and EVR C0 levels below 5ng/mL should be avoided to lower the risk of rejection.
- Blood EVR C0 concentrations above 10 ng/mL are associated with an increased risk of adverse events and should be avoided.
- ~~Early use of EVR as part of a CNI reduction or withdrawal strategy appears to be appropriate for the maintenance of immunosuppression and protection of renal function.~~
- ~~EVR-associated CAV benefit and reduction of CMV infections are also considerations for its early use after heart transplantation.~~
- ~~Late conversion to a EVR-based regimen may fail to show effects on renal function (>5 years post transplantation) and on CAV progression.~~

Commenté [LF15]: Not TDM to me

6.4 Lung Transplantation: EVR with CNI early after lung transplantation

Commenté [LF16]: Again. Only one new study in that part so I propose to restrict it.

Limited new information has been published on the topic since the last consensus document. In contrast to heart transplantation, after lung transplantation EVR is mainly used instead or with the cell cycle inhibitor MPA in CNI-based regimens but only individually in CNI-free regimens.

Experience with EVR in de novo lung transplantation is limited, possibly because of the negative outcome of two initial de novo lung transplantation studies with SRL. Both studies reported significant wound dehiscence and airway complications, leading to death in some patients [220,221]. It is, therefore, recommended to start mTOR inhibitor therapy after the anastomosis and airways are healed [222], because of the drugs' inhibitory effects on growth factors and fibroblast proliferation [223] Consequently, all study protocols for the use of EVR in lung transplantation have avoided early administration of the drug [224].

~~In a randomized control trial conversion to~~ EVR combined with CsA and GCs at month 3 in lung transplant recipients (RAD001 B159) demonstrated a slowing in loss of pulmonary function compared to azathioprine ~~(21.8% versus 33.9% occurrence of composite endpoint of efficacy failure in of AZA-treated and EVR-treated patients, respectively, at 12 months (P = 0.046), but there was~~ no difference ~~in this composite endpoint~~ at 24 months [225]. ~~Analyses of EVR pharmacokinetics and exposure-response relationships showed creatinine values increased without significance in the EVR group than in the AZA group: baseline 141 ± 48 µmol/L compared to 149±51 µmol/L, and after 12 month 226 ± 48 µmol/L compared to 186 ± 57 µmol/L, respectively [226]. The relevant increase in creatinine in this study was probably related to the long time need for CsA reduction, up to 12 month, to reach the target levels.~~ Conversion to mTOR inhibitors and reduced CNI improvement in renal function after conversion in patients with an eGFR of ≤29 mL/min (median eGFR from 24 to 33 mL/min, n = 29, P < 0.0001), but not for renal function in patients with an eGFR of 30–44 mL/min (median eGFR from 36 to 42mL/min, n = 26, P = 0.1032)[227]. One to 3 months EVR initiation with reduced CsA also compared favorably with enteric-coated MPS (with standard CsA) with similar renal function and less acute rejection[228]. However, h~~H~~igh EVR dosing during the first 2 months after initiation could aggravate CNI-related nephrotoxicity. Improvement of renal function was higher in patients without proteinuria at baseline

~~A retrospective study in over 100 patients with renal insufficiency (median eGFR of 30mL/min/1.73 m2), evaluated the differences in the decrease in eGFR after conversion to mTOR inhibitors (predominantly EVR-treated) and reduced CNI according to baseline eGFR [227]. Results of a sub-analysis showed an improvement in renal function after conversion in patients with an eGFR of ≤29 mL/min (median eGFR from 24 to 33 mL/min, n = 29, P < 0.0001), but not for renal function in patients with an eGFR of 30–44 mL/min (median eGFR from 36 to 42mL/min, n = 26, P = 0.1032). Improvement of renal function was higher in patients without proteinuria at baseline.~~

~~The CeMyLungs study was a 3-year randomized, multicentre, investigator-driven superiority study comparing de novo enteric-coated MPS with delayed-onset EVR (1 to 3 months post-transplant) both together with standard CsA or reduced CsA, respectively [228]. Both study groups demonstrated equivalent efficacy in preventing the primary outcome of BOS at 3 years after lung transplantation. However, a significantly lower rate of acute rejection was observed with EVR + reduced CNI versus MPS + standard CNI. Renal function between study groups were comparable three years post-transplant: mean creatinine level 152±98 µmol/L in the EVR group versus 160 ± 112 µmol/L in the MMF group (P = 0.67).~~

More recently, in a single center prospective study a EVR-based regimen was studied for the prevention of freedom from BOS. EVR was introduced once wound healing was established between 1 to 3 months after lung transplantation. The patients (n=190) received either EVR (6–8 ng/mL) and reduced CsA (C0 150-200 ng/mL, after month 6, 100–150 mg/mL after 12 months) or MMF in combination with standard CsA (C0 200-250 ng/mL after month 6, 150–200 mg/mL after 12 months) and prednisolone in both groups [229]. Freedom from BOS was significant higher in the EVR treatment arm in the per protocol population at 24 months follow-up. Interestingly, renal function decreased in both groups of approximately 50% within 6 months post-transplant with comparable eGFR after 24 months between the study groups (EVR-group: baseline eGFR 103 mL/min and 52 mL/min after 24 months versus baseline eGFR 96mL/min and 56 mL/min after 24 months in the MMF-group). Incidences of BPAR were also lower in the EVR group versus the MMF-group, demonstrating reduction of CsA exposure in the EVR arm did not lead to a higher incidence rejection.

The randomized, multicenter 4EVERLUNG study in Germany explored a quadruple drug regimen in patients with mild-to-moderate renal insufficiency (eGFR of 50–90 mL/min/1.73 mL2). Patients in the study group (n=67) received EVR (C0 3-5 ng/mL) with reduced CNI (TAC 3-5 ng/mL or CsA 25-75 ng/mL), and a fixed dose of either MPA or AZA plus prednisone. In the control group (n=63) patients received either CNI (TAC C0 >5 ng/mL, or CsA >100 ng/mL) and a fixed dose of either MPA or AZA plus prednisone 3 to 18 months after lung transplantation [230]. The eGFR of patients in the quadruple regimen was significantly higher than the eGFR in the control group (64.5 mL/min versus 55 mL/min/1.73 mL2, respectively) after 12 months follow-up. No differences were observed between the study groups regarding acute rejection and CLAD as well as safety parameters.

6.4.1 Recommendations: EVR in the early phase after lung transplantation

- EVR should be initiation until bronchial suture healing (endoscopic confirmation) or until at least 3 months after transplantation.
- When EVR is administered in combination with CNI, target EVR C0 levels should be 3–8 ng/mL in a triple regimen, and 3-5 ng/mL in a quadruple reduced CNI regimen to lower the risk of infections.
- There are insufficient clinical data to enable recommendations for appropriate therapeutic concentrations of EVR in CNI-free immunosuppressive regimens in lung transplant recipients.
- It seems to be favorable to initiate EVR early in the development of chronic kidney disease (mild to moderate impairment), preferably in the first year.
- However, there is no clearly defined stage of CKD for mTOR inhibitor indication, nor does severe CKD preclude the improvement of kidney function under mTOR inhibitors.
- Baseline proteinuria may be a negative predictor of favorable kidney response after the introduction of EVR.
- ~~A favorable effect on early initiation of EVR post-transplant on the incidence BOS in the long-term needs to be further explored.~~

6.4.2 EVR with CNI late after lung transplantation

In the NOCTET study (s. above) 92 lung transplants maintenance recipients were randomized to continue their current CNI-based immunosuppression or to a quadruple EVR regimen. Mean change in mGFR from baseline to month 12 and to month 24 was significantly in favor of the quadruple EVR regimen compared with the standard control regimen. In the quadruple regimen the between-group difference in the change of mGFR from month 0 to 12 was 2.3 mL/min (P=0.07) and to month 24 significant with a mean difference of 6.0 mL/min (P=0.02) [212]. However, after 60 months the benefit on renal function (mGFR) in EVR treated recipients vanished due to higher CNI levels in the EVR group compared to the period until 24 months. No difference in the efficacy was observed between the groups with a significant decrease in forced vitality capacity from randomization to last follow-up. Over the 60 months study period one or more adverse events occurred in the EVR compared to the control group. Pneumonia occurred more frequently as an adverse event (18.3% vs. 6.4%) and as a serious adverse event (9.8% vs. 3.2%) in the EVR group than in the control group [231].

~~A sub-analysis of lung transplant recipients with preexisting moderate or severe renal failure (mGFR 30–59 or 20–29 mL/min/1.73m2, respectively) in the NOCTET study showed, similar to heart transplant recipients, that in the EVR quadruple group renal function significantly improved. However, this beneficial effect was limited to patients undergoing conversion within 5 years of transplantation [213].~~

Commenté [LF17]: Already said

A similar benefit on renal function of EVR treated recipients with severe impaired renal function (eGFR of ≤ 29 mL/min/1.73m2) was ~~observed~~ replicated in a retrospective, observational study patients [232].

~~Two small retrospective studies characterized effects of EVR containing immunosuppressive regimens after lung transplantation in Chile. The creatinine level increased in the first 3 months after the addition of EVR and reduction of tacrolimus dosing before spontaneous return to baseline in the initial study [233]. In the follow-up period (mean 25 months), renal function remained stable in patients treated with EVR plus low-dose CNIs [234].~~

Commenté [LF18]: Old, small, retrospective studies.

~~In the EVERODATA registry study in Spain 65 maintenance patients (mean time post-transplant time of 10.2 ± 7.9 months) were converted to EVR because of mostly bronchiolitis obliterans syndrome (BOS) or renal insufficiency. The data confirmed a stabilization of the kidney function (eGFR) without significant improvement 12 month after conversion to a EVR based regimen with reduced CNI or without CNI [235].~~

In the 4EVERLUNG five years follow-up, however, no differences were observed on renal function between the study groups (123 randomized patients) [235]: ITT: eGFR 56 [48–73] versus 58 [48–69] mL/min; P=0.951; and PP: eGFR 59 [range: 50–73] versus 59 [range: 48–69] mL/min; P=0.946). In the quadruple EVR-group more patients were switched from their immunosuppressive regimen due to a significantly higher incidence of de novo donor specific antibodies and thromboembolic events (ITT:

11% versus 24%, P=0.048; PP: 11% versus 22%, P=0.162) compared with the triple control group. There was a trend for a higher CLAD–free survival for the quadruple EVR regimen in the per protocol population (P=0.082).

The less effective renal sparing regimens using EVR in lung transplantation compared to the ones using EVR in heart transplantation may be explained by the fact that the CsA C0 level in the reduced regimen is in general higher as the reduced CsA C0 levels studied in heart transplant recipients. Thus, confirming that CNI reduction is the main driver to spare the nephron. Of note, the lung is the more immunocompetent organ compared to heart, and, therefore requiring a higher immunosuppression including CNI exposure.

6.4.3 Recommendations: EVR in the late phase after Lung Transplantation

- Current data support the introduction of EVR in maintenance patients in quadruple immunosuppressive regimen or with reduces CNI exposure to improve renal function, especially in patients with severe GFR (<29 mL/min/1.73m2). The time post-transplant to EVR initiation should be less than 5 years after transplantation.
- ~~Pneumonia is a common adverse event of long-term EVR therapy.~~
- ~~Patients with EVR combined with other cell inhibitors seem to have a higher risk of thromboembolic events.~~
- At present, there are no conclusive data of a benefit of a long time EVR therapy to reduce the progression of BOS.

6.4 Pancreatic or Islet Transplantation

Commenté [LF19]: I do think this part is highly relevant. However, what I do miss is drug exposure and TDM data. We are supposed to end up with recommendations on these aspects.

Approximately 80% of pancreatic transplants are classified as simultaneous pancreatic-kidney transplants for patients with end-stage renal failure associated with insulin secretion deficiency. Additionally, approximately 15% of pancreatic transplant recipients receive pancreatic transplantation after renal transplantation; therefore, approximately 95% of pancreatic transplant recipients have a history of severe diabetes with renal dysfunction [236]. Considering their background, pancreatic transplant recipients should be treated as compromised hosts who were at high risk for post-transplant infection [237-240].

In this context, the purpose of everolimus for pancreatic transplantation is as follows.

1. As a first-line immunosuppressive agent with a CNI or MMF-free protocol.

2. As a second-line agent in cases treated with low-dose CNI, considering improving transplanted renal function, as an alternative to MMF to control infection, and to complement the reduction or discontinuation of medications to treat the side effects of CNI or MMF.

~~However, in a recent comprehensive review, evidence from various *in vivo* and *in vitro* studies has shown that mTOR inhibitors, including everolimus and sirolimus, impair the β-cell function, induce β-cell apoptosis, and suppress β-cell proliferation [241]. Although these basic scientific findings have been presented, in the clinical setting, an analysis of pancreatic transplants in the UNOS database from 1987 to 2016 showed that the use of m-TOR inhibitors was associated with a 7% reduction in the risk of allograft failure and significantly higher patient survival up to 10 years after transplantation compared to immunosuppression without the use of m-TOR inhibitors [242].~~

The First World Consensus Conference on Pancreas Transplantation was convened to complete the comprehensive evidence-based guidelines for the practice of pancreas transplantation in 2019, which collected expert opinion on pancreas transplantation [243]. According to these guidelines, data on the benefits (as immunosuppressants) and side effects of m-TOR inhibitors, including everolimus, compared to CNI or MMF are scarce; therefore, further research is required on the role of m-TOR inhibitors in pancreatic transplantation [244].

In a randomized trial that compared the effects of immunosuppression with sirolimus, another m-TOR inhibitor, and tacrolimus [245], despite the non-inferiority of sirolimus compared to tacrolimus with respect to graft survival, sirolimus treatment was associated with a high discontinuation rate in simultaneous pancreatic and kidney transplantation due to an intolerable adverse event. However, in a single-center study with an observation period of more than 10 years, Ciancio et al. [246] reported that sirolimus in combination with tacrolimus was better tolerated and more effective than MMF.

In the absence of data on the first-line use of everolimus in pancreatic transplantation, Sageshima et al. [247] conducted a single-center study comparing everolimus with a low-dose tacrolimus protocol and the use of enteric-coated mycophenolate sodium in pancreas transplantation. They reported that no rejection was observed in patients with EVR and that there were no significant differences between the groups in serum creatinine, HbA1c, surgical complications, or medical complications. ~~However, it is recommended that m-TOR inhibitors should not be used in the first few months after transplantation, due to the higher incidence of surgical complications, including delayed wound healing, lymphocele, and incisional hernia [244].~~

Commenté [LF20]: Well known.

There are several reports [248-253] of trials of early conversion of everolimus after kidney transplantation. These studies have reported that everolimus and low-dose CNI are associated with improved post-transplant renal function. Consequently, the use of everolimus as a second-line agent should bring the same benefit in pancreas transplant recipients because > 90% of pancreas transplant recipients have a kidney graft. Marcella-Neto et al. [254] found that, among 535 pancreatic transplant recipients, 13 patients had complications associated with CNI or MMF that forced them to switch to m-TOR inhibitors, although the pancreatic graft was lost after conversion in approximately 20% of the patients, the kidney and pancreas function of the graft were well maintained in the remaining patients. Aida et al. (reference) reported that in cases in which the dose of MMF was reduced or stopped due to MMF complications and complementary administration of EVR was

performed, DSA production was suppressed, and the endocrine function of the pancreatic graft was not affected.

On the other hand, in islet transplantation, mTOR inhibitors have long been the mainstay of immunosuppressants due to the short-term success of the Edmonton protocol [255]; however, sirolimus was often selected. However, due to the high incidence of side effects of high-dose sirolimus and the consideration of impairment of β-cell regeneration, the use of mTOR inhibitors in islet transplantation decreased from 83.6% in 1999 to 2002 to 46.8% in 2011–2014 21.

The results of the pancreatic islet transplantation clinical trials conducted by the GRAGIL network in Switzerland and France showed that sirolimus-based immunosuppressive regimens improve graft function, despite the lack of sustained insulin independence in most patients [256]. The Australian Islet Transplant Consortium chose to switch the immunosuppressive protocol from TAC/MMF-based immunosuppression to sirolimus 6 months after islet transplantation; however, half of the patients did not switch. Maffi et al. [257] reported a single center study that showed that CNI was required for at least the first few months after islet transplantation to prevent graft loss due to allorejection. Multivariate analysis of the Collaborative Islet Transplant Registry revealed that mTOR inhibitors were a favorable factor for the achievement of insulin independence after the first islet infusion, but not for the achievement of insulin independence or retention of graft function after the last islet infusion [258]. All islet transplantation data was based on sirolimus not everolimus; however, these clinical data led to a decrease in the use of mTOR inhibitors, including sirolimus and everolimus.

Thus, in pancreatic/islet transplantation, since the late 1990s, mTOR inhibitors - including everolimus- have transitioned from first line immunosuppressants to second line agents, which are now mainly used for patients who have CNI or MMF intolerance, CNI-related nephrotoxicity, or malignancy.

6.5 Oncology

Within the solid organ transplant field, everolimus is considered a narrow therapeutic index drug, for which TDM is routinely applied. In oncology the situation is quite different. Standard dosing, aiming at the maximum tolerated dose within the population is the norm. TDM of everolimus has therefore not been taken up in patient management for most oncologic indications. Everolimus is currently applied in metastatic renal cell carcinoma, with or without lenvatinib, metastatic neuroendocrine tumors, advanced hormone sensitive HER-2 negative breast cancer, subependymal giant cell astrocytoma and renal angiomyolipoma associated with tuberous sclerosis complex [259]. For the last two a dose titration to attain a trough concentration of 5-15 ng/ml is recommended [12].

Dose interruptions and dose reductions due to severe adverse events however occur in up to 60% of patients when standard dosing is applied. Since toxicity is related to high everolimus blood concentrations this offers the opportunity to implement dose reductions based on drug exposure,

prior to the occurrence of toxicity. An example are the pulmonary adverse events related to everolimus, that have clearly been shown to be related to higher everolimus concentrations. [260]. ~~Potentially also~~ Efficacy might also be improved if patients with low exposure have higher rates of progression. In the previous consensus paper we concluded that: "Further studies are required to determine the clinical utility of TDM in non-transplantation settings." [4]. We here present an update.

Oncologic PK and TDM data on everolimus are largely from studies in metastatic renal cell carcinoma (mRCC) and breast cancer. In a series of 40 patients with mRCC everolimus exposure (AUC 0-24h) was measured within the first two weeks of treatment [137]. It was shown that patients who required a dose reduction (n = 18) due to toxicity at any time during treatment had significantly higher everolimus exposures [mean AUC = 600 vs. 395 ng*h/mL (p = 0.008)] than patients without a dose reduction (n = 22). This study confirmed the data obtained in an earlier pooled analysis of data from five phase 2/3 studies, in which higher everolimus exposure was linked with the incidence of grade 3 or 4 pneumonitis, stomatitis and metabolic events [133]. The latter study also showed that the likelihood of tumor size reduction increased with increasing everolimus trough concentrations. Moreover, the retrospective analysis based on data from large randomized controlled trials showed superior median progression free survival in the C-through window of 10 – 30 ng/mL compared to <10 ng/mL and >30 ng/mL for a number of different populations treated for tumor types including carcinoid, non-small cell lung cancer, renal cell carcinoma and pancreatic neuroendocrine tumor [133].

More support for a concentration – effect relationship comes from a study that reported a 4-fold increased risk of toxicity (HR = 4.12, IC95% = [1.48–11.5], p = 0.0067) if everolimus trough concentrations were above 26.3 ng/mL, whereas troughs below 11.9 ng/mL were associated with a 3-fold increased risk of progression (HR = 3.2, IC95% = [1.33–7.81],p = 0.001) in patients with breast, renal and neuroendocrine tumors [131]. Fukudo et al showed in their study, in 19 metastatic breast cancer patients, that the median PFS was numerically longer in patients who maintained a steady state C-trough below the threshold of 17.3 ng/ml than in those who did not (327 days [95 % CI 103–355 days] vs. 194 days [95 % CI 45 days–not estimable]; P = 0.35)estimable]; P = 0.35) [261]. In this study the cumulative incidence of dose limiting toxicity was significantly higher in patients with C-trough ≥17.3 ng/ml than in other patients (sub-hazard ratio 4.87, 95 % confidence interval [CI] 1.53-15.5; P = 0.007) [261].

A strong impact of drug exposure was also observed in a study in patients with mRCC, that compared patients with everolimus exposure either below or above the median [132]. The median everolimus trough concentration of 42 patients was 14.1 ng/mL. Fourteen patients (67 %) with everolimus concentration above the median were free from progression at 6 months, compared to 8 (38 %) patients with everolimus concentration below 14.1 ng/mL (p = 0.06).

Several studies reported associations between drug exposure and toxicity, without an effect on efficacy. In a small Japanese study, the median everolimus concentration at day 8 was substantially higher in patients in whom everolimus had to be discontinued or reduced compared to patients in

whom everolimus dose could be maintained (median, 18.0 vs 8.2 ng/mL; P = 0.0139) [262]. Among 44 breast cancer patients, the geometric mean (GM) everolimus trough concentration was higher in patients with toxicity compared to patients without (17.4 versus 12.3 ng/mL (p = 0.02) [134]. The optimal cut-off value to predict toxicity was a trough of 19.2 ng/mL. In this study the trough concentration in patients with and without progressive disease within 3 months was not significantly different (12.0 versus 15.2 ng/mL (p = 0.118)). A post-hoc analysis of the phase 3 EVEREST study also identified significant associations between everolimus exposure and probability of toxicity [263].

Given the narrow therapeutic index, highly variable inter-individual pharmacokinetics and positive exposure-efficacy relationship there is a strong rationale for therapeutic drug monitoring (TDM) of everolimus in oncology. ~~However, the oncology community will only accept and implement TDM dosing strategies if the evidence shows improved clinical outcome with TDM, and if the TDM method itself does not obstruct the daily workflow. Therefore, repetitive steady-state trough measurements or single sample measurements analyzed in a population pharmacokinetic model form a more attractive approach than AUC based dosing. Furthermore, the trough versus AUC correlation of everolimus is relatively good [20].~~ Based on the data presented above a therapeutic window for the trough concentration between 12 and 20 ng/ml could be defined. There may however be a difference in optimal target range between everolimus monotherapy regimens and combined treatment regimens [135].

Ideally randomized trials comparing standard dosing with TDM based dosing should be performed to demonstrate a reduced incidence of toxicity. Feasibility will be a challenge since pharma companies currently do not want to invest in such trials. In view of the high frequency of toxicity-based dose reductions the benefit of TDM can be shown in relatively small study populations (less than 100 patients). For improvements in efficacy, it is likely that sample size will need to be higher, but not more than 300-400 patients. Novel microsampling techniques, such as dried blood spot sampling or volumetric absorptive microsampling, can facilitate blood collection at multiple time points and allow for availability of TDM results prior to the visit of the patient to the outpatient clinic [264]. We encourage oncologists to embark on this type of studies to improve patient outcomes.

6.6 Pharmacogenetics

Commenté [LF21]: I will put this part before the TDM

~~Pharmacogenomics aims to personalize drug treatment by selecting drugs and drug regimens based on genetic variations identified in patients. Several international groups, including the Dutch Pharmacogenetics Working Group (DPWG)[265] and the Clinical Pharmacogenetics Implementation Consortium (CPIC)[266], have systematically reviewed over a 100 gene–drug interactions resulting in over 50 guidelines providing therapeutic recommendations based on pharmacogenomic information. Concerning immunosuppressive drugs, the available guidelines thus far only provide limited recommendations concerning the starting dose of tacrolimus in patients based on the expected expression of the cytochrome P450 (CYP) enzyme 3A5 [267]. Here, we summarize the available information for EVR.~~

EVR is metabolized primarily by CYP3A4 with minor contributions from CYP3A5 and CYP2C8. Metabolism includes demethylation, hydroxylation and ring degradation [12,268,269]. There is also evidence that EVR is a substrate for ABCB1 (P-glycoprotein, encoded by the ABCB1 gene) [270-272].

6.6.1 CYP3A4

There is little information regarding the effect of CYP3A4 genetic variants on EVR PK. Two single nucleotide polymorphisms (SNP), the CYP3A4*1.001 allele (rs2740574; c.-392G>A,(previously called CYP3A4*1B [273]) and CYP3A4*22 (rs35599367; c.522-191C>T) have been evaluated so far.

The contribution of the CYP3A4*1.001 allelic variant to EVR PK variabilities is controversial. *In vitro* studies suggested that it is associated with increased CYP3A4 transcriptional activity [274,275] while other do not [276]. It was first hypothesized the discrepancy was due to differences expression system used in the studies [274 276]. It has also been suggested that the CYP3A4 and CYP3A5 haplotypes are closely linked and some effects originally thought to be due to a CYP3A4 allele are probably actually due to a CYP3A5 allele in linkage disequilibrium [277,278]. In lung transplant recipients, no association with EVR dose was found for CYP3A4*1.001 [279], suggesting that if any differences in CYP3A4 transcription do exist the clinical effect is minimal.

In contrast, the CYP3A4*22 variant allele has been associated with decreased hepatic activity *in vitro* [280] and *in vivo*, as assessed with CYP3A phenotyping probes in cancer patients [281]. In a study evaluating the effect of this SNP in patients, of the 97 EVR treated renal transplant patients included in the study, 8 were heterozygous and 1 homozygous, which is in accordance with the reported minor allele frequency (~5%) [142]. However, no significant influence on EVR PK was observed in relation to the variant, possibly due to the relatively small numbers of patients in the carriers of the defective allele in that study [142].

6.6.2 CYP3A5

The CYP3A5*3 (rs776746; c.219-237G>A) allele results in a splicing defect, leading to the production of a truncated protein with no enzyme activity. Several studies have shown no association between the common CYP3A5*3 allele and EVR blood concentrations, dose requirement or PK parameters estimated using population PK approaches [141,145,269,279,142 282 283 284]. Which given that it plays little role in the overall metabolism [12,268,269] of EVR this may not be a surprising finding.

One study described a possible effect between the CYP3A4*1.001–CYP3A5*3 haplotype and EVR and SRL related adverse effects [282]. In 184 kidney transplant recipients receiving either EVR or SRL, there was a significantly higher frequency of the CYP3A4*1–CYP3A5*1 (AA-GA) haplotype 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 EVR or SRL C0/dose/kg ratios between the haplotype groups. However, a confounding factor was that the patients were receiving CNIs, either de novo (44.5%) or as rescue therapy (55.5%).

6.6.3 CYP2C8

The CYP2C8*3 variant allele is denoted by two highly linked variants: rs11572080 (c.416G>A; p.R139K) and rs10509681 (c.1196A>G; p.K399R). Other variants are CYP2C8*2 (rs11572103: c.805A>T; p.Ile199Phe) and CYP2C8*4 (rs1058930: c.792C>G; p.Ile264Met)[279]. The effect of these alleles have been studied in a range of transplant types [141,279,284]. None of these studies, however, found a significant relationship between EVR dosing or blood concentrations and CYP2C8 genotype, which is consistent with its minor role in EVR metabolism [269]

6.6.4 ABCB1

EVR is extensively eliminated in the bile [20] and therefore canalicular excretion of the drug or its metabolites involving ABCB1 is likely important. There are several well described ABCB1 variants. Among the most studied ABCB1 SNPs are three SNP in strong linkage disequilibrium [(rs1128503 c.1236C>T, pGly412), (rs2032582, c.2677G>T/A, p. Ser893Ala/Thr), (rs1045642, c.3435C>T, p. Ile1145Ile)[285] but the results reported so far are inconsistent and, overall, the real functional impact of these SNPs remains controversial [286]. Other less frequent SNPs have been described for ABCB1 (rs3213619 c.-129T>C) and (rs2229109 c.1199G>A, Ser400Asn) [285] but results regard to their functionality are no more conclusive.

In a study including kidney transplant recipients switched from a triple therapy (CsA, MMF, prednisolone) to a CNI-free dual therapy including EVR twice daily and prednisolone, no influence of ABCB1 c.1236C>T, 2677G>A/T, 3435C>T or c.-129T>C was found for EVR apparent clearance (CL/F), Vd or first-order absorption rate constant using a population pharmacokinetics approach [141]. Similarly, no effect of the ABCB1 SNP c.3435C>Ton EVR PK was seen using a similar study approach in heart transplant recipients [145], and no effect was seen on the ABCB1 haplotype c.1236C>T -, 2677G>A/T -, 3435C>T on EVR steady-state dose-normalized C0 in lung transplant recipients [279]. However, it is now generally accepted that drug transporter activity has more influence on the local drug distribution than on the systemic exposure of their substrates. Unfortunately, information is lacking regarding the impact of ABCB1 polymorphisms on EVR tissue distribution, and more specifically on the uptake of the drug by T-cells.

6.6.5 PG-PD relationships

SNPs in the genes encoding mTOR (especially in the FRB domain) or the proteins of the EVR signaling pathway (FK-BP12, p70S6K, raptor) might theoretically confer a resistance phenotype to the drug, as demonstrated in vitro in mammalian cell lines [287]. However, to our knowledge, no significant associations between any genetic variants in PD genes and EVR effects has been reported [288]

6.6.6 Recommendations

- There is insufficient evidence to recommend the prospective genotyping of *CYP3A5* and *CYP3A4* in solid-organ transplant recipients for a priori dose adjustment of EVR.
- There may be evidence to consider evaluation of combined *CYP3A5*3* and *CYP3A4*22* genotyping to identify patients with high or low CYP3A total activity for the retrospective documentation of cases with unexpected EVR blood concentrations or adverse effects, combined with comprehensive exploration of potential drug–drug or food interactions.
- There is insufficient evidence to recommend genotyping for *ABCB1*, mTOR or any genes implicated in the immune signaling pathway targeted by EVR.
-

6.7 Pediatrics

Everolimus PK has been evaluated in several pediatric populations including neurofibromatosis type 1, tuberous sclerosis complex, solid tumors, and kidney and liver transplant recipients [289-293]. All of these pediatric studies showed large between patient variability in pre-dose trough concentration and exposure with substantial numbers of patients below or above the target range, even with relatively wide ranges of 5-15 ng/mL used in some studies. Earlier trials in patients with TSC (EXIST-3 trial) had studied once daily oral everolimus titrated to achieve a target trough level of 3–7 ng/mL (low target) or 9–15 ng/mL (high target) [294]. Recent efforts by the originator company have focused on a modeling and simulation approach that incorporated population PK, pharmacodynamics (PD), and physiologically-based pharmacokinetics (PBPK) modeling to predict everolimus exposure in infants and children. TSC-associated seizures affect newborns and infants, so a pediatric everolimus PBPK model was developed to predict a clinically relevant reduction in the disease symptoms including seizures in a population of very young infants [295]. As everolimus is both a CYP3A4 and P-gp substrate the model included a combination of changes resulting from both physiology and maturation (ontogeny factors) as a function of the child's age. Importantly, infants with TSC are at very high risk for developing epilepsy with a poor prognosis despite existing treatments. Clinical trials are ongoing to evaluate whether early intervention by initiating preventative therapy with sirolimus or everolimus using a MIPD approach would alter the natural course of TSC before seizures begin (ClinicalTrials.gov Identifier: NCT04595513 [296]).

Commenté [LF22]: Define

7. PHARMACODYNAMICS

Commenté [LLJP23]: This is a very long section and MUST be cut down

It takes up 11 pages and the conclusions are that is not yet useful.

7.1 Pharmacodynamic (PD) monitoring

Immunosuppression can be achieved by depleting lymphocytes, diverting lymphocyte traffic, or blocking response pathways. Everolimus is an immunosuppressive drug that is intended to reduce lymphocyte proliferation~~, whereas CN inhibits cell activation~~. The activation of mTORC1 promotes cell growth by activating the synthesis of proteins, lipids, and nucleotides and the biogenesis of miRNAs. The mTORC2 complex regulates cell survival and metabolism. Sirolimus mainly inhibits the mTORC1 complex, and in the case of chronic exposure, it can also inhibit mTORC2 in certain cell types [297]. As a result, treatment with sirolimus or everolimus decreases translational activity, cell cycle progression, and cell proliferation (see Chapter 3, mechanism of action).

As already stated in the first Consensus Report, 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. As of 2016 only limited data were available regarding PD monitoring of everolimus in transplantation medicine and oncology. At that time, there was no evidence of an association between everolimus PD markers and clinical outcome. We performed a literature search covering the time period between January 2015 and August 2022 to update the information available.

7.2 Non specific PD Monitoring of Everolimus

Approaches used for non specific PD monitoring of everolimus include cell proliferation assays with lymphocytes or tumor cells; cytokine production in lymphocytes and T-cells; intracellular production of ATP in CD4+ T-cells; cell-free DNA, surface activation markers on T-cells, miRNA expression and changes in the proportions of lymphocyte subsets [4,298].

7.2.1 Solid Organ Transplantation

7.2.1.1 Inhibition of cell proliferation

As already mentioned in the first consensus document cell proliferation inhibition of everolimus has mainly been monitored using the thiazolyl blue tetrazolium bromide (MTT) assay. The tetrazolium salt used in the MTT assay is reduced to formazan by metabolic active cells. Thus, the MTT assay can be used to measure the viability or proliferation of cells after treatment with chemical substances such as drugs. When using the MTT assay to measure the effects of everolimus treatment, anti-CD3 antibodies are used to stimulate the T-cell receptor. Thus, it is possible to identify differences in T-cell inhibition due to everolimus treatment. The dose-dependent inhibition of T-cell proliferation by everolimus in isolated peripheral blood mononuclear cells (PBMCs) was reported for blood obtained from kidney allograft recipients [299]. The *ex vivo* proliferation of PBMCs in response to T-cell receptor stimulation was inhibited for up to 10 hours after a single everolimus dose of 0.75-1.5 mg.

In a B-cell immunity assay, the effects of everolimus on proliferation and differentiation of peripheral B cells into plasma cells were compared to mycophenolic acid (MPA) and prednisolone at three different time points. Eeverolimus showed a prolonged anti-proliferative effect compared to MPA but not as long as high concentrations of steroids. These findings may be helpful to identify the optimal drug combination therapy after organ transplantation [300].

In the context of clarifying the role of everolimus in the development of tubulitis, a sign of rejection after kidney transplantation, an *in vitro* assay was developed by Demmers et al. involving recipient's PBMCs and donor-derived renal tubular epithelial cells [301]. Both cell types were co-cultured for 7 days before measuring the proliferation of CD4+ T-cells via [3H]thymidine incorporation. Although, clinically relevant concentrations of everolimus inhibited overall CD4+ T-cell proliferation, there was no effect on the expression of the CD4+/CD28null memory T-cell subset, which was different compared to the effects of MPA and steroids [301].

A case series observed everolimus effects in kidney transplant recipients with belatacept-resistant rejection. CD8+ effector memory T-cells (TEM) with a CD28low/HLA-DRhigh/CD38high/CD45RO+ phenotype were characterized by flow cytometry. As far as these TEM cells activate the mTOR pathway, everolimus treatment led to a decrease of *in vivo* proliferation of TEM cells and their ex vivo alloreactivity. Consequently, all rejections resolved [302].

The optimal time point of CNI withdrawal to preserve the kidney function without increasing the incidence of rejection after organ transplantation is a major concern. In PBMCs, the proliferation of CD3+ T-cells was increased in everolimus-treated liver transplant recipients after CNI withdrawal. Such proliferation of PD markers may be helpful to effectively guide CNI withdrawal ([303]. A flow-cytometric quantification of T-cell subsets, as it was performed by Mathew and colleagues [303], would be helpful to measure the inhibition of T-cell proliferation. A special feature was the absolute quantification of cell numbers per µL of blood by flow cytometry in that study. Usually, relative numbers are calculated in flow cytometric analyses.

7.2.1.2 T cell subpopulations

Newer data have emerged for nonspecific PD monitoring of everolimus using T-cell populations. Despite being a T-cell proliferation inhibitor, everolimus treatment has shown preferential expansion of the regulatory T-cells (Tregs). These cells constitute about 1-5% of the total circulating T-cells and are recognized by their CD4+CD25hiFoxP3+ status. It has been increasingly understood that they are capable of inducing operational tolerance by inhibiting the effector immune cells that trigger the graft rejection. Hence, increase in the Tregs is considered therapeutically advantageous in organ transplantation. While the other ISDs like CNIs have shown to decrease the Tregs in-vitro as well as in-vitro studies, Everolimus has shown to increase these cells [304-306]. This potentially provides the dual advantage of reduced CNI toxicity due to their dose reduction in combination therapy and reducing the requirement of immunosuppression by inducing tolerance. Exploiting this concept, everolimus has also been used for ex-vivo expansion of Tregs and re-infusing them to patients as adoptive immunosuppressive therapy.

The mTOR pathway is critical for lymphocyte differentiation and polarization, and mTOR-deficient CD4+ T-cell fail to differentiate into Th1 and Th17 subsets under activating condition and develop into FoxP3+ regulatory cells [307]. By blocking mTOR activity, sirolimus and everolimus inhibits effector CD4+ cell differentiation, promotes the generation of Th2 CD4+ T cells, inhibits formation of resident memory cells in intestinal mucosa, increase the generation of Treg cells, and enhances quality and quantity of CD8+ memory cells, and all of these phenotypic changes participate in the immunosuppressive effects of mTOR inhibitors [308].

Klaeske et al. established an immunological monitoring in long-term heart transplant recipients, who either were treated with a CNI-based or a everolimus-based, CNI-free regimen. Peripheral blood

samples were analyzed by flow cytometry for subsets of dendritic cells and Tregs. A principal component analysis showed that distinct subsets of dendritic cells as well as total Tregs could distinguish between non-rejectors and rejectors in everolimus-treated patients [309]. This is the first report that calculated an algorithm of different markers to assess the effects of immunosuppressive drugs such as everolimus in transplant recipients.

The mechanisms leading to upregulation of Tregs is rather well studied in the context of sirolimus. The differential effect on the T-regs subpopulation compared to the other T-cells is presumably because of mTOR inhibitors in the later phase of post-activation signaling in the T-cells (PI3K/mTOR and STAT5 pathways) which are less functional in Tregs, compared to others. It has been found in-vitro studies that everolimus is more potent than sirolimus for their effect on the T-reg generation and expansion [310]. However, another in-vitro study by Gerady et al. found that this capability is similar in the mTOR inhibitors, even their immunosuppressive effects were also similar [311]. Nevertheless, better bioavailability and adverse event profile make everolimus the preferred agent of choice. An everolimus based therapeutic regimen was associated with an increased frequency of Tregs in peripheral blood as determined by flow-cytometry immunophenotyping of Tregs (CD4+ CD25+ CD127− FoxP3+) and functional assays to prove their immunosuppressive capacity [312]. Treg function was assessed using TCR-activated total peripheral blood mononuclear cells (PBMNCs) from patients at a PBMNC to Treg ratio of 2:1. CD4+CD25+ T cells from patients were isolated from PBMNC and co-cultured for 48h with PBMNC activated with anti-CD3 and anti-CD28 antibodies. 3H-Thymidine was used to measure proliferation inhibition.

The effects of everolimus on immune response and particularly on T-cell subsets have been recently evaluated by Diaz-Molina et al. in heart transplant recipients after everolimus conversion from MMF. Their results showed an increase in cytotoxic CD8+ T-cells, allowing a greater number of IFN-γ producing cells under activation and a reduction in the expression of inhibitory receptors in NK-cells, enhancing its lytic capacity [313]. It is well known that CD8+T-cells and NK cells play a determinant role in long-term rejection and donor specific antibody mediated rejection [314], respectively. In addition, a significant increase in the percentage of Treg cells during the first month after everolimus conversion was observed.

The therapy with mTOR inhibitors is associated with a reduction in the incidence of CMV infection in transplant recipients who are CMV positive (R+). Kaminski H et al. [64] suggests that a multimodal functional action on both αβ and γδ effector T cells is involved. CMV-specific αβ and γδ effector T-cells were measured by flow cytometry. A higher proportion of T-cells with a dysfunctional phenotype in patients who are R+ were unable to control CMV infection without antiviral treatment. mTOR is improved the functional phenotype (decreased inhibitory receptors, increased functionality) of effector T-cells, together with a lower incidence and severity of CMV infection after transplantation. In vitro, a low dose of mTORi induced a long-lived cell profile, which was associated with increased proliferation, viability and IFN-g production in both αβ and γδ effector T cells. These characteristics were previously associated with improved functionality of effector T-cells [315]. Furthermore, mTORi-treated T-cells improved TCR signaling. This is of particular interest because

late effector memory cells are mostly dependent on TCR signaling, rather than cytokine signals, to persist during chronic infection [316,317]. Altogether, these findings have highlighted the involvement of dysfunctional T-cells in CMV infection risk after transplantation, and the effect of mTOR is on the restoration of CMV-specific late effector T-cells.

7.2.1.3 Cytokines

There are few studies that evaluate the effect of everolimus on the production of cytokines and chemokines. It is well known that everolimus does not block the synthesis of IL-2. Results from Iwasaki K et al. [318] showed that after stimulation of PBMC from healthy controls with anti-CD3/28 microbeads everolimus showed a moderate inhibitory effect on IL-10, IL-21 and IFN-γ production. In combination experiments with everolimus and Coni, everolimus was believed to exert an addictive effect at low CNi concentrations, which clinically suggested a supplementary effect on insufficient cytokine suppression caused by low dose CNi. It was reported that mTOR could regulate AO-1 activity, thus only under the condition of insufficient calcineurin repression due to low concentrations of CNi, mTOR inhibition might exhibit an additional effect on AP-1-NFAT cooperation in the promoter region of cytokine genes.

7.2.1.4 miRNAs

An emerging non-specific biomarker for PD monitoring is miRNAs expression in blood and tissues. Numerous studies indicate that mTOR and its signaling pathway is regulated by miRNAs. mTOR inhibitors may have homogenous effects on the mTOR pathway while acknowledging a link between particular miRNA and mTOR signaling. Taken together, miRX-136-5p and miR-150-5p seem to be connected to the mTOR pathway. The expression miR-150-5p seems to be a potential biomarker in the field of transplantation. miR-150 is an essential miRNA in immune regulatory functions, such as proliferation, activation, and apoptosis of B and T lymphocytes, and is selectively expressed in lymph nodes, spleen, and mature B T-cells; it is involved in innate and adaptive immune responses [319]. This miRNA is involved in Tregs cell differentiation through the regulation of mTOR expression [320] and also regulates NF-kB activation in T lymphocytes. In this sense, miR-150 has been proposed as a marker of lymphocyte activation [321]. The expression of miR-150-5p was significantly increased in allograft biopsies in patients with rejection [322]. An elevation of plasma miR-150-5p was associated with graft rejection and destruction of β cells in mice [323]. Future studies will be necessary to understand the actual relevance of miR-150-5p in acute graft rejection and its potential as biomarkers of immunosuppressive treatment. ~~The hallmark method for miRNA analysis is currently quantitative reverse-transcription PCR. Depending on the biological matrix used, isolated RNA is reversed transcribed into cDNA. Kits from different vendors such as Qiagen (Hilden, Germany or Applied Biosystems™, Thermo Fisher Scientific (Waltham, Massachusetts, USA) are commercially available. However, next generation sequencing (NGS) also provides a powerful tool for the analysis of miRNA transcription profiles and their target genes in specific clinical events in both solid tissues and biofluids.~~

7.2.1.5 Donor-derived cell-free DNA (dd-cfDNA)

Donor-derived cell-free DNA (dd-cfDNA) is a noninvasive biomarker which has been shown to be useful for early detection or exclusion of allograft injury from multiple causes, including rejection, in all transplanted solid organs [298,324-326]. ~~The use of dd-cfDNA as a biomarker is based on the theory that organ transplants are also genome transplants. The ability to accurately assess the amount of graft derived DNA in the circulation of an organ recipient using digital PCR or next generation sequencing makes it possible to perform serial, minimally invasive monitoring for allograft injury [327]. Most published studies to date have involved transplant patients treated with tacrolimus alone or in combination with other ISDs.~~ A literature search failed to identify published studies that systematically investigated the links between everolimus exposure and dd-cfDNA. However, in a few reports that measured dd-cfDNA, everolimus was given in combination with other immunosuppressants. For example, the recipient of a marginal liver graft who was monitored using serial dd-cfDNA was switched from tacrolimus to everolimus [328]. At the time of the switch, there was a substantial under-immunosuppression resulting in an acute rejection that was associated with a major increase of dd-cfDNA. A subsequent positive clinical response to a steroid bolus was confirmed by a rapid decrease of dd-cfDNA and changes in dd-cfDNA were monitored as treatment with everolimus was optimized. In another report, a heart transplant patient treated with cyclosporin and everolimus undergoing an attempted immunosuppression minimization between days 280 and 366 a highly significant increase of dd-cfDNA was observed, suggesting under-immunosuppression. This resulted in a biopsy that demonstrated an acute rejection [329]. Results in this patient suggested that immunosuppression minimization could have been better guided by serial dd-cfDNA monitoring. The successful use of dd-cfDNA monitoring was also reported in a study of cardiac allograft rejection [330]. Everolimus was also used in some patients (12/189) in a prospective study on the use of absolute quantification of dd-cfDNA [328]. In conclusion, dd-cfDNA appears to have the potential, when combined with TDM, to better guide everolimus dose adjustments to achieve more effective, personalized immunosuppression. Assessment of its clinical utility in large, prospective outcome studies of patients treated with everolimus seems to be warranted.

~~Immune monitoring by measuring intracellular ATP using the Immuknow® assay (Cy-lex Inc, Columbia, MD) has not been pursued during the recent four years. No new data have been generated concerning surface marker expression on T cells as a PD tool to monitor everolimus therapy.~~

7.2.2 Oncology

7.2.2.1 Inhibition of cell proliferation

Isolated tumor cells of three patients with renal cell carcinoma (RCC) and the RCC cell line 786-O were incubated *ex vivo* with different concentrations of everolimus to assess the 50% inhibition of cell proliferation using the MTT assay. The half-maximal inhibitory concentration (IC50) of the RCC cell line 786-O for everolimus was 10 µM ± 1 µM. The ex vivo sensitivity of RCC cells to everolimus was equivalent with the clinical response [331]. Further, it was stated that ex vivo tests may be suitable to evaluate the best first-line treatment for patient-specific management of RCC. Proliferation

inhibition of cancer cell lines of RCC (786-O, ACHN), breast cancer (MDA-MB-231, MDA-MB-361) and lung cancer (PC-9, NCI-H1975) by everolimus were performed with the same assay [332].

7.2.2.2 Positron emission tomography

In the meantime everolimus is increasingly used in oncology and [18F]-fluorodeoxyglucose positron emission tomography (FDG-PET) has been introduced as a drug non specific pharmacodynamic marker [333,334]. The ability of FDG-PET to predict everolimus efficacy was observed *in vitro* and *in vivo* for lung neuroendocrine tumor (NETs) [335] as well as other non-neuroendocrine cancer models [336]. However, in a recent investigation with 66 patients suffering from neuroendocrine neoplasia and treated either with 5 or 10 mg everolimus no significant difference in clinical outcomes was observed between patients with positive or negative FDG-PET findings. The authors conclude that patients should not be exempted from everolimus therapy despite positive FDG-PET findings [337].

7.2.2.3 Gene and protein expression

The expression of different genes and proteins can be influenced by everolimus treatment. Studies in the field of transplantation or oncology demonstrated the effect of everolimus on gene and protein expression in vitro and in animal models. For example, in a cardiac allograft vasculopathy (CAV) model, the exposure with 1000 ng/mL everolimus induced human leukocyte (HLA)-G, which was measured by Western blot densitometric analysis [338]. The upregulation of HLA-G inhibits the proliferation of human coronary artery muscle cells and tumor necrosis factor α-stimulated neutrophil adhesion to endothelial cells. Both events are critical in the development of CAV.

In the field of oncology, a few studies reported the effects of everolimus on gene and protein expression. Eeverolimus is a treatment option for advanced neuroendocrine G1- and G2-graded tumors, which are subgroups of pancreatic NETs (pNET). Recently, immunohistochemical analyses revealed that angiotensin II type I receptor (AT1R), an independent prognosticator of esophageal squamous cell carcinoma, correlated with mTOR activation which promotes cell proliferation [339]. Further, the authors reported that everolimus was able to inhibit the proliferation of tumor cells *in vitro* and in a xenograft mouse model [339].

A study analyzed the effects of everolimus on the expression of peroxiredoxins, which are antioxidants and involved in oncogenic signaling pathways, by quantitative real-time PCR and immunohistochemistry [340]. They used the human QGP-1 pNET cell line, patient-derived pNET tissue and a xenograft mouse model and found that the expression of peroxiredoxin-2 could serve as a biomarker for the therapeutic response or resistance to everolimus in pNET.

Resistance to single-drug therapy in breast cancer is common, because different pathways including androgen receptor (AR) and the HER2/PI3K/mTOR are involved. Combination therapies, however, have the potential to overcome resistance, and, additionally, PD biomarkers may be helpful to optimize combination therapy. *In vitro* studies in the breast cancer cell lines BT474-HR20 and

MDAMB453 demonstrated an increase in AR protein and gene expression after addition of everolimus for 48 hours, which were synergized by adding AR antagonists. *In vivo* experiments in breast cancer xenografts confirmed the drug synergy [341].

Another study evaluated the expression of tumor suppressor candidate gene nitrogen permease regulator-like 2 (NPRL2), which is involved in mTOR signaling and drug resistance in several cancers. Using an in vitro model of castration-resistant prostate cancer, the NPRL2 expression was evaluated by immunohistochemistry, quantitative real time-PCR, and Western blot [342]. Results showed an increase of autophagy because of NPRL2-promoted everolimus resistance. This implies that NPRL2 could be a biomarker for predicting resistance to everolimus in castration-resistant prostate cancer.

The mentioned studies identified several potential biomarkers to monitor the effect of everolimus treatment in transplant recipients and oncologic patients. However, the described effects of everolimus on the expression of HLA-G, AT1R, peroxiredoxin-2, AR and NPRL2 can be affected by a larger spectrum of other factors and need to be carefully investigated for their predictive value.

It is known that nuclear expression of Y-box–binding protein (YBX1) is closely correlated with poor clinical outcomes and drug resistance in cancer [343]. Everolimus can suppress YBX1 activation by inhibiting its phosphorylation via the AKT/mTOR/p70S6K signaling pathway, thereby overcoming the antiestrogen resistance [344]. Recently, everolimus inhibited YBX1 phosphorylation, and, therefore, overcame antiestrogen resistance in vitro and in a mouse model [344]. The relative quantification of YBX1 phosphorylation was quantified by Western Blot and immunohistochemistry. Further clinical studies need to proof, if YBX1 is a useful PD marker to start everolimus therapy.

7.2.2.4 miRNA

An increased expression of miR was observed in liver cancer cells treated with everolimus and sirolimus [345]. In liver cancer, the administration of immunosuppressants yields a specific pattern/signature of miRNAs in tumoral cells. Tacrolimus and mTOR inhibitors (everolimus and sirolimus) decreased miR-92a-1-5p, miR-197-3p, miR-483-3p and miR-720, and increased miR-22-3p, miR-376a-3p, miR-663b, miR-886-5p, miR-1300 and miR-1303 expressions in HepG2 cells [345]. The down-regulation of miR-483-3p might be relevant for its antitumoral properties. By contrast, the upregulation of miR-663b, miR-22-3p and miR-1303 has been related to oncogenic properties. More studies are required to understand the role of miRNAs in the cellular response observed upon the treatments with mTOR inhibitors in hepatoma cells.

7.2.3 Other Diseases

Tuberous sclerosis complex is a disease characterized by a considerable altered serum miRNA profile. In addition, these alterations seem to be mTOR-dependent, as they are partially reversed by treatment with everolimus [346]. Pawlik et al. [347] found 11 miRNAs to be dysregulated in serum in the same directions following both treatments, everolimus or sirolimus, with the most significant

changes in expression being observed for miR-136-5p, miR-376a-3p, and miR-150-5p. These 3 miRNAs were found upregulated in tuberous sclerosis complex patients treated with everolimus or sirolimus. This indicated that both mTOR inhibitors may have homogenous effects on the mTOR pathway while acknowledging a link between particular miRNA and mTOR signaling.

Budde et al. used. angiogenesis-related molecules such as collagen-IV, VEGF-A, and VEGF for nonspecific PD monitoring in 118 patients with angiomyolipoma associated with tuberous sclerosis complex (TSC) or sporadic lymphangioleiomyomatosis (sLAM). The authors found after 24 weeks of treatment with 10 mg RVR daily a reduction of vascular endothelial growth factor D (VEGF-D) and collagen type IV (COL-IV). In contrast, VEGF-A was increased in plasma. In addition, baseline VEGF-D and COL-IV levels were associated with baseline angiomyolipoma size and with angiomyolipoma response to everolimus treatment. The authors suggest that these biomarkers may be used for monitoring the PD effects of everolimus [348].

The nonspecific biomarkers for PD monitoring of everolimus therapy in transplantation and oncology are summarized in table 1.

7.3 Specific PD Monitoring of Everolimus

As elaborated in the first consensus document for drug-specific PD monitoring of everolimus, 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) [4]. However, in the field of transplantation no new evidence was generated which supported specific PD monitoring of enviroximes. For the methods used to perform drug-specific PD monitoring the reader is referred to the first consensus document [4].

In oncology, most of the drug-specific PD biomarkers were applied in various studies and diverse diseases. In the following section the data extracted from the literature are presented according to the drug-specific biomarker. This means that studies which used a combined panel of PD biomarkers will be mentioned repeatedly. The drug-specific biomarkers used for PD monitoring of everolimus therapy in oncology are summarized in table 2.

7.3.1 Phosphorylated mTOR (P-mTOR)

P-mTOR indicates activation of the mTOR pathway and may be used to select patients who benefit from a therapy with everolimus. A large study that included 342 patients with primary renal cell carcinoma (RCC) and 90 patients with metastases observed a differential expression of phosphorylated mTOR by immunohistochemistry [349]. High levels of mTOR phosphorylation correlated with impaired overall survival and cancer-specific survival. Rausch and colleagues decided to classify the stained sections by independent reviewers and quantified the relative amount of tumor cells stained for phosphorylated mTOR (grading from 0.0 to 1.0) and the staining intensity (grading from 0 to 3). A further study by Li et al. with patients suffering from anti-VEGF-refractory

Commenté [LLJP24]: Considering this states in the subheading

NOT specific for everolimus I suggest the entire section be removed.

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metastatic RCC investigated the expression levels of phosphorylated AKT, mTOR, eukaryotic initiation factor 4E binding protein-1 (P-4E-BP1) and 40S ribosomal S6 (rS6P) by immunohistochemistry. The outcome of everolimus therapy was more beneficial if the expression of P-mTOR was upregulated in tumor tissue [350].

In a trial with 23 patients with resectable non-small cell lung cancer (NSCLC) changes in the expression of active phosphorylated forms of mTOR, Akt, S6, eIF4e, p70S6K, 4EBP1 were determined by immunohistochemistry in tissue and the metabolic response by 18F-FDG PET/CT [351]. There was a significant negative correlation between metabolic response on PET imaging and percent change in immunoscore for nuclear p70S6K1 and cytoplasmic p70S6K1 expression in samples collected before and after treatment. The effect of everolimus on the metabolic response or anatomic tumor shrinkage as well in modulating key signaling proteins in the PI3K/AKT/mTOR pathway was dose dependent with a dose of 10 mg being more effective than 5 mg. The 5 mg dose of everolimus induced a weaker p-AKT expression concomitant with a lesser reduction in the downstream read-outs of pathway inhibition in comparison to the 10 mg dose [351].

Fukiudo et al. followed the PD effect by measuring mTOR activity in isolated peripheral blood mononuclear cells (PBMNCs) in 19 patients with metastatic breast cancer and treated with 10 mg/d everolimus once daily in combination with exemestane (25 mg orally once daily). The activity of mTOR was directly measured using the Kinase-linked immunosorbent assay (KLISA) mTOR activity kit (CBA055, Calbiochem, USA) by assessing phosphorylation of the specific mTOR substrate p70S6 kinase at Thr389. Pharmacodynamic could be recorded in 11 patients and a significant correlation was observed between blood everolimus concentrations and mTOR activity in PBMCs. However, everolimus did not completely inhibit mTOR activity at therapeutic concentrations [261].

Chronic lung allograft dysfunction (CLAD) is a limiting factor of long-term outcome after lung transplantation. To reduce everolimus side effects encapsulating it inside liposomes may be an option. Therefore phosphorylation of mTOR using anti-p-mTOR(Ser2448) and Western Blotting has been used ex vivo vitro as a read out to compare the efficacy of liposomes loaded with Everolimus and coated with hyaluronic acid in lung fibroblasts (LF). LFs were isolated from BAL of patients affected by chronic progressive fibrosis. It could be shown by inhibition of mTOR phosphorylation that the everolimus encapsulated in liposomes was able to vehicle everolimus inside the cells [352].

In neuroendocrine bronchial carcinoids Gagliano et al. showed by Western blot analysis that both the total and phosphorylated forms of the mTOR protein were higher in tumor tissue in a group of patients which were everolimus sensitive compared to patients which were considered resistant [353].

7.3.2 Phosphorylated p70S6-kinase (P-p70S6K1)

In the immunohistochemical analysis of bronchial NETs in 21 patients by Gagliano and colleagues mentioned above, besides P-mTOR also P-p70S6K was upregulated in everolimus sensitive patients

compared to everolimus resistant patients [353]. The efficacy of monitoring intra-tumor P-p70S6K was suggested by Benselama et al. [354].

In the publication by Gagliano et al. concerning human bronchial carcinoids p70S6-kinase activity was monitored in cell lines by using the AlphaScreen SureFire p-p70S6K (Thr389) assay (Perkin Elmer) and was inhibited by everolimus treatment [353].

7.3.3 Phosphorylated ribosomal S6 protein (P-rS6P)

Especially, patients with RCC, who are not suitable for a surgical therapy, may profit from a targeted drug therapy consisting of everolimus plus selumetinib, a highly selective MEK1 inhibitor [355]. Western blot analysis was used to test the expression of downstream molecules of mTOR and MEK pathways in RCC cells. Phosphorylated rS6P was completely inhibited by everolimus in combination with selumetinib. The results suggest that P- rS6P, a key effector of inhibition of the mTOR and MEK signaling pathways in RCC, serve as a PD marker for a targeted therapy. The study by Li et al. with RCC patients investigating the phosphorylation status of mTOR, P-4E-BP1 and rS6P by immunohistochemical staining supported also the suitability of P-rS6P as potential efficacy predictors for everolimus therapy in RCC [350].

As mentioned above P-rS6P was also assessed in cancer tissue of patients with NSCLC and treated with everolimus by Owonikoko et al. and was part of an immunoscore which predicted the sensitivity to a therapy with everolimus [351].

Kuo and colleagues showed that immunofluorescence staining of P-4EBP1 Thr37/46 and P-rS6P Ser424 can predict everolimus sensitivity in breast cancer cell models [356].

Inhibition of mTORC1 signaling has been shown to diminish growth of meningiomas and schwannomas. Everolimus may slow tumor progression particularly in patients with neurofibromatosis type 2 (NF2) with vestibular schwannoma. In a recent study published by Karajannis et al. measured everolimus blood concentration, tumor tissue drug concentrations and P-rS6P expression in 10 patients who received 10 mg everolimus daily for 10 days immediately prior to surgery. Median pre- and postoperative blood levels of everolimus were in the upper therapeutic range (17.4 ng/mL and 9.4 ng/mL, respectively) and the drug concentration in tumor tissue was 24.3 pg/mg as determined by mass spectrometry. However, only partial inhibition of phospho-S6 in the tumor tissue was noted by immunohistochemistry on formalin fixed tissue sections using a rabbit anti-human P-rS6P antibody suggesting incomplete target inhibition ([357].

7.3.4 Phosphorylated eukaryotic initiation Factor 4E binding protein-1 (P-4E-BP1)

In the study by Li et al. paraffin-embedded tumor tissue specimens derived from 18 RCC and were also analyzed for P-4E-BP-1 expression and progression-free survival time under the therapy with

everolimus was as already mentioned for the other mTOR downstream target molecules correlated with the expression levels [350]. As already mentioned for the PD target P-rS6P in the study by Zou et al. with everolimus plus selumetinib in RCC the same effect was seen on P-4E-BP1 [355]. P-4E-BP1 was also used as a PD marker reflecting mTor targets in the study with NSCLC by Owonikoko et al [351] and in bronchial carcinoids [353].

7.4 Summary: PD Monitoring

PD monitoring to guide everolimus therapy is still not yet established in clinical routine. PD monitoring was primarily an issue in solid organ transplantation and oncology. New drug non-specific PD biomarkers emerged during the last six years such as miRNA or graft derived cel-free DNA in transplantation whereas drug-specific biomarkers such phosphorylated mTOR, mTOR activity or phosphorylated ribosomal protein S6 were monitored in tumor tissue to monitor the everolimus effects. In transplantation T-cell subsets were studied mainly with a focus on Treg cells which are supposed to be preserved under mTOR therapy in contrast to immunosuppressive regimens based on CNI. Assessment of proliferation inhibition has been used in oncology for drug non-specific PD monitoring of everolimus action on tumor cells ex vivo and in vitro. Furthermore, fluorodeoxyglucose positron emission has been applied to monitor everolimus efficacy in vivo in oncology.

8. CONCLUSIONS

Conclusions will be written immediately after the rest of the manuscript is collected.

9. FIGURE LEGENDS

Figure 1. Cellular mechanism of action of everolimus.

Circled P refers to phosphorylated protein; blunt arrows (⊥) indicate inhibition while sharp arrows

(→) indicate stimulation.

Everolimus modifies the cell responses by associating with the intracellular protein FKBP12. The FKBP12-EVR complex binds directly to mTORC1 and inhibits its functions resulting in dephosphorylation and inactivation of HIF-1 leading to inhibition of angiogenesis and cell proliferation; inactivation of ULK1 leading to the promotion of autophagy, and inactivation of S6K1 and 4EBP1 leading to G1 cell cycle arrest.

IL-2, interleukin-2; IGF-1, insulin-like growth factor-1; EGF, epidermal growth factor; RTK, receptor tyrosine kinase; JAK, janus kinase; PI3K, phosphoinositide-3-kinase; PIP2, phosphatidylinositol 4,5 bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PDKD1, phosphoinositide-dependent protein kinase-1; AKT/PKB, protein kinase B; Ras, rat sarcoma virus; Raf, rapidly accelerated fibrosarcoma; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; TSC, tuberous sclerosis complex; GAP, GTPase-activating proteins; Rheb, Ras homolog enriched in brain; GTP, guanosine-5'-triphosphate; GDP, guanosine-5'-diphosphate; FKBP12, FK 506 binding protein-12; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; RAPTOR, regulatory-associated protein of mTOR; PRAS40, proline-rich AKT substrate 40; Deptor, DEP domain-containing mTOR-interacting protein; mLST8; mammalian lethal with Sec13 protein 8; mTORC2, mTOR complex 2; RICTOR, rapamycin-insensitive companion of mTOR; PROTOR, protein observed with RICTOR; HIF-1, hypoxia-inducible factor-1; ULK1, unc-51 like autophagy activating kinase 1; S6K1, ribosomal protein S6 kinase beta-1; rS6P, ribosomal S6 protein; 4EBP1, eukaryotic translation initiation factor 4E–binding protein 1; eIF, eukaryotic translation initiation factor; m7G, 7-methylguanosine.

10. ACKNOWLEDGEMENTS

11. TABLES

Table 1. Analytical performance of currently available EVR methods

Method	Analytical platform	Measurement range*	Total method imprecision	Inaccuracy (method comparison with a validated LC-MS/MS method**)	Specificity for the parent drug % crossreactivity with metabolites relative to EVR	References
LC-MS/MS***	Various LC-MS/MS platforms	0.5 – 50 µg/L	≤ 10% over the measurement range	Typically serves as the reference method	Specific for the parent drug	[94]
QMS® Thermo Scientific	General chemistry analyzers spectrophotometric detection	1.5 – 20 µg/L	< 10% at concentrations ≥3.9 µg/L	intercept: KTx: -0.005, HTx: -0.15, LTx: 0.98 slope: KTx: 1.11, HTx: 1.00, LTx: 0.98 r=0.93 – 0.96 [a]Clinical samples (KTx, n=150; HTx, n=41; LTx, n=111)	At a concentration of 20 µg/L 45,46-hydrohy-: 2.0% 24-hydroxy-: 5.0% 25-hydroxy-: 22.0% RAD SA: 2.0% RAD PSA: 16.0% RAD PC: 59.0	[358]
ECLIA Roche Diagnostics	Cobas platform: e modules	1.0-30 µg/l	< 8.5% at concentrations ≥2.8 µg/L	intercept: 0.478 slope: 1.21 r=0.91 [b]Clinical samples	At a concentration of 25 µg/l 45,46-hydrohy-: 6.0% 24-hydroxy-: 21.3% 25-hydroxy-: 15.4% RAD SA: 9.8% RAD PSA: 10.1% RAD PC: 109.3%	[102,106]

				(n=784 from KTx, HTx and LTx, pooled data from 5 European laboratories)		
ACMIA Siemens Healthcare Diagnostics	Dimension® and Dimension Vista® clinical chemistry systems	1.0-25 µg/L	< 7% at concentrations ≥3.0 µg/L	(n=295, transplanted organ not reported) intercept: -0.10 slope: 1.06 r=0.965 [c]Clinical samples	At a concentration of 5 µg/L 45,46-hydrohy-: 14.0% 24-hydroxy-: 26.0% 25-hydroxy-: 74.0% RAD SA: 52% RAD PSA: 54% RAD PC: 64-98% At a concentration of 20 µg/l RAD SA, RAD PSA or 25-hydroxy-EVR test results are suppressed, RAD PC 96%.	[359]

*the lower limit is defined either by the limit of quantification or by the functional sensitivity respective to what is reported by the manufacturer; ** appropriately validated LC-MS/MS method that serves as the reference, data shown are representative examples that may vary between patient populations e.g. with different type of transplantation; ***performance characteristic for state of the art methods; [a] Passing-Bablok regression; [b] Deming regression; [c] not stated; LC-MS/MS= liquid chromatography-tandem mass spectrometry; ACMIA= affinity chrome-mediated immunoassay; CMIA = Chemiluminescent Microparticle Immunoassay; ECLIA= Electrochemiluminescence Immunoassay; QMS= Quantitative Microsphere System; LTx=liver transplantation; KTx= kidney transplantation; HTx=heart transplantation.

Table 2. Average pharmacokinetic parameters for everolimus (EVR) in sub-populations for organ transplantations and cancer treatments.

		therapeutic range	references	Tmax	Cmax (µg/L)	C0 (µg/L)	t1/2	AUC24	references
healthy volunteers		NA		0.5 - 4 hrs	17.9 (±5.9) (single dose 2 mg)		31.5 (± 6.4) hrs	122 (± 52) µgxhr/L (single dose 2 mg; fasting)	[360,361]
Renal Tx	With reduced CNI:	3 - 8 µg/L	[166] [159,362-367]	1 - 3 hrs	8 - 14 µg/L (/dose ≤1mg) 21 - 55 µg/L (/dose ≥2.5mg)	2 - 7 µg/L	28 (±7) hrs	40-120 µgxhr/L (dose normalized) ~450 µgxhr/L (at steady state and 2.5mg/day)	[360,361] [159,367-369]
	Without CNI:	6 - 10 µg/L	[163,249] [250]						
lung heart	With reduced CNI:	3 - 8 µg/L	[213,230,370]	1.7 - 2.4 hrs	10 - 20 µg/L (dose: 1.5-3mg)	5 - 10 µg/L (dose: 1.5-3mg)	25 hrs	80 - 160 µgxhr/L (dose 1.5--3mg)	[370]
liver	With reduced CNI:	≥3 µg/L	[173]	1 - 4 hrs	10 - 14 µg/L (dose ≤2.5mg) 41 - 53 µg/L (dose 7.5mg)		25 - 40 hrs	107 - 137 µgxhr/L (dose normalized) ~750 µgxhr/L (at steady state and ≤7.5mg/day)	[371] [116]
	Without CNI:	5 - 12 µg/L	[176]						
pediatric	With CNI (CsA):	≥3 µg/L	[372]	1 - 2 hrs	10 - 20 µg/L (dose: max 3mg)	4.4 ± 1.7 µg/L	29.7±11.1 hrs	66 - 220 µgxhr/L (dose 0.8-1.6 mg/m2; max 3mg)	[115,373] [372]
Cancer	(10mgx1)	12 - 20 µg/L	"Cut-off": [261] LOWER: [131-133,375] UPPER: [133,134] [131,375] SUMMARY: [135]	1 - 6 hrs	62 (±18) µg/L	8 - 38 µg/L	26 - 38 hrs	435 - 565 µgxhr/L	[131,261,374] [134]

Table 3. Summary of published everolimus population PK models (including PBPK and Machine learning algorithms).

Patient population	Number of patients	Mean age (Range)	PK data and Model development	Covariates	Major findings	Reference
Renal transplantation	508 (train and test dataset) & From 500 to 10035 simulated PK profiles [141] 114 (external validation)	45 (35-57) 50 (40-59)	Everolimus AUC estimates (based on pre-dose, ~1 h, and ~2 h whole blood concentrations) and dose recommendation requests received on the ISBA website since 2018. https://abis.chu-limoges.fr/login Development of machine learning algorithms using XGBoost compared with Maximum A posteriori Probability Bayesian Estimation (MAP-BE). Simulated data were generated with the mrgsolve R package.	Not applicable. Evaluation of the contribution and limits of simulated data to train ML models to estimate everolimus $AUC_{0\text{-}12h}$.	The best results were obtained with XGBoost trained on 5016 simulated profiles. AUC estimation achieved in an external dataset of 114 full- PK profiles was excellent (root mean squared error [RMSE] = 10.8 µg*h/L) and slightly better than MAP-BE (RMSE = 11.9 µg*h/L). Using more profiles or combining with "real" profiles did not improve the ML algorithm performance.	150
Renal transplantation, metastatic thyroid cancer, or breast cancer	173	52.2 (19.6–79.0)	Whole-blood concentration data including 2933 C0 and 322 AUC_{0-12} from routine TDM. NONMEM using the stochastic approximation expectation–maximization algorithm followed by Monte Carlo Importance Sampling for objective function assessment (SAEM-IMP). Mechanistic population pharmacometric model [143]	Allometric scaling and hematocrit function (Model accounted for saturable erythrocyte binding of everolimus in whole-blood and estimated the pharmacokinetic parameters in plasma from paired observations of whole-blood concentrations and hematocrit)	The model was able to accurately and precisely predict future everolimus exposure from prior PK measurements. In addition, authors illustrated the potential added value of performing everolimus therapeutic drug monitoring with hematocrit-normalized whole-blood concentrations.	144
Tuberous sclerosis complex	531	2 years and older	6649 concentrations from patients enrolled in three Phase-3 studies. NONMEM 2-compartment model with first order absorption. Sequential PopPK/PD model fitting and simulations were performed in Monolix Suite 2016R1 using the SAEM algorithm. Everolimus effect was implemented as an inhibitory Emax function of C_{min} on the seizure mean	Tested: age, body surface area (BSA), race, sex, height, weight, and comedications (CYP450 3A and P-gp inducers). Retained: BSA, CYP3A and P-gp inducers Used drug inducer status on BSA adjusted clearance.	The results supported the recommended target concentration range for everolimus of 5-15 ng/mL to ensure treatment efficacy. The responder rate increased with increasing C_{min}	147

Renal transplantation	24	64 (40-76)	Rich PK and PBMC sampling (11 samples per PK profile). Non-parametric adaptive grid approach implemented in R software (Pmetrics). Bayesian Estimator and Limited Sampling Strategy (LSS). Two-compartment model with whole blood and PBMC as the first and second compartment, respectively, and a double gamma distribution to describe the absorption.	None of the tested covariates (age, sex, albumin, hematocrit, fat-free mass and genetic variants (CYP3A5*1, ABCB1 haplotype, PPARA*42, PPARA*48, and POR*28) were retained in the final model	Successful development of a popPK model and Bayesian estimator based on an LSS for whole blood and PBMC concentrations. Accurate model-informed determination of whole blood and PBMC exposure of everolimus using 3 optimally timed samples.	[149]
Breast cancer Renal transplantation	71 55	61 (37-80) 53 (19-74)	Rich PK sampling with eight (or more) sampling occasions for each subject. Total number of samples 1240. NONMEM using SAEM-IMP. Semi-physiological 2-compartmental liver model with transit compartment absorption.	Tested: patient demographics, fat free mass, formulation, dose, CYP3A inducers. Retained: prednisolone dose Nonlinear erythrocyte binding of everolimus as function of hematocrit (Emax model)	For oncological indications, the results encourage the investigation of dosing everolimus 3.75 mg BID in terms of superiority in safety and noninferiority in efficacy.	[143]
Thyroid cancer Breast cancer	42 32	62 (37-80)	PK profiles based on limited sampling (0, 1, 2, and 3 h after drug intake) or extensive sampling (0, 1, 2, 3, 4, 5, 6, 7, and 8 h); 915 PK samples. NONMEM semi-physiological 2-compartmental liver model with transit compartment absorption [143] Implementation of previously developed PK/PD Emax model relating inhibition of S6K1 with unbound plasma concentrations of everolimus [376]	No formal covariate analysis reported. Whole blood to plasma concentrations based on hematocrit equation.	Predicted mTOR inhibition was at a plateau at the approved dose, dose reductions may have only a limited impact on mTOR inhibition. Hematocrit influences whole-blood concentrations With no effect on plasma concentrations. Exposure–treatment outcome relationships based on plasma data can be evaluated with the semi-physiological model.	[148]
Renal cell carcinoma	22	67 (21-82)	Two full 24-h PK profiles on days 1 and 8 after the start of everolimus therapy. NONMEM 2-compartment model with fixed absorption rate constant from literature [293]	Tested: body weight, ideal body weight, age, Serum creatinine, albumin, liver function tests, total bilirubin. None identified (small sample size)	First population pharmacokinetic parameters of everolimus in Japanese patients with Renal Cell Carcinoma. Monitoring of blood trough concentration and implementing corresponding everolimus dosage adjustments in anticancer therapy is recommended.	[146]

Thyroid cancer	40	63 (40-80)	Sparse (pre, 1, 2, and 3h) + more extensive (4,5,6,7, and 8h); 669 PK samples. NONMEM 2-compt with first order (Ka) absorption and first order elimination.	Tested: bilirubin, liver functions, creatinine, body surface area and hematocrit. Pharmacogenetics of CYP3A4, CYP3A5 and CYP2C8, P-gp. Retained: presence of at least one ABCB1 TTT haplotype.	Dose reductions and everolimus-induced stomatitis were strongly associated with systemic everolimus drug exposure The presence of at least one ABCB1 haplotype was associated with a decrease in everolimus exposure due to decreased absorption.	[137]
Renal transplantation	97	51 (22–71)	Routine TDM based on $AUC_{0–12\,h}$ at steady state at regular clinical visits scheduled at 32, 52, 78, and 104 weeks after transplantation. Target AUC 120 µg*h/L; 1807 PK samples. NONMEM two-compartment model with transit compartment absorption and first-order elimination.	Tested; patient demographics, combined CYP3A genotypes (CYP3A4 and CYP3A5), concomitant medications. Retained: ideal body weight, hematocrit, and CYP3A4*22 on CL/F	The effect of CYP3A4*22 on everolimus, clearance was less than 20. These data suggest that dose individualization of everolimus therapy based on CYP3A genotype is not indicated.	[142]
Renal transplantation	52	52 (23-71)	Routine TDM targeting an AUC0-12 of 120 µg*h/L. Sampling at t = 0, 1, 2, 3, 4, 5 and 6 h or t = 0, 1, 2 and 3 h); 783 concentrations. NONMEM 2-compt model first order absorption and lag-time.	Tested: demographics (age, total body weight, sex, ethnicity, height, ideal body weight [IBW], body surface area, body mass index, lean body weight, hematocrit, albumin, underlying disease and co-medications. Retained: IBW on volume. ABCB1, CYP3A5, CYP2C8 and PXR had no clinically relevant effect.	C2 as a limited sampling model can be used to accurately estimate everolimus systemic exposure, an improvement over the widely used Ctrough monitoring.	[141]
Heart transplantation	59	50 (17-80)	Routine TDM pre-dose trough data. 13 sampling points per subject (range, 3–36); a total of 775 concentrations. NONMEM 1-compartment PK model with first order absorption (fixed) and elimination.	Tested: patient demographics (body weight, age, size, sex), liver panel, creatinine, albumin, post-op days, concomitant medication (including corticosteroids and cyclosporine), CYP3A5 and ABCB1. Retained: Sex, bilirubinemia, (and presence of cyclosporine (CSA)	First population PK of everolimus in heart transplantation patients. Prediction of the everolimus dose using the population PK model requires monitoring of the bilirubinemia and presence of cyclosporine treatment.	[145]

				Trend of CYP3A5 polymorphism on clearance.		
De novo renal transplantation	673	44.1 (16-70)	PK data from two randomized, double-blind, multicenter efficacy trials; 53.4% morning troughs. 621 PK profiles in 261 patients. 12% of the data came from the 5 post-dose sampling times (1, 2, 5, and 8 hours). Overall, 7.8 samples per subject (range 1-18); total of 5260 PK samples. NONMEM 1-comptartment model with first-order absorption (fixed) and linear elimination.	Tested: demographics (age, weight, sex, and race/ethnicity), comedication. Retained: weight, age, race, and erythromycin use.	Dose adjustment based on weight does not appear necessary. Black patients in general had a 20% higher clearance and may need a higher dose to achieve exposure similar to that in nonblack patients. Concomitant administration of potent inhibitors of CYP3A may decrease clearance and increase everolimus exposure.	[140]
Pediatrics						
Adult liver transplantation with extrapolation to pediatric patients	87 adults 13 children	60 (20-77) 6 (0.75-16)	TDM sampling of pre-dose troughs; 937 concentrations from adult subjects; 373 PK samples from pediatric patients. NONMEM 1-comptartment model with first-order absorption (fixed) and linear elimination. Explored the model-based extrapolation to pediatric patients and relationship between estimated individual CL/F of everolimus and Dose/Concentration ratio of tacrolimus.	Tested: patient demographics (age, height, body weight, sex, postoperative day, clinical laboratory data (e.g., hematocrit, liver panel], albumin, estimated glomerular filtration rate [eGFR]), and concomitant drugs). Retained, allometric scaling, eGFR, sex, total daily dose, and concomitant, fluconazole use.	Model-informed simulations indicated. that median trough concentrations in several cases could be achieved with lower than the label dose of 1.0 mg twice daily. A moderate correlation between the individual clearance and the tacrolimus D/C ratio was observed. The accuracy of the final model prediction in children was high except in one patient.	[292]
Tuberous sclerosis complex	294	9 (2-18)	PK data from three Phase-3 studies as previously reported [147] M&S framework with PBPK (Simcyp) and population PKPD models everolimus in tuberous sclerosis complex (TSC) –associated treatment-refractory partial-onset seizures (POS).	PBPK modeling to extrapolate of exposure (trough plasma concentration (Cmin) after a dose of 6 mg/m^2 to evaluate likelihood of reduction in seizure frequency in children aged between 6 months and 2 years.	Based on modeling, everolimus at 6 mg/m^2 dose is expected to be efficacious in reducing daily SF associated with TSC-associated POS in patients 6 months to 2 years of age in both the short term (i.e., 12 weeks of exposure) and long term (i.e., up to 2 years of exposure).	[295]
Solid tumors	25 (PK data were obtained from 18 patients)	10 (3-21)	Phase 1 trial in children with recurrent or refractory solid tumors. 8 sampling points per subject (0.5, 1, 1.5, 2,. 4, 6, 6, 8, and 24 h) NONMEM 2-compartment model with first-order absorption and linear elimination.	Clearance was scaled by body surface area.	The study established the everolimus maximum tolerated dose as 5 mg/m^2/day orally in this population.	[293]

					Interpatient PK variability was significant, with an approximate seven-fold variation in apparent oral clearance.	

AUC, area under the concentration-time curve; BSA, body surface area; CL/F, apparent clearance; eGFR, estimated glomerular filtration rate; IBW, ideal body weight; LLS, limited sampling strategy; MAP-BE, maximum a posteriori probability Bayesian estimation; PBMC, peripheral blood mononuclear cells; PBPK, physiologically-based pharmacokinetics; PD, pharmacodynamics; PK, pharmacokinetics; POS, partial-onset seizures; RMSE, root mean squared error; SAEM, stochastic approximation expectation–maximization; TSC, tuberous sclerosis complex.

Table 4. Stepwise process of model-informed Bayesian estimation to generate individual AUC estimates [377,378]

Prior Probability	New Information - feedback	Objective function	Posterior Probability	Therapeutic Goal	Adaptive Control
Population PK model	Patient Demographics & Everolimus Concentration(s)	Consider Prior (PopPK model) & New Information	Individual Predicted PK Profile	Pre-dose trough or AUC Targets	Estimate Dose to Achieve the Therapeutic Target

Commenté [LF26]: Will skip that

Table 5: Nonspecific Biomarkers For PD Monitoring Of Everolimus Therapy In Transplantation And Oncology

PD Marker	Methods	Diseases	Cell/Tissue	References
Inhibition of proliferation	MTT-Assay	Kidney transplantation Renal cell carcinoma	PBMNCs Tumor cells Cancel cell lines	299 331 332
	HLA-G expression	Cardiac allograft vasculopathy	Smooth muscle cells	338
	[^{3}H]thymidine incorporation assay	Kidney transplantation	PBMNCs	301
	Gene expression, T cell subsets	Liver transplantation	Whole blood	303
	Dysfunctional T-cell phenotype, mTOR signaling	Kidney transplantation	Whole blood	64
	CD8+ effector memory T-cells, flow cytometry	Kidney transplantation	Whole blood	302
	BrdU Elisa	Esophageal squamous cell carcinoma	Tumor tissue, graft tissue, cell lines	339
T-cell subsets	Flow cytometry Treg function Surface marker expression	Kidney transplantation Liver transplantation Heart transplantation	Whole blood	304-310,312
Cytokines	ELISA IL-2, IL-10, IL-21, IFN-y	Liver transplantation	Ex vivo stimulated PBMNCs	318
miRNAs	qPCR,	Kidney transplantation	Kidney biopsies	322
	GeneChip (Affymterix)	Kidney transplantation	Kidney biopsies	322
	NanoString nCounter® miRNA Expression Assay and qPCR	Islet transplantation im mice	Islet grafts and plasma	323
	qPCR	Liver cancer	HepG2 cells	345
dd-cf DNA	Digital droplet PCR NGS	Kidney, heart and liver transplantation	Plasma	327-329
Protein expression	Immunohistochemistry angiotensin II type I receptor (AT1R), phosphorylated mammalian target of rapamycin (p-mTOR).	Esophageal squamous cell carcinoma	Tumor tissue, graft tissue, cell lines	339
	Reverse Phase Protein Array (RPPA)	Breast cancer	Breast cancer cell lines	341

	Nitrogen permease regulator-like 2 (NPRL2Western Blot	Prostate cancer	Cancer tissue, prosate cancer cell lines	[342]
Gene expression	Peroxiredoxin-2 expression	Neuroendocrine tumors	Human pancreatic neuroendocrine cells	[340]
	Nitrogen permease regulator-like 2 (NPRL2) RT-PCR	Prostate cancer	Cancer tissue, prosate cancer cell lines	[342]
Positron emission tomography	[18F]fluorodeoxyglucose PET (FDG-PET	Neuroendocrine neoplasias	Patients in vivo	[335]
		Different solid tumors		[334]
		Non-small cell lung cancer		[351]
		Gastric cancer	Xenograft-bearing mice	[336]

Table 6: Specific Biomarkers Used For PD Monitoring Of Everolimus Therapy In Oncology

PD Marker	Methods	Diseases	Cell/Tissue	References
Phoshorylated m-TOR (P-mTOR)	Immunohistochemistry	Renal cell carcinoma (RCC)	Tumor tissue	[349,350]
		Non-small cell lung cancer	Tumor tissue	[351]
	Western Blot	Neuroendocrine tumor (NET)	Tumor tissue (Bronchial carcinoid)	[353]
	Western Blot	Chronic lung allograft dysfunction	Lung fibroblasts	[352]
	Kinase linked immuosorbent assay (p70S6-kinase) in cell lysates	Metastatic breast cancer	PBMNCs	[353]
Phosphorylated p70S6-kinase (P-p70S6K1)	Western Blot Immunohistochemistry	Neuroendocrine tumors (NET)	Tumor tissue (Bronchial carcinoid, Carcinoid, VIPoma, Insulinoma, Glucagonoma)	[353,354]
	AlphaScreen SureFire (p-p70S6-kinase) in cell lysates	Neuroendocrine tumor (NET)	Cancer cell lines	[353]
Phosphorylated ribosomal S6 protein (P-rS6P)	Immunohistochemistry Western Blot	Renal cell carcinoma (RCC)	Tumor tissue Cancer cell lines Tumor xenografts	[350,355]
	Immunohistochemistry	Non-small cell lung cancer (NSCLC)	Tumor tissue	[351]
	Immunohistochemistry	Schwannoma	Tumor tisseue	[357]
	Immunofluorescence staining	Breast cancer	Cancer cell lines	[356]
Phosphorylated eukaryotic initiation Factor 4E binding protein-1 (P-4E-BP1)	Immunohistochemistry Western Blot	Renal cell carcinoma	Tumor tissue Cancer cell lines Tumor xenografts	[350,355]
	Immunohistochemistry Western Blot	Non-small cell lung cancer (NSCLC)	Tumor tissue	[351]
		Neuroendocrine tumors (NET)	Tumor tissue (Bronchial carcinoid)	[353]
	Immunofluorescence staining	Breast cancer	Cancer cell lines	[356]

REFERENCES

1. 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(6):642-654.
2. Brunet M, van Gelder T, Asberg A, et al. Therapeutic Drug Monitoring of Tacrolimus-Personalized Therapy: Second Consensus Report. *Ther Drug Monit.* 2019;41(3):261-307.
3. Bergan S, Brunet M, Hesselink DA, et al. Personalized Therapy for Mycophenolate: Consensus Report by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology. *Ther Drug Monit.* 2021;43(2):150-200.
4. Shipkova M, Hesselink DA, Holt DW, et al. Therapeutic Drug Monitoring of Everolimus: A Consensus Report. *Ther Drug Monit.* 2016;38(2):143-169.
5. Shaw LM, Holt DW, Keown P, Venkataramanan R, Yatscoff RW. Current opinions on therapeutic drug monitoring of immunosuppressive drugs. *Clin Ther.* 1999;21(10):1632-1652; discussion 1631.
6. Masuda S, Inui K. An up-date review on individualized dosage adjustment of calcineurin inhibitors in organ transplant patients. *Pharmacol Ther.* 2006;112(1):184-198.
7. Yamada T, Zhang M, Masuda S. Significance of Ethnic Factors in Immunosuppressive Therapy Management After Organ Transplantation. *Ther Drug Monit.* 2020;42(3):369-380.
8. Elens L, Langman LJ, Hesselink DA, et al. Pharmacologic Treatment of Transplant Recipients Infected With SARS-CoV-2: Considerations Regarding Therapeutic Drug Monitoring and Drug-Drug Interactions. *Ther Drug Monit.* 2020;42(3):360-368.
9. van Gelder T, Le Meur Y, Shaw LM, et al. Therapeutic drug monitoring of mycophenolate mofetil in transplantation. *Ther Drug Monit.* 2006;28(2):145-154.
10. van Gelder T, Fischer L, Shihab F, Shipkova M. Optimizing everolimus exposure when combined with calcineurin inhibitors in solid organ transplantation. *Transplant Rev (Orlando).* 2017;31(3):151-157.
11. Mabasa VH, Ensom MH. The role of therapeutic monitoring of everolimus in solid organ transplantation. *Ther Drug Monit.* 2005;27(5):666-676.
12. Everolimus In: IBM Micromedex® (electronic version). IBM Watson Health, Greenwood Village, Colorado, USA. Available at: https://www.micromedexsolutions.com/ Accessed April 6, 2023.
13. Tang Z, Yin L, Zhang Y, Yu W, Wang Q, Zhan Z. Preparation and study of two kinds of ophthalmic nano-preparations of everolimus. *Drug Deliv.* 2019;26(1):1235-1242.
14. Bierer BE, Mattila PS, Standaert RF, et al. Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc Natl Acad Sci U S A.* 1990;87(23):9231-9235.

15. Tong M, Jiang Y. FK506-Binding Proteins and Their Diverse Functions. *Curr Mol Pharmacol.* 2015;9(1):48-65.

16. Harding MW, Galat A, Uehling DE, Schreiber SL. A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature.* 1989;341(6244):758-760.

17. Siekierka JJ, Hung SH, Poe M, Lin CS, Sigal NH. A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature.* 1989;341(6244):755-757.

18. Wellstein A, Giaccone G, Atkins MB, Sausville EA. Pathway-Targeted Therapies: Monoclonal Antibodies, Protein Kinase Inhibitors, and Various Small Molecules. In: Brunton LL, Hilal-Dandan R, Knollmann BC, eds. *Goodman & Gilman's: The Pharmacological Basis of Therapeutics, 13e.* New York, NY: McGraw-Hill Education; 2017.

19. Hall MN. TOR and paradigm change: cell growth is controlled. *Mol Biol Cell.* 2016;27(18):2804-2806.

20. Kirchner GI, Meier-Wiedenbach I, Manns MP. Clinical pharmacokinetics of everolimus. *Clin Pharmacokinet.* 2004;43(2):83-95.

21. Schuler W, Sedrani R, Cottens S, et al. SDZ RAD, a new rapamycin derivative: pharmacological properties in vitro and in vivo. *Transplantation.* 1997;64(1):36-42.

22. Malek TR, Castro I. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity.* 2010;33(2):153-165.

23. Goodridge HS, Harnett MM. Introduction to immune cell signalling. *Parasitology.* 2005;130 Suppl:S3-9.

24. Potter CJ, Pedraza LG, Xu T. Akt regulates growth by directly phosphorylating Tsc2. *Nat Cell Biol.* 2002;4(9):658-665.

25. Puertollano R. mTOR and lysosome regulation. *F1000Prime Rep.* 2014;6:52.

26. Bhaoighill MN, Dunlop EA. Mechanistic target of rapamycin inhibitors: successes and challenges as cancer therapeutics. *Cancer Drug Resist.* 2019;2(4):1069-1085.

27. Sancak Y, Thoreen CC, Peterson TR, et al. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol Cell.* 2007;25(6):903-915.

28. Loewith R, Jacinto E, Wullschleger S, et al. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell.* 2002;10(3):457-468.

29. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell.* 2012;149(2):274-293.

30. Jewell JL, Russell RC, Guan KL. Amino acid signalling upstream of mTOR. *Nat Rev Mol Cell Biol.* 2013;14(3):133-139.

31. Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A.* 1998;95(4):1432-1437.

32. Dufner A, Thomas G. Ribosomal S6 kinase signaling and the control of translation. *Exp Cell Res.* 1999;253(1):100-109.

33. Gingras AC, Raught B, Sonenberg N. mTOR signaling to translation. *Curr Top Microbiol Immunol.* 2004;279:169-197.

34. Mayer C, Grummt I. Ribosome biogenesis and cell growth: mTOR coordinates transcription by all three classes of nuclear RNA polymerases. *Oncogene.* 2006;25(48):6384-6391.

35. Gingras AC, Raught B, Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem.* 1999;68:913-963.

36. Sonenberg N, Dever TE. Eukaryotic translation initiation factors and regulators. *Curr Opin Struct Biol.* 2003;13(1):56-63.

37. Richter JD, Sonenberg N. Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature.* 2005;433(7025):477-480.

38. Moorman NJ, Shenk T. Rapamycin-resistant mTORC1 kinase activity is required for herpesvirus replication. *J Virol.* 2010;84(10):5260-5269.

39. Abraham RT, Wiederrecht GJ. Immunopharmacology of rapamycin. *Annu Rev Immunol.* 1996;14:483-510.

40. Dure M, Macian F. IL-2 signaling prevents T cell anergy by inhibiting the expression of anergy-inducing genes. *Mol Immunol.* 2009;46(5):999-1006.

41. Waickman AT, Powell JD. Mammalian target of rapamycin integrates diverse inputs to guide the outcome of antigen recognition in T cells. *J Immunol.* 2012;188(10):4721-4729.

42. Dai H, Thomson AW. The "other" mTOR complex: New insights into mTORC2 immunobiology and their implications. *Am J Transplant.* 2019;19(6):1614-1621.

43. Warfel NA, Dolloff NG, Dicker DT, Malysz J, El-Deiry WS. CDK1 stabilizes HIF-1alpha via direct phosphorylation of Ser668 to promote tumor growth. *Cell Cycle.* 2013;12(23):3689-3701.

44. Ke Q, Costa M. Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol.* 2006;70(5):1469-1480.

45. Kurtzman D, Vleugels RA, Callen J. Immunosuppressive and Immunomodulatory Drugs. In: Kang S, Amagai M, Bruckner AL, et al., eds. *Fitzpatrick's Dermatology, 9e.* New York, NY: McGraw-Hill Education; 2019.

46. Motzer RJ, Escudier B, Oudard S, et al. Phase 3 trial of everolimus for metastatic renal cell carcinoma : final results and analysis of prognostic factors. *Cancer.* 2010;116(18):4256-4265.

47. Thomas GV, Tran C, Mellinghoff IK, et al. Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. *Nat Med.* 2006;12(1):122-127.

48. Alshaker H, Wang Q, Kawano Y, et al. Everolimus (RAD001) sensitizes prostate cancer cells to docetaxel by down-regulation of HIF-1alpha and sphingosine kinase 1. *Oncotarget.* 2016;7(49):80943-80956.

49. Harris AL. Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer.* 2002;2(1):38-47.

50. Beaver JA, Park BH. The BOLERO-2 trial: the addition of everolimus to exemestane in the treatment of postmenopausal hormone receptor-positive advanced breast cancer. *Future Oncol.* 2012;8(6):651-657.

51. McAndrew NP, Finn RS. Management of ER positive metastatic breast cancer. *Semin Oncol.* 2020;47(5):270-277.

52. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/results?cond=Cancer&term=mTOR&cntry=&state=&city=&dist=;%20last%20visited%206.7.2022. Accessed April 10, 2023.

53. Julich K, Sahin M. Mechanism-based treatment in tuberous sclerosis complex. *Pediatr Neurol.* 2014;50(4):290-296.

54. Kumar R, Ison MG. Opportunistic Infections in Transplant Patients. *Infect Dis Clin North Am.* 2019;33(4):1143-1157.

55. van Delden C, Stampf S, Hirsch HH, et al. Burden and Timeline of Infectious Diseases in the First Year After Solid Organ Transplantation in the Swiss Transplant Cohort Study. *Clin Infect Dis.* 2020;71(7):e159-e169.

56. Brennan DC, Aguado JM, Potena L, et al. Effect of maintenance immunosuppressive drugs on virus pathobiology: evidence and potential mechanisms. *Rev Med Virol.* 2013;23(2):97-125.

57. Nashan B, Gaston R, Emery V, et al. Review of cytomegalovirus infection findings with mammalian target of rapamycin inhibitor-based immunosuppressive therapy in de novo renal transplant recipients. *Transplantation.* 2012;93(11):1075-1085.

58. Kobashigawa J, Ross H, Bara C, et al. Everolimus is associated with a reduced incidence of cytomegalovirus infection following de novo cardiac transplantation. *Transpl Infect Dis.* 2013;15(2):150-162.

59. Bowman LJ, Brueckner AJ, Doligalski CT. The Role of mTOR Inhibitors in the Management of Viral Infections: A Review of Current Literature. *Transplantation.* 2018;102(2S Suppl 1):S50-S59.

60. Araki K, Turner AP, Shaffer VO, et al. mTOR regulates memory CD8 T-cell differentiation. *Nature.* 2009;460(7251):108-112.

61. Havenith SH, Yong SL, van Donselaar-van der Pant KA, van Lier RA, ten Berge IJ, Bemelman FJ. Everolimus-treated renal transplant recipients have a more robust CMV-specific CD8+ T-cell response compared with cyclosporine- or mycophenolate-treated patients. *Transplantation.* 2013;95(1):184-191.

62. Poglitsch M, Weichhart T, Hecking M, et al. CMV late phase-induced mTOR activation is essential for efficient virus replication in polarized human macrophages. *Am J Transplant.* 2012;12(6):1458-1468.

63. Roy J, Paquette JS, Fortin JF, Tremblay MJ. The immunosuppressant rapamycin represses human immunodeficiency virus type 1 replication. *Antimicrob Agents Chemother.* 2002;46(11):3447-3455.

64. Kaminski H, Marseres G, Yared N, et al. mTOR Inhibitors Prevent CMV Infection through the Restoration of Functional alphabeta and gammadelta T cells in Kidney Transplantation. *J Am Soc Nephrol.* 2022;33(1):121-137.

65. Tan L, Sato N, Shiraki A, et al. Everolimus delayed and suppressed cytomegalovirus DNA synthesis, spread of the infection, and alleviated cytomegalovirus infection. *Antiviral Res.* 2019;162:30-38.

66. Clippinger AJ, Maguire TG, Alwine JC. The changing role of mTOR kinase in the maintenance of protein synthesis during human cytomegalovirus infection. *J Virol.* 2011;85(8):3930-3939.

67. Kudchodkar SB, Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus infection induces rapamycin-insensitive phosphorylation of downstream effectors of mTOR kinase. *J Virol.* 2004;78(20):11030-11039.

68. Kudchodkar SB, Del Prete GQ, Maguire TG, Alwine JC. AMPK-mediated inhibition of mTOR kinase is circumvented during immediate-early times of human cytomegalovirus infection. *J Virol.* 2007;81(7):3649-3651.

69. Kudchodkar SB, Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus infection alters the substrate specificities and rapamycin sensitivities of raptor- and rictor-containing complexes. *Proc Natl Acad Sci U S A.* 2006;103(38):14182-14187.

70. Robertsen I, Vethe NT, Midtvedt K, Falck P, Christensen H, Asberg A. Closer to the Site of Action: Everolimus Concentrations in Peripheral Blood Mononuclear Cells Correlate Well With Whole Blood Concentrations. *Ther Drug Monit.* 2015;37(5):675-680.

71. Ghareeb M, Akhlaghi F. Alternative matrices for therapeutic drug monitoring of immunosuppressive agents using LC-MS/MS. *Bioanalysis.* 2015;7(8):1037-1058.

72. Zhang Y, Zhang R. Recent advances in analytical methods for the therapeutic drug monitoring of immunosuppressive drugs. *Drug Test Anal.* 2018;10(1):81-94.

73. Keevil BG. The analysis of dried blood spot samples using liquid chromatography tandem mass spectrometry. *Clin Biochem.* 2011;44(1):110-118.

74. Capiau S, Veenhof H, Koster RA, et al. Official International Association for Therapeutic Drug Monitoring and Clinical Toxicology Guideline: Development and Validation of Dried Blood Spot-Based Methods for Therapeutic Drug Monitoring. *Ther Drug Monit.* 2019;41(4):409-430.

75. Le J, Peng R, Yang SL, et al. Quantification of immunosuppressants from one 3.2 mm dried blood spot by a novel cold-induced phase separation based LC-MS/MS method. *Anal Chim Acta.* 2022;1210:339889.

76. Deprez S, Stove CP. Fully Automated Dried Blood Spot Extraction coupled to Liquid Chromatography-tandem Mass Spectrometry for Therapeutic Drug Monitoring of Immunosuppressants. *J Chromatogr A.* 2021;1653:462430.

77. Klak A, Pauwels S, Vermeersch P. Preanalytical considerations in therapeutic drug monitoring of immunosuppressants with dried blood spots. *Diagnosis (Berl).* 2019;6(1):57-68.

78. Knapen LM, Beer Y, Bruggemann RJM, et al. Development and validation of an analytical method using UPLC-MS/MS to quantify everolimus in dried blood spots in the oncology setting. *J Pharm Biomed Anal.* 2018;149:106-113.

79. Wilhelm AJ, den Burger JC, Swart EL. Therapeutic drug monitoring by dried blood spot: progress to date and future directions. *Clin Pharmacokinet.* 2014;53(11):961-973.

80. Veenhof H, Koster RA, Alffenaar JC, et al. Clinical application of a dried blood spot assay for sirolimus and everolimus in transplant patients. *Clin Chem Lab Med.* 2019;57(12):1854-1862.

81. Willemsen A, Knapen LM, de Beer YM, et al. Clinical validation study of dried blood spot for determining everolimus concentration in patients with cancer. *Eur J Clin Pharmacol.* 2018;74(4):465-471.

82. Bressan IG, Gimenez MI, Llesuy SF. Clinical validation of a liquid chromatography-tandem mass spectrometry method for the quantification of calcineurin and mTOR inhibitors in dried matrix on paper discs. *J Mass Spectrom Adv Clin Lab.* 2022;25:12-18.

83. Tey HY, See HH. A review of recent advances in microsampling techniques of biological fluids for therapeutic drug monitoring. *J Chromatogr A.* 2021;1635:461731.

84. Gruzdys V, Merrigan SD, Johnson-Davis KL. Feasibility of Immunosuppressant Drug Monitoring by a Microsampling Device. *J Appl Lab Med.* 2019;4(2):241-246.

85. Paniagua-Gonzalez L, Diaz-Louzao C, Lendoiro E, et al. Volumetric Absorptive Microsampling (VAMS) for assaying immunosuppressants from venous whole blood by LC-MS/MS using a novel atmospheric pressure ionization probe (UniSpray). *J Pharm Biomed Anal.* 2020;189:113422.

86. Koster RA, Niemeijer P, Veenhof H, Hateren KV, Alffenaar JC, Touw DJ. A volumetric absorptive microsampling LC-MS/MS method for five immunosuppressants and their hematocrit effects. *Bioanalysis.* 2019;11(6):495-508.

87. Verheijen RB, Thijssen B, Atrafi F, et al. Validation and clinical application of an LC-MS/MS method for the quantification of everolimus using volumetric absorptive microsampling. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2019;1104:234-239.

88. Paniagua-Gonzalez L, Lendoiro E, Otero-Anton E, et al. A multidrug LC-MS/MS method for the determination of five immunosuppressants in oral fluid. *Bioanalysis.* 2019;11(16):1509-1521.

89. Molenaar-Kuijsten L, Verheijen RB, Jacobs BAW, et al. Everolimus Concentration in Saliva to Predict Stomatitis: A Feasibility Study in Patients with Cancer. *Ther Drug Monit.* 2022;44(4):520-526.

90. Capron A, Haufroid V, Wallemacq P. Intra-cellular immunosuppressive drugs monitoring: A step forward towards better therapeutic efficacy after organ transplantation? *Pharmacol Res.* 2016;111:610-618.

91. Roullet-Renoleau F, Lemaitre F, Antignac M, Zahr N, Farinotti R, Fernandez C. Everolimus quantification in peripheral blood mononuclear cells using ultra high performance liquid chromatography tandem mass spectrometry. *J Pharm Biomed Anal.* 2012;66:278-281.

92. Pensi D, De Nicolo A, Pinon M, et al. First UHPLC-MS/MS method coupled with automated online SPE for quantification both of tacrolimus and everolimus in peripheral blood mononuclear cells and its application on samples from co-treated pediatric patients. *J Mass Spectrom.* 2017;52(3):187-195.

93. Lemaitre F, Antignac M, Verdier MC, Bellissant E, Fernandez C. Opportunity to monitor immunosuppressive drugs in peripheral blood mononuclear cells: where are we and where are we going? *Pharmacol Res.* 2013;74:109-112.

94. Seger C, Shipkova M, Christians U, et al. Assuring the Proper Analytical Performance of Measurement Procedures for Immunosuppressive Drug Concentrations in Clinical Practice: Recommendations of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology Immunosuppressive Drug Scientific Committee. *Ther Drug Monit.* 2016;38(2):170-189.

95. Morgan P, Nwafor M, Tredger M. Use of a small particle solid-core packing for improved efficiency and rapid measurement of sirolimus and everolimus by LC-MS/MS. *Biomed Chromatogr.* 2016;30(6):983-985.

96. Miyagi C, Tanaka R, Hirata K, et al. High-Sensitivity and High-Throughput Quantification of Everolimus in Human Whole Blood Using Ultrahigh-Performance Liquid Chromatography Coupled With Tandem Mass Spectrometry. *Ther Drug Monit.* 2022;44(5):633-640.

97. Kvamsoe MM, Hansen KR, Skadberg O, Vethe NT, Brede C. Salting Out-Assisted Liquid-Liquid Extraction for Liquid Chromatography-Tandem Mass Spectrometry Measurement of Tacrolimus, Sirolimus, Everolimus, and Cyclosporine a in Whole Blood. *Ther Drug Monit.* 2020;42(5):695-701.

98. Zheng M, Song J, Xue H, Li H, Lian K. Simultaneous Determination of Six Immunosuppressants in Human Whole Blood by HPLC-MS/MS Using a Modified QuEChERS Method. *Molecules.* 2022;27(13).

99. Horber S, Peter A, Lehmann R, Hoene M. Evaluation of the first immunosuppressive drug assay available on a fully automated LC-MS/MS-based clinical analyzer suggests a new era in laboratory medicine. *Clin Chem Lab Med.* 2021;59(5):913-920.

100. Bruns K, Monnikes R, Lackner KJ. Quantitative determination of four immunosuppressants by high resolution mass spectrometry (HRMS). *Clin Chem Lab Med.* 2016;54(7):1193-1200.

101. Taibon J, van Rooij M, Schmid R, et al. An isotope dilution LC-MS/MS based candidate reference method for the quantification of cyclosporine A, tacrolimus, sirolimus and everolimus in human whole blood. *Clin Biochem.* 2020;82:73-84.

102. Verstraete AG, Rigo-Bonnin R, Wallemacq P, et al. Multicenter Evaluation of a New Electrochemiluminescence Immunoassay for Everolimus Concentrations in Whole Blood. *Ther Drug Monit.* 2018;40(1):59-68.

103. Shipkova M, Rapp S, Rigo-Bonnin R, Wieland E, Peter A. Therapeutic Drug Monitoring of Everolimus: Comparability of Concentrations Determined by 2 Immunoassays and a Liquid Chromatography Tandem Mass Spectrometry Method. *Ther Drug Monit.* 2017;39(2):102-108.

104. Schniedewind B, Meyer EJ, Christians U. Long-Term Performance of Laboratory-Developed Liquid Chromatography-Tandem Mass Spectrometry Tests and a Food and Drug Administration-Approved Immunoassay for the Therapeutic Drug Monitoring of Everolimus. *Ther Drug Monit.* 2020;42(3):421-426.

105. Lee EJ, Kim HK, Ahn S, et al. Accuracy evaluation of automated electrochemiluminescence immunoassay for everolimus and sirolimus compared to liquid chromatography-tandem mass spectrometry. *J Clin Lab Anal.* 2019;33(7):e22941.

106. Akamine Y, Sato S, Kagaya H, Ohkubo T, Satoh S, Miura M. Comparison of electrochemiluminescence immunoassay and latex agglutination turbidimetric immunoassay for evaluation of everolimus blood concentrations in renal transplant patients. *J Clin Pharm Ther.* 2018;43(5):675-681.

107. Ialongo C, Sapio M, Angeloni A. Analytical Performance of the New Siemens Affinity Chrome-Mediated Immunoassay Everolimus Assay and Its Interchangeability With the Thermo Quantitative Microsphere System for Routine Therapeutic Drug Monitoring of Patients After Solid Organ Transplantation. *Ther Drug Monit.* 2023;45(2):217-222.

108. Brede C, Vethe NT, Skadberg O. The Question of Accuracy Versus Interlaboratory Agreement for Monitoring the Immunosuppressants Everolimus and Sirolimus. *Ther Drug Monit.* 2021;43(3):444-446.

109. Seger C, Salzmann L. After another decade: LC-MS/MS became routine in clinical diagnostics. *Clin Biochem.* 2020;82:2-11.

110. Miller WG, Greenberg N. Harmonization and Standardization: Where Are We Now? *J Appl Lab Med.* 2021;6(2):510-521.

111. Rigo-Bonnin R, Canalias F. Traceability of immunosuppressant's mass concentration results obtained using different commercial calibrators. *Clin Biochem.* 2019;63:113-120.

112. Giancaspro G, Adams KM, Bhavaraju S, et al. The qNMR Summit 5.0: Proceedings and Status of qNMR Technology. *Anal Chem.* 2021;93(36):12162-12169.

113. Rigo-Bonnin R, Diaz-Troyano N, Garcia-Tejada L, Marce-Galindo A, Valbuena-Asensio M, Canalias F. Estimation of the measurement uncertainty and practical suggestion for the description of the metrological traceability in clinical laboratories. *Biochem Med (Zagreb).* 2021;31(1):010501.

114. Rigo-Bonnin R, Alia P, Canalias F. Measurement uncertainty and metrological traceability of whole blood cyclosporin A mass concentration results obtained by UHPLC-MS/MS. *Clin Chem Lab Med.* 2018;56(9):1458-1468.

115. Kovarik JM, Noe A, Berthier S, et al. Clinical development of an everolimus pediatric formulation: relative bioavailability, food effect, and steady-state pharmacokinetics. *J Clin Pharmacol.* 2003;43(2):141-147.

116. Doyle RL, Hertz MI, Dunitz JM, et al. RAD in stable lung and heart/lung transplant recipients: safety, tolerability, pharmacokinetics, and impact of cystic fibrosis. *J Heart Lung Transplant.* 2001;20(3):330-339.

117. Kovarik JM, Beyer D, Schmouder RL. Everolimus drug interactions: application of a classification system for clinical decision making. *Biopharm Drug Dispos.* 2006;27(9):421-426.

118. Okihara M, Takeuchi H, Akiyama S, et al. Pharmacodynamic Drug-Drug Interaction on Human Peripheral Blood Mononuclear Cells Between Everolimus and Tacrolimus at the Therapeutic Concentration Range in Renal Transplantation. *Ann Transplant.* 2021;26:e928817.

119. 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(1):113-116.

120. 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(2):217-226.

121. Kovarik JM, Curtis JJ, Hricik DE, Pescovitz MD, Scantlebury V, Vasquez A. Differential pharmacokinetic interaction of tacrolimus and cyclosporine on everolimus. *Transplant Proc.* 2006;38(10):3456-3458.

122. Christians U, Jacobsen W, Benet LZ, Lampen A. Mechanisms of clinically relevant drug interactions associated with tacrolimus. *Clin Pharmacokinet.* 2002;41(11):813-851.

123. Lefeuvre S, Rebaudet S, Billaud EM, Wyplosz B. Management of rifamycins-everolimus drug-drug interactions in a liver-transplant patient with pulmonary tuberculosis. *Transpl Int.* 2012;25(11):e120-123.

124. 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(7):481-486.

125. Wang YC, Salvador NG, Lin CC, et al. Comparative analysis of the drug-drug interaction between immunosuppressants, safety and efficacy of rifabutin from rifampicin-based Anti-TB treatment in living donor liver transplant recipients with active tuberculosis. *Biomed J.* 2021;44(6 Suppl 2):S162-S170.

126. Molenaar-Kuijsten L, Van Balen DEM, Beijnen JH, Steeghs N, Huitema ADR. A Review of CYP3A Drug-Drug Interaction Studies: Practical Guidelines for Patients Using Targeted Oral Anticancer Drugs. *Front Pharmacol.* 2021;12:670862.

127. Marcath LA, Finley CM, Wong SF, Hertz DL. Drug-drug interactions in subjects enrolled in SWOG trials of oral chemotherapy. *BMC Cancer.* 2021;21(1):324.

128. Miklja Z, Yadav VN, Cartaxo RT, et al. Everolimus improves the efficacy of dasatinib in PDGFRalpha-driven glioma. *J Clin Invest.* 2020;130(10):5313-5325.

129. Page RL, 2nd, Allen LA, Kloner RA, et al. Medical Marijuana, Recreational Cannabis, and Cardiovascular Health: A Scientific Statement From the American Heart Association. *Circulation.* 2020;142(10):e131-e152.

130. Gilmartin CGS, Dowd Z, Parker APJ, Harijan P. Interaction of cannabidiol with other antiseizure medications: A narrative review. *Seizure.* 2021;86:189-196.

131. Deppenweiler M, Falkowski S, Saint-Marcoux F, et al. Towards therapeutic drug monitoring of everolimus in cancer? Results of an exploratory study of exposure-effect relationship. *Pharmacol Res.* 2017;121:138-144.

132. 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(5):999-1007.

133. Ravaud A, Urva SR, Grosch K, Cheung WK, Anak O, Sellami DB. Relationship between everolimus exposure and safety and efficacy: meta-analysis of clinical trials in oncology. *Eur J Cancer.* 2014;50(3):486-495.

134. Willemsen A, de Geus-Oei LF, de Boer M, et al. Everolimus Exposure and Early Metabolic Response as Predictors of Treatment Outcomes in Breast Cancer Patients Treated with Everolimus and Exemestane. *Target Oncol.* 2018;13(5):641-648.

135. Falkowski S, Woillard JB. Therapeutic Drug Monitoring of Everolimus in Oncology: Evidences and Perspectives. *Ther Drug Monit.* 2019;41(5):568-574.

136. Pritchard KI, Burris HA, 3rd, Ito Y, et al. Safety and efficacy of everolimus with exemestane vs. exemestane alone in elderly patients with HER2-negative, hormone receptor-positive breast cancer in BOLERO-2. *Clin Breast Cancer.* 2013;13(6):421-432 e428.

137. de Wit D, Schneider TC, Moes DJ, et al. Everolimus pharmacokinetics and its exposure-toxicity relationship in patients with thyroid cancer. *Cancer Chemother Pharmacol.* 2016;78(1):63-71.

138. Verheijen RB, Atrafi F, Schellens JHM, et al. Pharmacokinetic Optimization of Everolimus Dosing in Oncology: A Randomized Crossover Trial. *Clin Pharmacokinet.* 2018;57(5):637-644.

139. Krueger DA, Care MM, Holland K, et al. Everolimus for subependymal giant-cell astrocytomas in tuberous sclerosis. *N Engl J Med.* 2010;363(19):1801-1811.

140. Kovarik JM, Hsu CH, McMahon L, Berthier S, Rordorf C. Population pharmacokinetics of everolimus in de novo renal transplant patients: impact of ethnicity and comedications. *Clin Pharmacol Ther.* 2001;70(3):247-254.

141. Moes DJ, Press RR, den Hartigh J, van der Straaten T, de Fijter JW, Guchelaar HJ. Population pharmacokinetics and pharmacogenetics of everolimus in renal transplant patients. *Clin Pharmacokinet.* 2012;51(7):467-480.

142. 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.

143. Ter Heine R, van Erp NP, Guchelaar HJ, et al. A pharmacological rationale for improved everolimus dosing in oncology and transplant patients. *Br J Clin Pharmacol.* 2018;84(7):1575-1586.

144. Zwart TC, Moes D, van der Boog PJM, et al. Model-Informed Precision Dosing of Everolimus: External Validation in Adult Renal Transplant Recipients. *Clin Pharmacokinet.* 2021;60(2):191-203.

145. 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(6):686-694.

146. Tanaka A, Yano I, Shinsako K, et al. Population Pharmacokinetics of Everolimus in Relation to Clinical Outcomes in Patients With Advanced Renal Cell Carcinoma. *Ther Drug Monit.* 2016;38(6):663-669.

147. Combes FP, Baneyx G, Coello N, et al. Population pharmacokinetics-pharmacodynamics of oral everolimus in patients with seizures associated with tuberous sclerosis complex. *J Pharmacokinet Pharmacodyn.* 2018;45(5):707-719.

148. van Erp NP, van Herpen CM, de Wit D, et al. A Semi-Physiological Population Model to Quantify the Effect of Hematocrit on Everolimus Pharmacokinetics and Pharmacodynamics in Cancer Patients. *Clin Pharmacokinet.* 2016;55(11):1447-1456.

149. Robertsen I, Debord J, Asberg A, Marquet P, Woillard JB. A Limited Sampling Strategy to Estimate Exposure of Everolimus in Whole Blood and Peripheral Blood Mononuclear Cells in Renal Transplant Recipients Using Population Pharmacokinetic Modeling and Bayesian Estimators. *Clin Pharmacokinet.* 2018;57(11):1459-1469.

150. Labriffe M, Woillard JB, Debord J, Marquet P. Machine learning algorithms to estimate everolimus exposure trained on simulated and patient pharmacokinetic profiles. *CPT Pharmacometrics Syst Pharmacol.* 2022;11(8):1018-1028.

151. van Gelder T, Vinks AA. Machine Learning as a Novel Method to Support Therapeutic Drug Management and Precision Dosing. *Clin Pharmacol Ther.* 2021;110(2):273-276.

152. Barrett JS, Barrett RF, Vinks AA. Status Toward the Implementation of Precision Dosing in Children. *J Clin Pharmacol.* 2021;61 Suppl 1:S36-S51.

153. Darwich AS, Ogungbenro K, Vinks AA, et al. Why has model-informed precision dosing not yet become common clinical reality? lessons from the past and a roadmap for the future. *Clin Pharmacol Ther.* 2017;101(5):646-656.

154. Polasek TM, Rostami-Hodjegan A, Yim DS, et al. What Does it Take to Make Model-Informed Precision Dosing Common Practice? Report from the 1st Asian Symposium on Precision Dosing. *AAPS J.* 2019;21(2):17.

155. Mizuno T, Emoto C, Fukuda T, Hammill AM, Adams DM, Vinks AA. Model-based precision dosing of sirolimus in pediatric patients with vascular anomalies. *Eur J Pharm Sci.* 2017;109S:S124-S131.

156. Mizuno T, O'Brien MM, Vinks AA. Significant effect of infection and food intake on sirolimus pharmacokinetics and exposure in pediatric patients with acute lymphoblastic leukemia. *Eur J Pharm Sci.* 2019;128:209-214.

157. Immunosuppressants Bayesian Dose Adjustment (ISBA) - Centre Hospitalier Universitaire at Limoges. https://abis.chu-limoges.fr/login. Accessed April 6, 2023.

158. 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(6):920-925.

159. 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(5):499-505.

160. Qazi Y, Shaffer D, Kaplan B, et al. Efficacy and Safety of Everolimus Plus Low-Dose Tacrolimus Versus Mycophenolate Mofetil Plus Standard-Dose Tacrolimus in De Novo Renal Transplant Recipients: 12-Month Data. *Am J Transplant.* 2017;17(5):1358-1369.

161. Shihab F, Qazi Y, Mulgaonkar S, et al. Association of Clinical Events With Everolimus Exposure in Kidney Transplant Patients Receiving Low Doses of Tacrolimus. *Am J Transplant.* 2017;17(9):2363-2371.

162. 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(5):1192-1198.

163. de Fijter JW, Holdaas H, Oyen O, et al. Early Conversion From Calcineurin Inhibitor- to Everolimus-Based Therapy Following Kidney Transplantation: Results of the Randomized ELEVATE Trial. *Am J Transplant.* 2017;17(7):1853-1867.

164. Pascual J, Berger SP, Witzke O, et al. Everolimus with Reduced Calcineurin Inhibitor Exposure in Renal Transplantation. *J Am Soc Nephrol.* 2018;29(7):1979-1991.

165. Sommerer C, Suwelack B, Dragun D, et al. Design and rationale of the ATHENA study--A 12-month, multicentre, prospective study evaluating the outcomes of a de novo everolimus-based regimen in combination with reduced cyclosporine or tacrolimus versus a standard regimen in kidney transplant patients: study protocol for a randomised controlled trial. *Trials.* 2016;17:92.

166. Sommerer C, Suwelack B, Dragun D, et al. An open-label, randomized trial indicates that everolimus with tacrolimus or cyclosporine is comparable to standard immunosuppression in de novo kidney transplant patients. *Kidney Int.* 2019;96(1):231-244.

167. Chadban S, Tedesco-Silva H. ATHENA: wisdom and warfare in defining the role of de novo mTOR inhibition in kidney transplantation. *Kidney Int.* 2019;96(1):27-30.

168. Ahlenstiel-Grunow T, Liu X, Schild R, et al. Steering Transplant Immunosuppression by Measuring Virus-Specific T Cell Levels: The Randomized, Controlled IVIST Trial. *J Am Soc Nephrol.* 2021;32(2):502-516.

169. de Boer SE, Sanders JSF, Bemelman FJ, et al. Rationale and design of the OPTIMIZE trial: OPen label multicenter randomized trial comparing standard IMmunosuppression with tacrolimus and mycophenolate mofetil with a low exposure tacrolimus regimen In combination with everolimus in de novo renal transplantation in Elderly patients. *BMC Nephrol.* 2021;22(1):208.

170. de Boer SE, Berger SP, van Leer-Buter CC, et al. Enhanced Humoral Immune Response After COVID-19 Vaccination in Elderly Kidney Transplant Recipients on Everolimus Versus

Mycophenolate Mofetil-containing Immunosuppressive Regimens. *Transplantation.* 2022;106(8):1615-1621.

171. Watt KD, Charlton MR. Metabolic syndrome and liver transplantation: a review and guide to management. *J Hepatol.* 2010;53(1):199-206.

172. Tedesco-Silva H, Felipe C, Ferreira A, et al. Reduced Incidence of Cytomegalovirus Infection in Kidney Transplant Recipients Receiving Everolimus and Reduced Tacrolimus Doses. *Am J Transplant.* 2015;15(10):2655-2664.

173. Levy G, Schmidli H, Punch J, et al. Safety, tolerability, and efficacy of everolimus in de novo liver transplant recipients: 12- and 36-month results. *Liver Transpl.* 2006;12(11):1640-1648.

174. Nashan B, Schemmer P, Braun F, et al. Early Everolimus-Facilitated Reduced Tacrolimus in Liver Transplantation: Results From the Randomized HEPHAISTOS Trial. *Liver Transpl.* 2022;28(6):998-1010.

175. Saliba F, Fischer L, de Simone P, Bernhardt P, Bader G, Fung J. Association Between Renal Dysfunction and Major Adverse Cardiac Events After Liver Transplantation: Evidence from an International Randomized Trial of Everolimus-Based Immunosuppression. *Ann Transplant.* 2018;23:751-757.

176. Sterneck M, Kaiser GM, Heyne N, et al. Long-term follow-up of five yr shows superior renal function with everolimus plus early calcineurin inhibitor withdrawal in the PROTECT randomized liver transplantation study. *Clin Transplant.* 2016;30(6):741-748.

177. Gomez-Bravo M, Prieto Castillo M, Navasa M, et al. Effects of everolimus plus minimized tacrolimus on kidney function in liver transplantation: REDUCE, a prospective, randomized controlled study. *Rev Esp Enferm Dig.* 2022;114(6):335-342.

178. Jeng LB, Lee SG, Soin AS, et al. Efficacy and safety of everolimus with reduced tacrolimus in living-donor liver transplant recipients: 12-month results of a randomized multicenter study. *Am J Transplant.* 2018;18(6):1435-1446.

179. Saliba F, Dharancy S, Salame E, et al. Time to Conversion to an Everolimus-Based Regimen: Renal Outcomes in Liver Transplant Recipients From the EVEROLIVER Registry. *Liver Transpl.* 2020;26(11):1465-1476.

180. 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 Transpl.* 2009;15(10):1262-1269.

181. Lemaitre F, Tron C, Renard T, et al. Redefining Therapeutic Drug Monitoring of Tacrolimus in Patients Undergoing Liver Transplantation: A Target Trough Concentration of 4-7 ng/mL During the First Month After Liver Transplantation is Safe and Improves Graft and Renal Function. *Ther Drug Monit.* 2020;42(5):671-678.

182. Cillo U, Saracino L, Vitale A, et al. Very Early Introduction of Everolimus in De Novo Liver Transplantation: Results of a Multicenter, Prospective, Randomized Trial. *Liver Transpl.* 2019;25(2):242-251.

183. Saliba F, Duvoux C, Gugenheim J, et al. Efficacy and Safety of Everolimus and Mycophenolic Acid With Early Tacrolimus Withdrawal After Liver Transplantation: A Multicenter Randomized Trial. *Am J Transplant.* 2017;17(7):1843-1852.

184. 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(11):3008-3020.

185. Saliba F, Duvoux C, Dharancy S, et al. Five-year outcomes in liver transplant patients receiving everolimus with or without a calcineurin inhibitor: Results from the CERTITUDE study. *Liver Int.* 2022;42(11):2513-2523.

186. 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(7):1221-1225.

187. 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. *Japanese Journal of Therapeutic Drug Monitoring.* 2014;31(1):1-5.

188. Uebayashi EY, Okajima H, Yamamoto M, et al. The New Challenge in Pediatric Liver Transplantation: Chronic Antibody-Mediated Rejection. *J Clin Med.* 2022;11(16).

189. Bakouny Z, Assi T, El Rassy E, Nasr F. Second-line Treatments of Advanced Hepatocellular Carcinoma: Systematic Review and Network Meta-Analysis of Randomized Controlled Trials. *J Clin Gastroenterol.* 2019;53(4):251-261.

190. Global Cancer Observatory https://gco.iarc.fr/. Accessed June 5, 2023.

191. Kim JM. Can hepatocellular carcinoma recurrence be prevented after liver transplantation? *Clin Mol Hepatol.* 2021;27(4):562-563.

192. Nitta H, Younes A, El-Domiaty N, et al. High trough levels of everolimus combined to sorafenib improve patients survival after hepatocellular carcinoma recurrence in liver transplant recipients. *Transpl Int.* 2021;34(7):1293-1305.

193. Hoffman D, Mehta N. Recurrence of hepatocellular carcinoma following liver transplantation. *Expert Rev Gastroenterol Hepatol.* 2021;15(1):91-102.

194. Pelizzaro F, Gambato M, Gringeri E, et al. Management of Hepatocellular Carcinoma Recurrence after Liver Transplantation. *Cancers (Basel).* 2021;13(19).

195. 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(11):693-699.

196. Cholongitas E, Mamou C, Rodriguez-Castro KI, Burra P. Mammalian target of rapamycin inhibitors are associated with lower rates of hepatocellular carcinoma recurrence after liver transplantation: a systematic review. *Transpl Int.* 2014;27(10):1039-1049.

197. Ferrin G, Guerrero M, Amado V, Rodriguez-Peralvarez M, De la Mata M. Activation of mTOR Signaling Pathway in Hepatocellular Carcinoma. *Int J Mol Sci.* 2020;21(4).

198. Grigg SE, Sarri GL, Gow PJ, Yeomans ND. Systematic review with meta-analysis: sirolimus- or everolimus-based immunosuppression following liver transplantation for hepatocellular carcinoma. *Aliment Pharmacol Ther.* 2019;49(10):1260-1273.

199. Kang I, Lee JG, Choi SH, et al. Impact of everolimus on survival after liver transplantation for hepatocellular carcinoma. *Clin Mol Hepatol.* 2021;27(4):589-602.

200. Yan X, Huang S, Yang Y, et al. Sirolimus or Everolimus Improves Survival After Liver Transplantation for Hepatocellular Carcinoma: A Systematic Review and Meta-Analysis. *Liver Transpl.* 2022;28(6):1063-1077.

201. Cholongitas E. What Is the Impact of Mammalian Target of Rapamycin Inhibitors on Hepatocellular Carcinoma Recurrence After Liver Transplantation. *Transplantation.* 2022;106(3):e189.

202. Rajendran L, Ivanics T, Claasen MP, Muaddi H, Sapisochin G. The management of post-transplantation recurrence of hepatocellular carcinoma. *Clin Mol Hepatol.* 2022;28(1):1-16.

203. Rubin Suarez A, Bilbao Aguirre I, Fernandez-Castroagudin J, et al. Recommendations of everolimus use in liver transplant. *Gastroenterol Hepatol.* 2017;40(9):629-640.

204. Zhu AX, Kudo M, Assenat E, et al. Effect of everolimus on survival in advanced hepatocellular carcinoma after failure of sorafenib: the EVOLVE-1 randomized clinical trial. *JAMA.* 2014;312(1):57-67.

205. 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(9):847-858.

206. de Queiroz JM, Jr., Blanks JC, Ozler SA, Alfaro DV, Liggett PE. Subretinal perfluorocarbon liquids. An experimental study. *Retina.* 1992;12(3 Suppl):S33-39.

207. Muller-Lissner S. [Pathophysiology of constipation]. *Z Arztl Fortbild (Jena).* 1992;86(3):87-90.

208. Lehmkuhl 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(7):700-704.

209. 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(1):115-122.

210. Davis SJ, Teresi LM, Bradley WG, Burke JW. The "notch" sign: meniscal contour deformities as indicators of tear in MR imaging of the knee. *J Comput Assist Tomogr.* 1990;14(6):975-980.

211. Eisen HJ, Kobashigawa J, Starling RC, et al. Everolimus versus mycophenolate mofetil in heart transplantation: a randomized, multicenter trial. *Am J Transplant.* 2013;13(5):1203-1216.

212. 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(12):1581-1589.

213. 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(3):259-265.

214. Barten MJ, Hirt SW, Garbade J, et al. Comparing everolimus-based immunosuppression with reduction or withdrawal of calcineurin inhibitor reduction from six months after heart transplantation: the randomized MANDELA study. *Am J Transplant.* 2019.

215. Rothenburger M, Teerling E, Bruch C, et al. Calcineurin inhibitor-free immunosuppression using everolimus (Certican) in maintenance heart transplant recipients: 6 months' follow-up. *J Heart Lung Transplant.* 2007;26(3):250-257.

216. Gude E, Gullestad L, Arora S, et al. Benefit of early conversion from CNI-based to everolimus-based immunosuppression in heart transplantation. *J Heart Lung Transplant.* 2010;29(6):641-647.

217. 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(8):1828-1838.

218. Andreassen AK, Andersson B, Gustafsson F, et al. Everolimus Initiation With Early Calcineurin Inhibitor Withdrawal in De Novo Heart Transplant Recipients: Three-Year Results From the Randomized SCHEDULE Study. *Am J Transplant.* 2016;16(4):1238-1247.

219. Gustafsson F, Andreassen AK, Andersson B, et al. Everolimus Initiation With Early Calcineurin Inhibitor Withdrawal in De Novo Heart Transplant Recipients: Long-term Follow-up From the Randomized SCHEDULE Study. *Transplantation.* 2020;104(1):154-164.

220. King-Biggs MB, Dunitz JM, Park SJ, Kay Savik S, Hertz MI. Airway anastomotic dehiscence associated with use of sirolimus immediately after lung transplantation. *Transplantation.* 2003;75(9):1437-1443.

221. 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(5):632-638.

222. de Pablo A, Santos F, Sole A, et al. Recommendations on the use of everolimus in lung transplantation. *Transplant Rev (Orlando).* 2013;27(1):9-16.

223. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. *Crit Rev Oncol Hematol.* 2005;56(1):23-46.

224. Schmucki K, Hofmann P, Fehr T, Inci I, Kohler M, Schuurmans MM. Mammalian Target of Rapamycin Inhibitors and Kidney Function After Thoracic Transplantation: A Systematic Review and Recommendations for Management of Lung Transplant Recipients. *Transplantation.* 2023;107(1):53-73.

225. 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(1):169-177.

226. Kovarik JM, Snell GI, Valentine V, et al. Everolimus in pulmonary transplantation: pharmacokinetics and exposure-response relationships. *J Heart Lung Transplant.* 2006;25(4):440-446.

227. Schneer S, Kramer MR, Fox B, et al. Renal function preservation with the mTOR inhibitor, Everolimus, after lung transplant. *Clin Transplant.* 2014;28(6):662-668.

228. Glanville AR, Aboyoun 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(1):16-25.

229. Strueber M, Warnecke G, Fuge J, et al. Everolimus Versus Mycophenolate Mofetil De Novo After Lung Transplantation: A Prospective, Randomized, Open-Label Trial. *Am J Transplant.* 2016;16(11):3171-3180.

230. Gottlieb J, Neurohr C, Muller-Quernheim J, et al. A randomized trial of everolimus-based quadruple therapy vs standard triple therapy early after lung transplantation. *Am J Transplant.* 2019;19(6):1759-1769.

231. Gullestad L, Eiskjaer H, Gustafsson F, et al. Long-term outcomes of thoracic transplant recipients following conversion to everolimus with reduced calcineurin inhibitor in a multicenter, open-label, randomized trial. *Transpl Int.* 2016;29(7):819-829.

232. Bos S, De Sadeleer LJ, Yserbyt J, et al. Real life experience with mTOR-inhibitors after lung transplantation. *Int Immunopharmacol.* 2021;94:107501.

233. Parada MT, Alba A, Sepulveda C. Everolimus in lung transplantation in Chile. *Transplant Proc.* 2010;42(1):328-330.

234. Parada MT, Alba A, Sepulveda C, Melo J. Long-term use of everolimus in lung transplant patients. *Transplant Proc.* 2011;43(6):2313-2315.

235. Roman A, Ussetti P, Zurbano F, et al. A retrospective 12-month study of conversion to everolimus in lung transplant recipients. *Transplant Proc.* 2011;43(7):2693-2698.

236. Gruessner AC, Gruessner RW. Pancreas Transplantation of US and Non-US Cases from 2005 to 2014 as Reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR). *Rev Diabet Stud.* 2016;13(1):35-58.

237. Kawecki D, Kwiatkowski A, Michalak G, et al. Urinary tract infections in the early posttransplant period after simultaneous pancreas-kidney transplantation. *Transplant Proc.* 2009;41(8):3148-3150.

238. Lopez-Medrano F, Munoz de la Espada M, Perez-Jacoiste Asin MA, et al. Fluconazole versus micafungin for initial antifungal prophylaxis against Candida in pancreas transplant recipients: A comparative study of two consecutive periods. *Mycoses.* 2022;65(5):517-525.

239. Smets YF, van der Pijl JW, van Dissel JT, Ringers J, de Fijter JW, Lemkes HH. Infectious disease complications of simultaneous pancreas kidney transplantation. *Nephrol Dial Transplant.* 1997;12(4):764-771.

240. Vidal E, Torre-Cisneros J, Blanes M, et al. Bacterial urinary tract infection after solid organ transplantation in the RESITRA cohort. *Transpl Infect Dis.* 2012;14(6):595-603.

241. Barlow AD, Nicholson ML, Herbert TP. Evidence for rapamycin toxicity in pancreatic beta-cells and a review of the underlying molecular mechanisms. *Diabetes.* 2013;62(8):2674-2682.

242. Siskind EJ, Liu C, Collins DT, et al. Use of Mammalian Target of Rapamycin Inhibitors for Pancreas Transplant Immunosuppression Is Associated With Improved Allograft Survival and Improved Early Patient Survival. *Pancreas.* 2019;48(5):644-651.

243. Boggi U, Vistoli F, Marchetti P, Kandaswamy R, Berney T, World Consensus Group on Pancreas T. First world consensus conference on pancreas transplantation: Part I-Methods and results of literature search. *Am J Transplant.* 2021;21 Suppl 3(Suppl 3):1-16.

244. Boggi U, Vistoli F, Andres A, et al. First World Consensus Conference on pancreas transplantation: Part II - recommendations. *Am J Transplant.* 2021;21 Suppl 3(Suppl 3):17-59.

245. Cantarovich D, Kervella D, Karam G, et al. Tacrolimus- versus sirolimus-based immunosuppression after simultaneous pancreas and kidney transplantation: 5-year results of a randomized trial. *Am J Transplant.* 2020;20(6):1679-1690.

246. Ciancio G, Sageshima J, Chen L, et al. Advantage of rapamycin over mycophenolate mofetil when used with tacrolimus for simultaneous pancreas kidney transplants: randomized, single-center trial at 10 years. *Am J Transplant.* 2012;12(12):3363-3376.

247. Sageshima J, Ciancio G, Chen L, et al. Everolimus with low-dose tacrolimus in simultaneous pancreas and kidney transplantation. *Clin Transplant.* 2014;28(7):797-801.

248. Lehner F, Budde K, Zeier M, et al. Efficacy and safety of conversion from cyclosporine to everolimus in living-donor kidney transplant recipients: an analysis from the ZEUS study. *Transpl Int.* 2014;27(11):1192-1204.

249. Mjornstedt L, Schwartz Sorensen S, von Zur Muhlen 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(1):42-51.

250. 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(1):119-128.

251. Brakemeier S, Arns W, Lehner F, et al. Everolimus in de novo kidney transplant recipients participating in the Eurotransplant senior program: Results of a prospective randomized multicenter study (SENATOR). *PLoS One.* 2019;14(9):e0222730.

252. Tonshoff B, Tedesco-Silva H, Ettenger R, et al. Three-year outcomes from the CRADLE study in de novo pediatric kidney transplant recipients receiving everolimus with reduced tacrolimus and early steroid withdrawal. *Am J Transplant.* 2021;21(1):123-137.

253. Sommerer C, Budde K, Zeier M, et al. Early conversion from cyclosporine to everolimus following living-donor kidney transplantation: outcomes at 5 years posttransplant in the randomized ZEUS trial. *Clin Nephrol.* 2016;85(4):215-225.

254. Marcella-Neto R, de Sa JR, Melaragno CS, et al. Late Conversion to Sirolimus or Everolimus After Pancreas Transplant. *Transplant Proc.* 2020;52(5):1376-1379.

255. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med.* 2000;343(4):230-238.

256. Lablanche S, Borot S, Wojtusciszyn A, et al. Five-Year Metabolic, Functional, and Safety Results of Patients With Type 1 Diabetes Transplanted With Allogenic Islets Within the Swiss-French GRAGIL Network. *Diabetes Care.* 2015;38(9):1714-1722.

257. Maffi P, Berney T, Nano R, et al. Calcineurin inhibitor-free immunosuppressive regimen in type 1 diabetes patients receiving islet transplantation: single-group phase 1/2 trial. *Transplantation.* 2014;98(12):1301-1309.

258. Hering BJ, Clarke WR, Bridges ND, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. *Diabetes Care.* 2016;39(7):1230-1240.

259. Afinitor, European Medicines Agency https://www.ema.europa.eu/en/medicines/human/EPAR/afinitor. Accessed April 11, 2023.

260. Noguchi S, Shinohara N, Ito T, et al. Relationship between Pulmonary Adverse Events and Everolimus Exposure in Japanese and Non-Japanese Patients: A Meta-Analysis of Oncology Trials. *Oncology.* 2017;92(5):243-254.

261. Fukudo M, Ishibashi K, Kitada M. Real-world pharmacokinetics and pharmacodynamics of everolimus in metastatic breast cancer. *Invest New Drugs.* 2021;39(6):1707-1715.

262. Takasaki S, Yamaguchi H, Kawasaki Y, et al. Long-term relationship between everolimus blood concentration and clinical outcomes in Japanese patients with metastatic renal cell carcinoma: a prospective study. *J Pharm Health Care Sci.* 2019;5:6.

263. Synold TW, Plets M, Tangen CM, et al. Everolimus Exposure as a Predictor of Toxicity in Renal Cell Cancer Patients in the Adjuvant Setting: Results of a Pharmacokinetic Analysis for SWOG S0931 (EVEREST), a Phase III Study (NCT01120249). *Kidney Cancer.* 2019;3(2):111-118.

264. Mueller-Schoell A, Groenland SL, Scherf-Clavel O, et al. Therapeutic drug monitoring of oral targeted antineoplastic drugs. *Eur J Clin Pharmacol.* 2021;77(4):441-464.

265. The Dutch Pharmacogenetics Working Group (DPWG). https://www.pharmgkb.org/page/dpwg. Accessed Februrary 27, 2023.

266. CPIC Clinical Pharmacogenetics Implementation Consortium; Genes-Drugs. https://cpicpgx.org/genes-drugs/. Accessed Februrary 27, 2023.

267. Birdwell KA, Decker B, Barbarino JM, et al. Clinical Pharmacogenetics Implementation Consortium (CPIC) Guidelines for CYP3A5 Genotype and Tacrolimus Dosing. *Clin Pharmacol Ther.* 2015;98(1):19-24.

268. Jacobsen W, Serkova N, Hausen B, Morris RE, Benet LZ, Christians U. Comparison of the in vitro metabolism of the macrolide immunosuppressants sirolimus and RAD. *Transplant Proc.* 2001;33(1-2):514-515.

269. Picard N, Rouguieg-Malki K, Kamar N, Rostaing L, Marquet P. CYP3A5 genotype does not influence everolimus in vitro metabolism and clinical pharmacokinetics in renal transplant recipients. *Transplantation.* 2011;91(6):652-656.

270. 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(11):1666-1672.

271. Chu C, Abbara C, Noel-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(10):1629-1634.

272. Lamoureux F, Picard N, Boussera B, Sauvage FL, Marquet P. Sirolimus and everolimus intestinal absorption and interaction with calcineurin inhibitors: a differential effect between cyclosporine and tacrolimus. *Fundam Clin Pharmacol.* 2012;26(4):463-472.

273. The Pharmacogene Variation (PharmVar) Consortium - CYP3A4 https://www.pharmvar.org/gene/CYP3A4. Accessed Februrary 27, 2023.

274. Amirimani B, Walker AH, Weber BL, Rebbeck TR. RESPONSE: re: modification of clinical presentation of prostate tumors by a novel genetic variant in CYP3A4. *J Natl Cancer Inst.* 1999;91(18):1588-1590.

275. Amirimani B, Ning B, Deitz AC, Weber BL, Kadlubar FF, Rebbeck TR. Increased transcriptional activity of the CYP3A4*1B promoter variant. *Environ Mol Mutagen.* 2003;42(4):299-305.

276. 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(5):355-366.

277. Fukushima-Uesaka H, Saito Y, Watanabe H, et al. Haplotypes of CYP3A4 and their close linkage with CYP3A5 haplotypes in a Japanese population. *Hum Mutat.* 2004;23(1):100.

278. Miao J, Jin Y, Marunde RL, et al. Association of genotypes of the CYP3A cluster with midazolam disposition in vivo. *Pharmacogenomics J.* 2009;9(5):319-326.

279. Schoeppler KE, Aquilante CL, Kiser TH, Fish DN, Zamora MR. The impact of genetic polymorphisms, diltiazem, and demographic variables on everolimus trough concentrations in lung transplant recipients. *Clin Transplant.* 2014;28(5):590-597.

280. Wang D, Guo Y, Wrighton SA, Cooke GE, Sadee W. Intronic polymorphism in CYP3A4 affects hepatic expression and response to statin drugs. *Pharmacogenomics J.* 2011;11(4):274-286.

281. Elens L, Nieuweboer A, Clarke SJ, et al. CYP3A4 intron 6 C>T SNP (CYP3A4*22) encodes lower CYP3A4 activity in cancer patients, as measured with probes midazolam and erythromycin. *Pharmacogenomics.* 2013;14(2):137-149.

282. Bandur S, Petrasek J, Hribova P, Novotna E, Brabcova I, Viklicky O. Haplotypic arrangement in CYP3A locus is associated with side effects of proliferative signal inhibitors in renal transplant recipients. *Transplantation.* 2011;91(1):e1-2.

283. Kniepeiss 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(1):146-150.

284. Kniepeiss D, Wagner D, Wasler A, Tscheliessnigg KH, Renner W. The role of CYP2C8 genotypes in dose requirement and levels of everolimus after heart transplantation. *Wien Klin Wochenschr.* 2013;125(13-14):393-395.

285. Whirl-Carrillo M, Huddart R, Gong L, et al. An Evidence-Based Framework for Evaluating Pharmacogenomics Knowledge for Personalized Medicine. *Clin Pharmacol Ther.* 2021;110(3):563-572.

286. Haufroid V. Genetic polymorphisms of ATP-binding cassette transporters ABCB1 and ABCC2 and their impact on drug disposition. *Curr Drug Targets.* 2011;12(5):631-646.

287. Huang S, Bjornsti MA, Houghton PJ. Rapamycins: mechanism of action and cellular resistance. *Cancer Biol Ther.* 2003;2(3):222-232.

288. Woillard JB, Kamar N, Rousseau A, Rostaing L, Marquet P, Picard N. Association of sirolimus adverse effects with m-TOR, p70S6K or Raptor polymorphisms in kidney transplant recipients. *Pharmacogenet Genomics.* 2012;22(10):725-732.

289. Pape L, Ganschow R, Ahlenstiel T. Everolimus in pediatric transplantation. *Curr Opin Organ Transplant.* 2012;17(5):515-519.

290. Ullrich NJ, Prabhu SP, Reddy AT, et al. A phase II study of continuous oral mTOR inhibitor everolimus for recurrent, radiographic-progressive neurofibromatosis type 1-associated pediatric low-grade glioma: a Neurofibromatosis Clinical Trials Consortium study. *Neuro Oncol.* 2020;22(10):1527-1535.

291. Wright KD, Yao X, London WB, et al. A POETIC Phase II study of continuous oral everolimus in recurrent, radiographically progressive pediatric low-grade glioma. *Pediatr Blood Cancer.* 2021;68(2):e28787.

292. Itohara K, Yano I, Nakagawa S, et al. Population pharmacokinetics of everolimus in adult liver transplant patients: Comparison to tacrolimus disposition and extrapolation to pediatrics. *Clin Transl Sci.* 2022.

293. Fouladi M, Laningham F, Wu J, et al. Phase I study of everolimus in pediatric patients with refractory solid tumors. *J Clin Oncol.* 2007;25(30):4806-4812.

294. French JA, Lawson JA, Yapici Z, et al. Adjunctive everolimus therapy for treatment-resistant focal-onset seizures associated with tuberous sclerosis (EXIST-3): a phase 3, randomised, double-blind, placebo-controlled study. *Lancet.* 2016;388(10056):2153-2163.

295. Combes FP, Einolf HJ, Coello N, Heimbach T, He H, Grosch K. Model-Informed Drug Development for Everolimus Dosing Selection in Pediatric Infant Patients. *CPT Pharmacometrics Syst Pharmacol.* 2020;9(4):230-237.

296. Stopping TSC Onset and Progression 2: Epilepsy Prevention in TSC Infants (STOP2), ClinicalTrials.gov Identifier: NCT04595513 https://clinicaltrials.gov/ct2/show/NCT04595513. Accessed April 6, 2023.

297. Moes DJ, Guchelaar HJ, de Fijter JW. Sirolimus and everolimus in kidney transplantation. *Drug Discov Today.* 2015;20(10):1243-1249.

298. Filippone EJ, Farber JL. The Monitoring of Donor-derived Cell-free DNA in Kidney Transplantation. *Transplantation.* 2021;105(3):509-516.

299. Bohler 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(3):306-311.

300. Haneda M, Owaki M, Kuzuya T, Iwasaki K, Miwa Y, Kobayashi T. Comparative analysis of drug action on B-cell proliferation and differentiation for mycophenolic acid, everolimus, and prednisolone. *Transplantation.* 2014;97(4):405-412.

301. Demmers MW, Baan CC, Janssen M, et al. Substantial proliferation of human renal tubular epithelial cell-reactive CD4+CD28null memory T cells, which is resistant to tacrolimus and everolimus. *Transplantation.* 2014;97(1):47-55.

302. Castro-Rojas CM, Godarova A, Shi T, et al. mTOR Inhibitor Therapy Diminishes Circulating CD8+ CD28- Effector Memory T Cells and Improves Allograft Inflammation in Belatacept-refractory Renal Allograft Rejection. *Transplantation.* 2020;104(5):1058-1069.

303. Mathew JM, Kurian S, Cravedi P, et al. Immune and gene expression profiling during tacrolimus to everolimus conversion early after liver transplantation. *Hum Immunol.* 2021;82(2):81-88.

304. San Segundo D, Fernandez-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(8):2871-2873.

305. Sabbatini M, Ruggiero G, Palatucci AT, et al. Oscillatory mTOR inhibition and Treg increase in kidney transplantation. *Clin Exp Immunol.* 2015;182(2):230-240.

306. Roat E, De Biasi S, Bertoncelli L, et al. Immunological advantages of everolimus versus cyclosporin A in liver-transplanted recipients, as revealed by polychromatic flow cytometry. *Cytometry A.* 2012;81(4):303-311.

307. Pollizzi KN, Powell JD. Regulation of T cells by mTOR: the known knowns and the known unknowns. *Trends Immunol.* 2015;36(1):13-20.

308. Pallet N, Fernandez-Ramos AA, Loriot MA. Impact of Immunosuppressive Drugs on the Metabolism of T Cells. *Int Rev Cell Mol Biol.* 2018;341:169-200.

309. Klaeske K, Lehmann S, Palitzsch R, et al. Everolimus-Induced Immune Effects after Heart Transplantation: A Possible Tool for Clinicians to Monitor Patients at Risk for Transplant Rejection. *Life (Basel).* 2021;11(12).

310. Levitsky J, Miller J, Huang X, Gallon L, Leventhal JR, Mathew JM. Immunoregulatory Effects of Everolimus on In Vitro Alloimmune Responses. *PLoS One.* 2016;11(6):e0156535.

311. Gedaly R, De Stefano F, Turcios L, et al. mTOR Inhibitor Everolimus in Regulatory T Cell Expansion for Clinical Application in Transplantation. *Transplantation.* 2019;103(4):705-715.

312. Barjon C, Dahlqvist G, Ghazal K, et al. Influence of everolimus-based treatment on circulating regulatory T cells after liver transplantation: Comparative study with tacrolimus-based therapy. *Clin Res Hepatol Gastroenterol.* 2021;45(5):101559.

313. Diaz-Molina B, Diaz-Bulnes P, Carvajal Palao R, et al. Early Everolimus Initiation Fails to Counteract the Cytotoxic Response Mediated by CD8(+) T and NK Cells in Heart Transplant Patients. *Front Immunol.* 2018;9:2181.

314. Parkes MD, Halloran PF, Hidalgo LG. Mechanistic Sharing Between NK Cells in ABMR and Effector T Cells in TCMR. *Am J Transplant.* 2018;18(1):63-73.

315. Sen DR, Kaminski J, Barnitz RA, et al. The epigenetic landscape of T cell exhaustion. *Science.* 2016;354(6316):1165-1169.

316. Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A.* 2004;101(45):16004-16009.

317. Shin H, Blackburn SD, Blattman JN, Wherry EJ. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J Exp Med.* 2007;204(4):941-949.

318. Iwasaki K, Kitahata N, Miwa Y, et al. Suppressive Effect of Everolimus on IL-2, IL-10, IL-21, and IFNgamma Levels: Implications for the Successful Minimization of Calcineurin Inhibitor Use in Transplantation. *Ther Drug Monit.* 2019;41(3):371-375.

319. Smith NL, Wissink EM, Grimson A, Rudd BD. miR-150 Regulates Differentiation and Cytolytic Effector Function in CD8+ T cells. *Sci Rep.* 2015;5:16399.

320. Hippen KL, Loschi M, Nicholls J, MacDonald KPA, Blazar BR. Effects of MicroRNA on Regulatory T Cells and Implications for Adoptive Cellular Therapy to Ameliorate Graft-versus-Host Disease. *Front Immunol.* 2018;9:57.

321. de Candia P, Torri A, Gorletta T, et al. Intracellular modulation, extracellular disposal and serum increase of MiR-150 mark lymphocyte activation. *PLoS One.* 2013;8(9):e75348.

322. Tinel C, Lamarthee B, Callemeyn J, et al. Integrative Omics Analysis Unravels Microvascular Inflammation-Related Pathways in Kidney Allograft Biopsies. *Front Immunol.* 2021;12:738795.

323. Roat R, Hossain MM, Christopherson J, et al. Circulating miRNA-150-5p is associated with immune-mediated early beta-cell loss in a humanized mouse model. *Xenotransplantation.* 2019;26(2):e12474.

324. Oellerich M, Sherwood K, Keown P, et al. Liquid biopsies: donor-derived cell-free DNA for the detection of kidney allograft injury. *Nat Rev Nephrol.* 2021;17(9):591-603.

325. Knight SR, Thorne A, Lo Faro ML. Donor-specific Cell-free DNA as a Biomarker in Solid Organ Transplantation. A Systematic Review. *Transplantation.* 2019;103(2):273-283.

326. Kataria A, Kumar D, Gupta G. Donor-derived Cell-free DNA in Solid-organ Transplant Diagnostics: Indications, Limitations, and Future Directions. *Transplantation.* 2021;105(6):1203-1211.

327. De Vlaminck I, Valantine HA, Snyder TM, et al. Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. *Sci Transl Med.* 2014;6(241):241ra277.

328. Oellerich M, Shipkova M, Asendorf T, et al. Absolute quantification of donor-derived cell-free DNA as a marker of rejection and graft injury in kidney transplantation: Results from a prospective observational study. *Am J Transplant.* 2019;19(11):3087-3099.

329. Kanzow P, Kollmar O, Schutz E, et al. Graft-derived cell-free DNA as an early organ integrity biomarker after transplantation of a marginal HELLP syndrome donor liver. *Transplantation.* 2014;98(5):e43-45.

330. Knuttgen F, Beck J, Dittrich M, et al. Graft-derived Cell-free DNA as a Noninvasive Biomarker of Cardiac Allograft Rejection: A Cohort Study on Clinical Validity and Confounding Factors. *Transplantation.* 2022;106(3):615-622.

331. Grepin 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(3):e89449.

332. Raimondo L, D'Amato V, Servetto A, et al. Everolimus induces Met inactivation by disrupting the FKBP12/Met complex. *Oncotarget.* 2016;7(26):40073-40084.

333. Ciunci CA, Perini RF, Avadhani AN, et al. Phase 1 and pharmacodynamic trial of everolimus in combination with cetuximab in patients with advanced cancer. *Cancer.* 2014;120(1):77-85.

334. Nogova L, Mattonet C, Scheffler M, et al. Sorafenib and everolimus in patients with advanced solid tumors and KRAS-mutated NSCLC: A phase I trial with early pharmacodynamic FDG-PET assessment. *Cancer Med.* 2020;9(14):4991-5007.

335. Johnbeck CB, Munk Jensen M, Haagen Nielsen C, Fisker Hag AM, Knigge U, Kjaer A. 18F-FDG and 18F-FLT-PET imaging for monitoring everolimus effect on tumor-growth in neuroendocrine tumors: studies in human tumor xenografts in mice. *PLoS One.* 2014;9(3):e91387.

336. Cejka D, Kuntner C, Preusser M, et al. FDG uptake is a surrogate marker for defining the optimal biological dose of the mTOR inhibitor everolimus in vivo. *Br J Cancer.* 2009;100(11):1739-1745.

337. Rinzivillo M, Prosperi D, Mazzuca F, et al. [(18)F]FDG-PET/CT and long-term responses to everolimus in advanced neuroendocrine neoplasia. *J Endocrinol Invest.* 2021;44(4):811-818.

338. Mociornita AG, Adamson MB, Tumiati LC, Ross HJ, Rao V, Delgado DH. Effects of everolimus and HLA-G on cellular proliferation and neutrophil adhesion in an in vitro model of cardiac allograft vasculopathy. *Am J Transplant.* 2018;18(12):3038-3044.

339. Li SH, Lu HI, Chang AY, et al. Angiotensin II type I receptor (AT1R) is an independent prognosticator of esophageal squamous cell carcinoma and promotes cells proliferation via mTOR activation. *Oncotarget.* 2016;7(41):67150-67165.

340. Kim EJ, Kim YJ, Lee HI, Jeong SH, Nam HJ, Cho JH. Upregulation of Peroxiredoxin-2 in Well-Differentiated Pancreatic Neuroendocrine Tumors and Its Utility as a Biomarker for Predicting the Response to Everolimus. *Antioxidants (Basel).* 2020;9(11).

341. Gordon MA, D'Amato NC, Gu H, et al. Synergy between Androgen Receptor Antagonism and Inhibition of mTOR and HER2 in Breast Cancer. *Mol Cancer Ther.* 2017;16(7):1389-1400.

342. Chen Z, Jiang Q, Zhu P, et al. NPRL2 enhances autophagy and the resistance to Everolimus in castration-resistant prostate cancer. *Prostate.* 2019;79(1):44-53.

343. Kuwano M, Shibata T, Watari K, Ono M. Oncogenic Y-box binding protein-1 as an effective therapeutic target in drug-resistant cancer. *Cancer Sci.* 2019;110(5):1536-1543.

344. Shibata T, Watari K, Kawahara A, et al. Targeting Phosphorylation of Y-Box-Binding Protein YBX1 by TAS0612 and Everolimus in Overcoming Antiestrogen Resistance. *Mol Cancer Ther.* 2020;19(3):882-894.

345. Navarro-Villaran E, de la Cruz-Ojeda P, Contreras L, et al. Molecular Pathways Leading to Induction of Cell Death and Anti-Proliferative Properties by Tacrolimus and mTOR Inhibitors in Liver Cancer Cells. *Cell Physiol Biochem.* 2020;54(3):457-473.

346. Totary-Jain H, Marks AR. MicroRNAs and the cellular response to rapamycin: potential role in diagnosis and therapy. *Cell Cycle.* 2013;12(6):861-862.

347. Pawlik B, Smyczynska U, Grabia S, et al. mTOR Inhibitor Treatment in Patients with Tuberous Sclerosis Complex Is Associated with Specific Changes in microRNA Serum Profile. *J Clin Med.* 2022;11(12).

348. Budde K, Zonnenberg BA, Frost M, et al. Pharmacokinetics and pharmacodynamics of everolimus in patients with renal angiomyolipoma and tuberous sclerosis complex or lymphangioleiomyomatosis. *Br J Clin Pharmacol.* 2016;81(5):958-970.

349. Rausch S, Schollenberger D, Hennenlotter J, et al. mTOR and mTOR phosphorylation status in primary and metastatic renal cell carcinoma tissue: differential expression and clinical relevance. *J Cancer Res Clin Oncol.* 2019;145(1):153-163.

350. Li S, Kong Y, Si L, et al. Phosphorylation of mTOR and S6RP predicts the efficacy of everolimus in patients with metastatic renal cell carcinoma. *BMC Cancer.* 2014;14:376.

351. Owonikoko TK, Ramalingam SS, Miller DL, et al. A Translational, Pharmacodynamic, and Pharmacokinetic Phase IB Clinical Study of Everolimus in Resectable Non-Small Cell Lung Cancer. *Clin Cancer Res.* 2015;21(8):1859-1868.

352. Pandolfi L, Marengo A, Japiassu KB, et al. Liposomes Loaded with Everolimus and Coated with Hyaluronic Acid: A Promising Approach for Lung Fibrosis. *Int J Mol Sci.* 2021;22(14).

353. Gagliano T, Bellio M, Gentilin E, et al. mTOR, p70S6K, AKT, and ERK1/2 levels predict sensitivity to mTOR and PI3K/mTOR inhibitors in human bronchial carcinoids. *Endocr Relat Cancer.* 2013;20(4):463-475.

354. Benslama N, Bollard J, Vercherat C, et al. Prediction of response to everolimus in neuroendocrine tumors: evaluation of clinical, biological and histological factors. *Invest New Drugs.* 2016;34(5):654-662.

355. Zou Y, Wang J, Leng X, et al. The selective MEK1 inhibitor Selumetinib enhances the antitumor activity of everolimus against renal cell carcinoma in vitro and in vivo. *Oncotarget.* 2017;8(13):20825-20833.

356. Kuo CT, Chen CL, Li CC, et al. Immunofluorescence can assess the efficacy of mTOR pathway therapeutic agent Everolimus in breast cancer models. *Sci Rep.* 2019;9(1):10898.

357. Karajannis MA, Mauguen A, Maloku E, et al. Phase 0 Clinical Trial of Everolimus in Patients with Vestibular Schwannoma or Meningioma. *Mol Cancer Ther.* 2021;20(9):1584-1591.

358. Thermo Fisher Scientific Inc. QMS Everolimus Immunoassay Package Insert; Revision 1. 2010. In.

359. Siemens Healthcare Diagnostics Inc. ACMIA Everolimus Immunoassay Package Insert; Revision 1. 2021. In.

360. Monchaud C, Marquet P. Pharmacokinetic optimization of immunosuppressive therapy in thoracic transplantation: part II. *Clin Pharmacokinet.* 2009;48(8):489-516.

361. 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(2):154-159.

362. Berger SP, Sommerer C, Witzke O, et al. Two-year outcomes in de novo renal transplant recipients receiving everolimus-facilitated calcineurin inhibitor reduction regimen from the TRANSFORM study. *Am J Transplant.* 2019;19(11):3018-3034.

363. Takahashi K, Uchida K, Yoshimura N, et al. Efficacy and safety of concentration-controlled everolimus with reduced-dose cyclosporine in Japanese de novo renal transplant patients: 12-month results. *Transplant Res.* 2013;2(1):14.

364. 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(7):933-942.

365. Novoa PA, Grinyo JM, Ramos FJ, et al. De novo use of everolimus with elimination or minimization of cyclosporine in renal transplant recipients. *Transplant Proc.* 2011;43(9):3331-3339.

366. 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(10):1194-1202.

367. Chan L, Hartmann E, Cibrik D, Cooper M, Shaw LM. Optimal everolimus concentration is associated with risk reduction for acute rejection in de novo renal transplant recipients. *Transplantation.* 2010;90(1):31-37.

368. Kahan BD, Wong RL, Carter C, et al. A phase I study of a 4-week course of SDZ-RAD (RAD) quiescent cyclosporine-prednisone-treated renal transplant recipients. *Transplantation.* 1999;68(8):1100-1106.

369. Rostaing L, Christiaans MH, Kovarik JM, Pascual J. 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.

370. 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(10):1117-1125.

371. Levy GA, Grant D, Paradis K, Campestrini J, Smith T, Kovarik JM. Pharmacokinetics and tolerability of 40-0-[2-hydroxyethyl]rapamycin in de novo liver transplant recipients. *Transplantation.* 2001;71(1):160-163.

372. Hoyer PF, Ettenger R, Kovarik JM, et al. Everolimus in pediatric de nova renal transplant patients. *Transplantation.* 2003;75(12):2082-2085.

373. Van Damme-Lombaerts R, Webb NA, Hoyer PF, et al. Single-dose pharmacokinetics and tolerability of everolimus in stable pediatric renal transplant patients. *Pediatr Transplant.* 2002;6(2):147-152.

374. O'Donnell A, Faivre S, Burris HA, 3rd, 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(10):1588-1595.

375. Schoch LK, Asiama A, Zahurak M, et al. Pharmacokinetically-targeted dosed everolimus maintenance therapy in lymphoma patients. *Cancer Chemother Pharmacol.* 2018;81(2):347-354.

376. Tanaka C, O'Reilly T, Kovarik JM, et al. Identifying optimal biologic doses of everolimus (RAD001) in patients with cancer based on the modeling of preclinical and clinical pharmacokinetic and pharmacodynamic data. *J Clin Oncol.* 2008;26(10):1596-1602.

377. Jelliffe RW, Schumitzky A, Bayard D, et al. Model-based, goal-oriented, individualised drug therapy. Linkage of population modelling, new 'multiple model' dosage design, bayesian feedback and individualised target goals. *Clin Pharmacokinet.* 1998;34(1):57-77.

378. Jelliffe RW, Schumitzky A, Van Guilder M, et al. Individualizing drug dosage regimens: roles of population pharmacokinetic and dynamic models, Bayesian fitting, and adaptive control. *Ther Drug Monit.* 1993;15(5):380-393.