Calcineurin Inhibitor Toxicity in Solid Organ Transplantation

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Abstract

Calcineurin inhibitors (CNIs) have a substantial role in maintaining immunosuppression after solid organ transplantation (SOT). These drugs have a narrow therapeutic window, and individual doses and drug treatment monitoring are necessary. Still, a substantial proportion of patients suffer from short- or long-term calcineurin inhibitor toxicity (CNT), including kidney function impairment, hypertension, neurotoxicity, and metabolic disturbances. The authors discuss pathophysiology, clinical presentation, and histological features of CNT, with focus on renal manifestations. Furthermore, we elucidate recent and ongoing attempts to reduce the burden of CNT in SOT including CNI-sparing and CNI-free regimens.

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Introduction

Clinical Evidence for Calcineurin Inhibitor Toxicity

Solid organ transplantation (SOT) has rapidly evolved since the 1960s as the primary treatment for patients with advanced and end-stage renal, liver, heart, and lung diseases [1, 2]. Early outcomes were complicated with acute

rejection episodes and rapid deterioration of allograft function and subsequent loss [3]. With the introduction of individualized immunological matching at allocation [4] and the modern immunosuppressive regimens, allograft and patient outcome have substantially improved ever since. Calcineurin inhibitors (CNIs) including cyclosporine A (CsA) and tacrolimus (Tac) were approved as an immunosuppressive treatment in 1983 and 1999, respectively, by the US Food and Drug Administration [5, 6] and have substantially contributed to long-term control of antiallograft immunity. However, CNIs have a narrow therapeutic window and are responsible for substantial side effects, including nephrotoxicity, hypertension, neurotoxicity, tremor, and metabolic disturbances [7]. Indeed, CNIs may induce acute and dose-dependent renal dysfunction with the histological correlate of tubular vacuolization and epithelial necrosis, rarely accompanied with thrombotic microangiopathy (TMA). Alternatively, CNIs induce a rather dose-independent chronic renal toxicity with progressive athero- and arteriohyalinosis and interstitial fibrosis and tubular atrophy (IFTA) [8]. In a landmark article, Nankivell demonstrated that histological features of chronic calcineurin inhibitor toxicity (CNT) are present in virtually all per-protocol biopsies in kidney allografts 10 years after transplantation, and advanced lesions are associated with inferior renal function and premature graft failure [9].

CNT not only affects kidney transplanted patients but also patients under long-term CNI treatment due to non-

renal SOT or autoimmune diseases. Ojo et al. [10] showed that chronic renal disease also occurs with a 5-year cumulative incidence in nonrenal SOT with 10.9% after a heart transplantation and 18.1% after liver transplantation. Nonrenal transplant recipients showing chronic renal failure under CNI treatment also have a 4 times higher risk of death [10]. The progressive estimated glomerular filtration rate (eGFR) decline is at least partially caused by CNI treatment, since prospective trials with CNI-avoiding regimens show superior long-term kidney function in patients randomized to the CNI-free arm (see below) [11].

CNI-Sparing and CNI-Avoiding Treatment Options in SOT

For many years, Tac was claimed to induce less nephrotoxicity, mainly due to its higher potency and less likely off-target effects [12]. Yet, Stegall et al. [13] recently demonstrated that Tac-based immunosuppression induces similar CNT lesions in the majority of patients and is responsible for substantial nonimmune-mediated nephrotoxicity after SOT. Since CNT is likely due to high peak levels of CNIs, researches have speculated that extended-release (ER) Tac drugs may reduce CNI-mediated toxicity in long-term exposure [14]. Currently, only limited research is available to support this hypothesis. In a small cross-over trial, Tremblay et al. [15] compared ER (Advagraf/Astagraf) and MeltDose formulations of Tac (LCPT-Tac or Envarsus). Tac ER is administrated once a day. Its absorption is slower than that of other Tac formulations; however, it provides the same exposure of Tac at the same dosage. MeltDose is a new technology with a higher bioavailability due to the usage of the small particle size. Reduced particle size enhances dissolution and absorption [14]. The ASTCOFF (all Tac formulation) study showed the highest Tac exposure for LCPT (Table 1) [15]. Studies have shown that CNI-induced tremor in transplanted patients was less observed in the LCPT-Envarsus-treated group [15]. Both ER formulations are safe and effective in the prevention of kidney transplant rejection with noninferiority to immediate-release Tac for de novo kidney transplantation and secondary conversion [16–18].

Given the narrow therapeutic window of CNIs, many investigators have attempted to introduce CNI-avoiding or CNI-withdrawing regimens in SOT, primarily by the substitution of CNI with the mammalian target of rapamycin (mTOR) inhibitors [19]. However, several large randomized studies showed an unacceptable high risk for early rejection after kidney transplantation in patients

treated de novo with mTOR-inhibitor-based and CNI-free regimens (Table 1) [20–22]. Similarly, early or late conversion from CNI-containing regimens to mTOR inhibitors led to a surge in mild to moderate rejection events with increased risk for structural and sustained allograft damage (Table 1) [23–25]. Furthermore, high dose of mTOR inhibitor treatment was ill tolerated in many patients with treatment failure and reconversion to CNIs. Consequently, the hopes for CNI-free and purely mTOR-based immunosuppression had to be abandoned as a treatment path for unselected kidney transplant recipients.

Therefore, in an alternative approach, reduced CNI exposure was tested in conjunction with mTOR inhibitors for de novo transplantation. In this landmark article, Pascual et al. [26] showed with the TRANSFORM trial in de novo transplanted patients that treatment with low-dose CNI combined with everolimus is non-inferior to standard CNI (CsA or Tac) and mycophenolate mofetil treatment (Table 1). Allograft survival, achieved transplant function, incidence of de novo donor-specific antibodies, and acute antibody-mediated rejection (ABMR) were similar among the groups after 12 months of treatment. Intriguingly, the CNI + everolimus group showed significantly less infections than the CNI + mycophenolate mofetil group, notably cytomegalovirus (3.6 vs. 13.3%) and polyomavirus 1 (BK virus) (4.3 vs. 8%) [26]. Also, the 2-year outcomes were among the 2 groups, meaning rejection rates and viral infections are low under combined treatment of CNI and everolimus [27].

While CNI seemed indispensable in kidney transplantation for decades, recent studies have promoted hope in novel CNI-avoiding regimens. Belatacept is an affinity- and stability-engineered CTLA4-Fc (cytotoxic T-lymphocyte-associated protein 4) fusion protein and effectively blocks B7.1/B7.2-mediated costimulation in T cells [28]. In a first randomized trial, Vincenti et al. [29] showed that belatacept was noninferior compared with cyclosporine A for preventing acute kidney rejection. The BENEFIT (belatacept for kidney transplantation) and BENEFIT-EXT trials investigated belatacept compared to CNI-based immunosuppression in de novo kidney transplantation (Table 1) [30]. Overall outcome was excellent and belatacept-randomized patients showed superior kidney function as well as fewer chronic allograft nephropathy in the short- (1 year) [30] and long-term follow-up (7 years) [31]. However, early rejection and posttransplant lymphoproliferative disease were more frequent in the patient groups receiving belatacept [30,

Table 1. Overview of clinical trials investigating CNI sparing and avoidance treatment in kidney transplanted patients

Trial name	CNI sparing/avoidance	Study findings
ASTCOFF [15–18]	De novo or conversion to Tac-extended release To MeltDose formulations of Tac	+ Non-inferiority for allograft survival, rejection, and function + Reduced dose with similar exposure for MeltDose Tac + MeltDose-treated patients showed less CNI-induced tremor
ELITE-Symphony, Orion [20–22]	De novo to mTOR inhibitors	+ Good outcome for overall allograft survival – High rates of mild to moderate biopsy proven acute rejection with mTOR inhibitors – High rate of (limiting) intolerability in the mTOR inhibitor group
CONCEPT, CONVERT, ZEUS, ASCERTAIN [23–25]	Early or late conversion to mTOR inhibitors	+ Superior renal function in the outcome of the allograft (ZEUS) + Similar allograft survival – Increased risk for acute rejection in the mTOR inhibitor group – High rate of (limiting) intolerability in the mTOR inhibitor group
Transform [27]	De novo Low dose of CNI + mTOR inhibitor	+ CNI sparing is possible when CNI effects are potentiated with mTOR inhibitors + Less viral infections + Similar allograft survival – High rate of (limiting) intolerability in the mTOR inhibitor group
Benefit benefit-ext [30–33]	De novo or conversion to belatacept	+ Improved mid- and long-term kidney function in the belatacept group + Reduced risk for de novo DSA – Increased risk of early rejections in the belatacept group – Increased risk for PTLN, notably in patients with EBV risk constellation (donor+/recipient–)
Cirrus I [35, 37–41]	De novo or conversion to iscalimab (CFZ533)	+ Good immunosuppression + Low prevalence of chronic allograft nephropathy in 1 year per protocol biopsy

Tac, tacrolimus; DSA, donor-specific antibodies; PTLN, posttransplant lymphoproliferative disease; CNI, calcineurin inhibitor; mTOR, mammalian target of rapamycin.

32]. Due to the increased risk of developing posttransplant lymphoproliferative disease when under belatacept treatment, only EBV seropositive patients should be treated with belatacept.

Recently, monoclonal antibodies blocking CD40/CD40L pathway have been evaluated in SOT [33]. CD40/CD40L is the costimulatory signal needed to fully activate T-cells. It has been shown that blockage of the CD40L/CD40 axis is effective to prevent allograft rejection and graft loss in nonhuman primate kidney transplantation [34]. Yet, antibodies directed against CD40L (CD154) increased the risk for thromboembolic events, which resulted in termination of clinical trials [35]. Meanwhile, blockage of CD40 with humanized nondepleting monoclonal antibodies, also known as iscalimab, showed to be safe in clinical phase I and II studies (Table 1) [36–40]. In 2019, first results from a phase II trial in kidney transplant patients under iscalimab treatment were demonstrated [36]. Currently, the Cirrus I trial studying the efficacy, safety, tolerability, and pharmacokinetic and dynamic properties of CD40 blockage in kidney transplantation is ongoing (NCT03663335, clinicaltrials.gov) [37].

Histological Features/Transcriptomic Profiles

Many debates have been held about the histological correlates of chronic CNT and their specificity. Longitudinal per-protocol biopsies in kidney transplant patients under CNI-containing regimens show progressive arterio- (ah) and arteriolohyalinosis (aah) (Table 2) (eccentric hyaline matrix deposition in the small renal arteries) and IFTA [9]. Furthermore, severe and progressive ah/aah and IFTA can be observed in allografts with active acute and chronic ABMR [41]. Furthermore, the degree of ah/aah lesions and IFTA seems not to correlate with the cumulative CNI dosage, although no detailed studies in this respect have been performed [42]. These lesions are not highly specific for CNI-induced changes since it was observed that kidneys from diseased donors are more prone to ah/aah lesions than kidneys from healthy donors [43]. Furthermore, other authors have suggested that predisposition to CNI nephrotoxicity is dependent on other donor- and recipient-derived factors, including genetic signatures and polymorphisms, since the histological and clinical presentation of CNT has shown high interindividual variations in patients (such as CYP3A5*3/*3 or TGFβ polymorphism in codon 10) with similar CNI reg-

Table 2. Overview of the main characteristics of CNT in the kidney

Stage of CNT	Diagnostic criteria
Acute CNT	
Hemodynamic effects	Vasoconstriction
Endothelial toxicity	TMA
Tubular toxicity	Tubular vacuolization
	Epithelial necrosis
	Loss of brush border
Chronic CNT	Arthero- and arteriohyalinosis
	Tubular atrophy
	Interstitial fibrosis (IFTA)

TMA, thrombotic microangiopathy; CNT, calcineurin inhibitor toxicity; IFTA, interstitial fibrosis and tubular atrophy.

imens [44, 45]. That donor- and recipient-derived factors might influence ah/aah lesions was shown by Einecke who showed an independent association of ah/aah lesions with donor age and chronic glomerular disease independent of immunosuppression [46]. Similarly, uncontrolled hypertension, diabetes, recurrence of original disease, and other factors may influence predisposition to arteriohyalinosis.

To date, no serological or radiological marker to predict or measure CNT has been developed and validated. A method to assess rejection and injury in the transplanted kidney was developed by Halloran et al. The group developed the Molecular Microscope Diagnostic System, which is a microassay-based mRNA assessment. It is used in biopsies from transplants to assess the diagnosis, such as T-cell-mediated rejection and ABMR. Biopsies from kidney transplants are suitable to be evaluated by MMDx, as rejection of a kidney allograft is well characterized by histology and mRNA expression. The mRNA transcript change within the biopsies may represent the transcription change in cells [47]. To our knowledge, no study has investigated molecular signatures of kidney biopsies in patients with CNI-based and CNI-avoiding regimens (e.g., mTOR inhibitors, belatacept, and isaclimab) in patients with renal and nonrenal SOT.

Hemodynamic Effects of Cyclosporine A

Murray et al. [48] discovered that CsA treatment initiates vasoconstriction of the afferent arterioles [48, 49]. This constriction leads to a reduced diameter of the afferent arteriole, leading to less perfused kidneys (lower GFR) (Table 2). Furthermore, it has been shown that these vascular deteriorations occur due to CsA's activation of the renin angiotensin system (RAS). The RAS is activated

when blood pressure is low, which induces a renin release. Renin cleaves from angiotensinogen the peptide angiotensin I, which is then converted to angiotensin II by the angiotensin-converting enzyme (ACE) [50, 51]. But not only angiotensin II but also other mediators such as endothelin, which is increased in rat mesenteric artery after CsA treatment [52], and thromboxane [53] contribute to vasoconstriction. Moreover, CsA suppresses prostaglandin synthesis and the nitric oxide system, which acts as a vasodilator [54]. Therefore, much evidence points to the notion that CsA has a predominant functional effect on renal hemodynamic, comparable to ACE inhibitors or angiotensin-II-receptor antagonists (sartans), and that these compounds lead to a (partially reversible) deterioration of the eGFR [55, 56].

Endothelial Toxicity

Clearly, in certain circumstances, CNI can induce severe endothelial dysfunction with TMA and occlusion of the renal microvasculature during the early phase, 3–6 months, posttransplantation (Table 2). Unfortunately, half of the patients with TMA lose their renal allograft 2 years after TMA onset. Commonly, a dysregulation of the alternative complement pathway together with ABMR and/or high immunosuppressive treatment (CNI) is the underlying cause of TMA. The pathophysiology of CNI-associated TMA is incompletely understood, yet likely involves activation of prothrombotic factors due to CNI treatment and ischemia-reperfusion endothelial injury [57, 58]. For around one-third of the patients, TMA can only be diagnosed in the renal allograft through biopsy as TMA manifests within the grafts without any hemolysis and thrombocytopenia. A promising treatment of TMA is discontinuation of CNI treatment and potentially plasma and/or eculizumab, which inhibits the C5 protein of the complement system [59, 60].

Tubular Toxicity

Early experience with high doses of CNI demonstrated that acute CNI exposure may include nephrotoxicity with the histological correlation of tubular vacuolization and loss of brush borders. Such repeated events may have induced chronic CNT with tubulointerstitial fibrosis, arteriohyalinosis, and tubular atrophy (Table 2). Studies have shown that due to reduced diameter of the small vasculature, the renal parenchyma undergoes degenerative changes [61, 62]. Furthermore, these regions of the kidneys are more susceptible to injury due to their limited oxygen availability [62, 63]. Reduced oxygen in the renal tissue promotes the development of chronic kidney dis-

Fig. 1. CsA treatment induces pro-inflammatory and fibrotic signatures in vivo and in vitro. **a** Expression of selected genes involved in inflammation and fibrosis measured by RNA array (which 1) in kidneys after exposure of animals to 100 mg/kg CsA for 3 consecutive days. **b** Induction of Fn14 in total lysates and as **c** surface receptor expression on MCT treated with indicated concentrations of CsA (unpublished). CsA, cyclosporine A; MCT, murine tubular epithelial cells.

ease additionally to the nephrotoxicity induced by CNI treatment. Low oxygen leads to renal hypoxia and therefore also to ATP starvation. These will then activate fibrosis pathways. Interstitial fibrosis around the tubules in the kidney augments the diffusion distance between blood and tissue. Cippa showed that also patients with high domicile altitude show more progressive ah lesions than patients with lower residential altitude [64].

Common Endpoints/Pathophysiology of CNT

Repeated acute tubular toxicity and impaired renal hemodynamics induce vicious cycles of proinflammatory stress signals, such as cytokines, and effector molecules for tissue remodeling and fibrosis, including TGF β , IL4,

and IL13 [65]. TGF β 1 is a cytokine involved in cell proliferation and cell differentiation as well as wound healing [66, 67]. The cytokine is upregulated after tissue injury and contributes to tissue fibrosis development. Earlier studies showed that CsA treatment increases mRNA [68] as well as protein levels [69] of TGF β 1. Furthermore, TGF β 1 staining in kidney biopsies correlates with chronic changes in the vasculature and the kidney allograft outcome. Furthermore, TGF β 1 is a major stimulator for epithelial mesenchymal transition. Due to cytokine stimulus, epithelial cells start to express less epithelial specific markers but more fibroblast markers. Epithelial cells then differentiate into myofibroblasts [70, 71]. Indeed, we and others have demonstrated that exposure of mice to CsA

induces a strong proinflammatory and profibrotic signature in the kidneys. In an *in vivo* model, mice (C57BL/6JRj) were treated with high dose of CsA (100 mg/kg). mRNA from the kidney was extracted and analyzed with a gene array for nephrotoxicity. The heatmap (Fig. 1a) showed significant upregulation of nephrotoxic genes involved in fibrosis and inflammation, including Fn14 (TNFRSF12A), vimentin, TIMP1, and CD44. Our group has recently elucidated the role of the TWEAK/Fn14 pathways in CNT. TWEAK (TNF-related weak inducer of apoptosis) is produced by myeloid cells and will bind to the surface receptor Fn14 (fibroblast growth factor-inducible 14) on epithelial cells. We could show that the protein Fn14 is time- (Fig. 1b) and dose-dependently (Fig. 1c) induced on kidney epithelial cells upon CsA treatment. The increase of the protein Fn14 was either measured by Western blot analysis (Fig. 1b) or the mean fluorescence intensity of Fn14 on the surface of the kidney epithelial cells using fluorescence-activated cell sorting (Fig. 1c) (unpublished). The binding of TWEAK to the Fn14 receptor will activate proinflammatory and profibrotic pathways that then contribute to CNT progression [72]. Furthermore, deficiency of TWEAK was sufficient to protect animals from acute CNT [73]. Fibrosis in the kidney leads to malfunctioning of the glomerulus, nephrons, and capillaries. Furthermore, the deposition of the extracellular matrix will further stimulate immune cells that are secreting more cytokines and therefore contributing further to inflammation [74]. That kind of inflammatory environment imposed by alloreactivity immunity promotes a more aggressive CNT. Inflammation and fibrosis in the kidney decrease the survival of the transplanted kidney allograft.

Treatment

Currently, no specific treatments to prevent, halt, or reverse CNT are available. Research in this field is limited, mainly due to the fact that modeling chronic CNT is cumbersome in experimental animals. Inbred mice show a high resistance toward chronic CNI-induced kidney lesions, even when treated at (supra)maximal doses and for prolonged time frames. The reason for this is unknown, but is likely due to high renal reserves. This reserve gives the kidney the possibility to adapt to stress stimuli and therefore increase the eGFR [75]. Furthermore, inbred mice (such as C57BL/6) have only 1 renin gene and therefore less activation of the RAS, which essentially would lead to less vasoconstriction [76]. Conversely, inbred rats (Wistar) develop arteriohyalinosis, but long treatment regimens with repeated injections or osmotic pumps are needed.

Several approaches have been conducted to ameliorate CNT. Andoh et al. [77] showed that severe salt restriction may accelerate CNT in rats. Rats treated with CsA and on a low salt diet developed features of chronic nephrotoxicity compared with rats receiving the same dose of CsA but standard salt diet. Then CsA treatment was stopped, and all groups received high salt diets. Interestingly, only rats that developed prior CNT showed an increase in blood pressure. However, the mechanisms of disease are unclear. It is possible that salt restriction induces intrarenal fibrotic loops. Alternatively, it is possible that the profibrotic effect is related to decreased blood pressure, hemodynamic-mediated deterioration of renal perfusion, and subsequent induction of chronic inflammation and fibrosis [77].

Other authors have suggested that CNT can be alleviated by dietary potassium and magnesium supplementation. An *in vivo* study with rats investigated if supplementation of potassium or magnesium would ameliorate CsA-induced hypertension and nephrotoxicity. Both dietary supplementation showed a decrease of CNT. However, magnesium supplementation was much more beneficial against CNT than potassium supplementation. Interestingly, the combination of potassium and magnesium showed the best protection against CNT [78]. The beneficial effect of potassium supplementation is due to natriuresis and less sodium-induced elevation of blood pressure [79]. Other studies have shown that magnesium induces vasodilatation in arteries and therefore serves as a protection against CNT [80, 81].

It was shown that Ca^{2+} channel blockers prevent CsA-associated hypertension [82] as well as renal hypoperfusion, meaning there is a decrease in renal blood flow [83]. Early on, it was shown that lacidipine (a calcium channel blocker) prevents the decrease in GFR in renal allografts when administered additionally to CsA. It was observed that GFR decreased as soon as lacidipine was discontinued [83]. Recently, a retrospective study (FAVORIT, NCT00064753) evaluating the benefit of calcium channel blocking agents after renal transplantation was performed. In this analysis, it was found that calcium channel blockers did not significantly increase the survival of the kidney allograft [84]. Strikingly, the same results were shown in another clinical trial with 210 patients with renal allografts. However, this study showed that overall the group receiving calcium channel blockers had a significantly better renal function [85]. Conclusively, administration of Ca^{2+} channel blockers with CNI ameliorates the renal function but not the overall survival of the renal allograft.

Finally, others have suggested that the combined effect of CNI and blockers of the RAS may ameliorate CNT in transplanted patients. First, it was shown that patients with chronic kidney disease show a slower loss of kidney function when under ACE inhibitors. It has been shown that ACE inhibitor administration in rats improves the survival of the transplanted allograft. The treatment showed lower systemic and glomerular capillary pressure. On the histological level, it was observed that ACE inhibitors prevented atherosclerosis development [86].

Other potential drugs minimizing the toxicity of CNI on the renal allograft are mineralocorticoid receptor antagonists such as aldosterone inhibitors (eplerenone and spironolactone). Rats receiving in addition to CsA treatment aldosterone inhibitors showed less arteriolopathy and less tubulointerstitial fibrosis [87]. The SPIREN trial evaluated the effect of spironolactone in patients with renal allograft. It could be shown that administration of a mineralocorticoid receptor antagonism is beneficial for the renal allograft outcome (fibrosis) and survival (NCT01021943) [88].

Conclusion

Clinical and subclinical toxicity of CNIs is almost uniformly dominant in solid organ transplant recipients. Notably, progressive nonimmune-mediated deterioration of kidney function is common. Clearly, the predisposition to develop clinically relevant CNT is highly variable and may depend on comedication, comorbidity, and genetic predisposition. Currently, the precise mechanisms of action for CNT development are not known, neither for kidney disease nor extra-renal disease. To date, no

measures to prevent, alleviate, or revert CNT are known. Given the notion that CNIs remain a dominant pillar of immunosuppressive regimens in renal and nonrenal transplanted patients, CNT will remain a critical momentum for severe morbidity within the SOT community. For the future, either novel immunosuppressive therapy which allows CNI avoidance or protective/preemptive or therapeutic measures to cope with CNT are needed.

Statement of Ethics

This study protocol was reviewed and approved by the Veterinärmedizin der Universität Bern, Sekretariat Tierversuche (approval number BE63/19).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Andrea Karolin: wrote the paper and performed the literature search. Vera Genitsch: wrote the paper and discussed the literature search. Daniel Sidler: established the layout, performed the literature search, and wrote the paper.

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