

Calcineurin Inhibitor Nephrotoxicity

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The use of the calcineurin inhibitors cyclosporine and tacrolimus led to major advances in the field of transplantation, with excellent short-term outcome. However, the chronic nephrotoxicity of these drugs is the Achilles' heel of current immunosuppressive regimens. In this review, the authors summarize the clinical features and histologic appearance of both acute and chronic calcineurin inhibitor nephrotoxicity in renal and nonrenal transplantation, together with the pitfalls in its diagnosis. The authors also review the available literature on the physiologic and molecular mechanisms underlying acute and chronic calcineurin inhibitor nephrotoxicity, and demonstrate that its development is related to both reversible alterations and irreversible damage to all compartments of the kidneys, including glomeruli, arterioles, and tubulo-interstitium. The main question—whether nephrotoxicity is secondary to the actions of cyclosporine and tacrolimus on the calcineurin-NFAT pathway—remains largely unanswered. The authors critically review the current evidence relating systemic blood levels of cyclosporine and tacrolimus to calcineurin inhibitor nephrotoxicity, and summarize the data suggesting that local exposure to cyclosporine or tacrolimus could be more important than systemic exposure. Finally, other local susceptibility factors for calcineurin inhibitor nephrotoxicity are reviewed, including variability in P-glycoprotein and CYP3A4/5 expression or activity, older kidney age, salt depletion, the use of nonsteroidal anti-inflammatory drugs, and genetic polymorphisms in genes like *TGF-β* and *ACE*. Better insight into the mechanisms underlying calcineurin inhibitor nephrotoxicity might pave the way toward more targeted therapy or prevention of calcineurin inhibitor nephrotoxicity.

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The introduction of the calcineurin inhibitor (CNI) cyclosporine in human kidney transplantation in the late 1970s revolutionized transplantation medicine, and made transplantation a preferable therapeutic intervention for end-stage renal diseases (1,2). In 1984, the potent immunosuppressive properties of another CNI, tacrolimus, were discovered, and tacrolimus was used successfully in human liver, kidney, and heart allograft recipients (3,4). Currently, 94% of kidney transplant recipients are discharged after transplantation with a CNI-based immunosuppressive regimen (5).

The immunosuppressive properties of cyclosporine and tacrolimus result from inhibition of calcineurin, a calcium- and calmodulin-dependent phosphatase (protein phosphatase 3 [PPP3C], formerly PP2B). Intracellularly, these completely different molecules bind to cyclophilin and FKBP12 for cyclosporine and tacrolimus, respectively (6–9). The competitive binding of cyclosporine-cyclophilin and tacrolimus-FKBP12 complexes to calcineurin inhibits phosphatase activity of calcineurin. This inhibition then suppresses the transcription of IL-2 via inhibition of the dephosphorylation and impaired translocation of the nuclear factor of activated T cells (NFAT) (10–12), which regulates IL-2 transcription and thus T cell activation (13–15). The current insights into the role of calcium, calcineurin and NFAT

in the regulation of T cell development and function are summarized in a review by Macian *et al.* (16).

Calcineurin (17–19) and NFAT isoforms (five different isoforms; only NFAT5 is not calcineurin dependent) (20) are, however, not T cell specific, and inhibition of this pathway by cyclosporine and tacrolimus gives rise to toxicity beyond immunosuppression (21). The considerable side effects that accompany treatment with cyclosporine and tacrolimus hamper long-term kidney graft and patient survival, and cause major additional morbidity. This is the case not only for kidney transplantation, in which the direct nephrotoxicity of these agents represents a huge challenge for clinicians, pathologists, and scientists. The nephrotoxic effects of cyclosporine and tacrolimus are also a major concern in nonrenal solid organ transplantation and many other diseases requiring therapy with these drugs (22–25).

In this article, we discuss the clinical and histologic aspects of CNI nephrotoxicity, together with the molecular mechanisms and clinical risk factors associated with acute and chronic CNI nephrotoxicity. We also provide a brief overview on current and potential future approaches to prevent and treat CNI nephrotoxicity.

Pathogenesis, Histology, and Clinical Features of CNI Nephrotoxicity

Because cyclosporine has been used for a much longer time, most data in this field pertain to cyclosporine. The effects of tacrolimus are considered to be similar (see below). The nephrotoxicity of cyclosporine was reported in the first publications on the clinical use of cyclosporine in humans after renal transplanta-

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tion (1,2), whereas prior animal studies had not observed this important side effect (26,27). The first experiences with cyclosporine nephrotoxicity suggested that this phenomenon was attributable to functional changes and was thus reversible (28–30). This reversible, hemodynamically mediated renal dysfunction is now recognized as “acute CNI nephrotoxicity.” However, in 1984, Myers *et al.* were the first to demonstrate that the long-term use of cyclosporine in heart transplant recipients was associated not only with a reversible decrease in GFR, but also with irreversible renal functional deterioration as a result of irreversible and progressive tubulo-interstitial injury and glomerulosclerosis (22), called

“chronic CNI nephrotoxicity.” These chronic effects of long-term cyclosporine use were then confirmed by others, both for cyclosporine (23,31,32) and for tacrolimus (33,34).

Table 1 provides a summary of the histologic lesions typically associated with the use of cyclosporine and tacrolimus. Figure 1 depicts the direct and indirect effects of CNI use on renal physiology, histology and function.

Acute CNI Nephrotoxicity

Vascular Effects: “Acute Arteriopathy.” In 1985, it was suggested that the reversible functional impairment associated

Table 1. Histological lesions associated with calcineurin inhibitor (CNI) use, and the differential diagnosis of CNI nephrotoxicity in post-transplantation biopsies

Lesions associated with calcineurin inhibitor use	Differential diagnosis in post-transplantation biopsies
Acute CNI nephrotoxicity	
Acute arteriopathy = renal dysfunction without histological alterations	Other causes of altered renal hemodynamics (e.g. drugs interfering with renal vascular resistance and prerenal azotemia)
Tubular vacuolization (isometric)	Osmotic nephrosis due to other agents like mannitol, inulin, glucose, sucrose, dextran, hydroxyethylstarch, urea and radiocontrast agents and also secondary to intravenous immunoglobulins; other causes of tubular ischemia.
Thrombotic microangiopathy (TMA)	Recurrent disease (primary HUS/TTP) and other risk factors for TMA like ischemia-reperfusion endothelial injury, renal infections, vascular rejection, anticardiolipin antibodies, malignancies and various other drugs (e.g., mTOR inhibitors, antiviral agents)
Chronic CNI nephrotoxicity	
Interstitial fibrosis and tubular atrophy (typically striped)	Pre-existing donor injury, aging, ischemia-reperfusion injury, tubulo-interstitial rejection, infection (e.g., UTI, polyomavirus, CMV), chronic ischemia (e.g., renal artery stenosis, size discrepancy in pediatric transplantation), chronic postrenal obstruction, diabetes mellitus
Medial arteriolar hyalinosis	Pre-existing donor injury, aging, diabetes mellitus, hypertension (in these cases more subendothelial deposition)
Glomerular capsular fibrosis	Glomerular ischemia (e.g., renal artery stenosis, chronic arteriolar vasoconstriction, or arteriolar hyalinosis) and other causes of atubular glomeruli (i.e., causes of tubular atrophy)
Global glomerulosclerosis	Pre-existing donor injury, aging, chronic glomerular ischemia (e.g., renal artery stenosis, arteriolar vasoconstriction, or hyalinosis), recurrent primary disease, <i>de novo</i> glomerular disease, hypertension secondary to tubular atrophy in a late stage
Focal segmental glomerulosclerosis (FSGS)	Recurrent primary disease; donor–recipient size discrepancy with hyperfiltration injury; FSGS secondary to other causes of glomerulosclerosis
Juxtaglomerular apparatus hyperplasia	Not well established, but likely other causes of hyperreninemia (e.g., transplant renal artery stenosis)
Tubular microcalcifications	Pre-existing donor injury, ischemic tubular injury and acute tubular necrosis, bone and mineral metabolism imbalance, proteinuria

HUS, hemolytic uremic syndrome; TTP, thrombotic thrombocytopenic purpura; TMA, thrombotic microangiopathy; CNI, calcineurin inhibitor; UTI, urinary tract infection; CMV, cytomegalovirus; FSGS, focal segmental glomerulosclerosis.

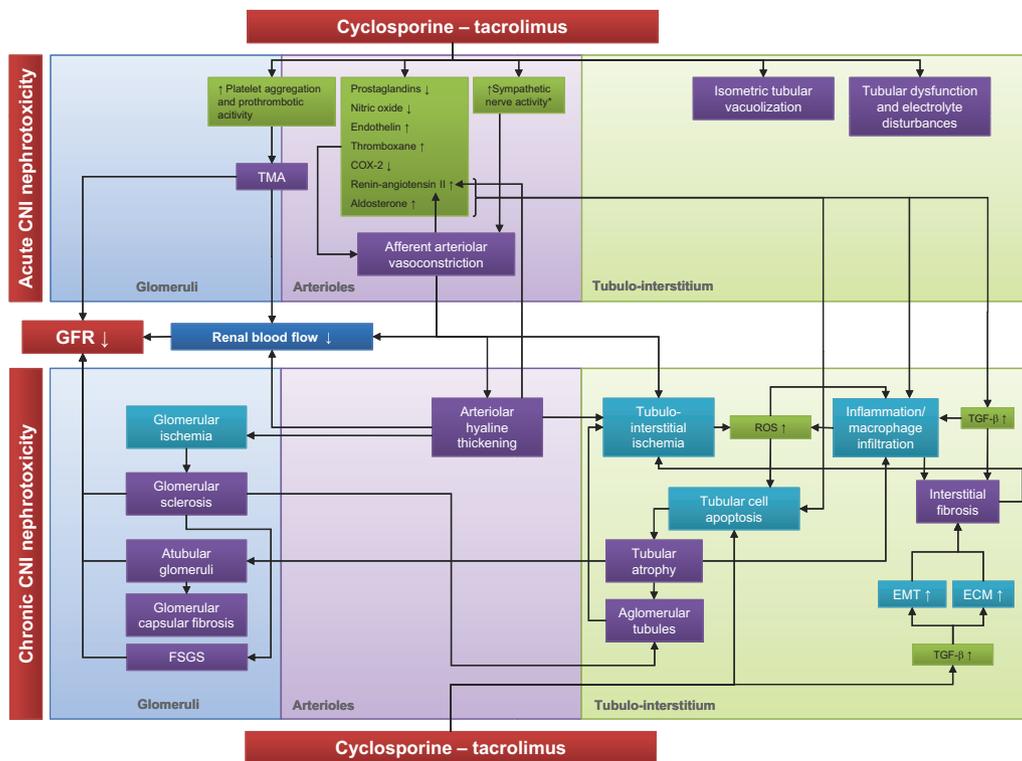


Figure 1. Schematic representation of the etiology of calcineurin inhibitor nephrotoxicity. CNI, calcineurin inhibitor; TMA, thrombotic microangiopathy; EMT, epithelial mesenchymal transition; ECM, extracellular matrix; GFR, glomerular filtration rate; FSGS, focal segmental glomerulosclerosis; ROS, reactive oxygen species. *Only in native kidneys.

with the use of cyclosporine is due to vasoconstriction of the afferent arterioles (35), which was first demonstrated by Murray *et al.* (36). The importance of renal vascular resistance and blood flow in the afferent and efferent arterioles for the acute functional impairment associated with the use of cyclosporine was confirmed by other groups, which unequivocally showed the profound alterations in vascular flow (37–39) and even reduced diameter of the afferent arterioles (40) with cyclosporine treatment.

Several studies indicate that vascular dysfunction results from an increase in vasoconstrictor factors that include endothelin and thromboxane and activation of the renin-angiotensin system (RAS), as well as a reduction of vasodilator factors like prostacyclin, prostaglandin E2, and nitric oxide (NO) (reviewed in 25,41,42). In addition, free radical formation plays a role in acute CNI nephrotoxicity, as well as sympathetic nerve activation in native kidneys. Finally, reversible tubular dysfunction is also recognized as a feature of acute CNI nephrotoxicity. It is currently not known whether these effects are related to calcineurin/NFAT-dependent mechanisms, but the similarities between cyclosporine and tacrolimus, which have a completely different molecular structure and intracellular binding site but a similar toxicity profile, suggest that this may be the case.

Endothelin is a potent vasoconstrictor widely released in the kidney and vascular beds (43). Endothelin was linked to acute CNI nephrotoxicity when it was found that cyclosporine stimulates endothelin release from cultured renal epithelial cells (44). This was later confirmed in animal studies (45) and in

humans (46). The pivotal role of endothelin in CNI-associated renal dysfunction is further illustrated by the finding that anti-endothelin antibodies block the functional renal impairment of acute CNI nephrotoxicity, at least in rats (47). The mechanism underlying CNI-associated increase in endothelin production is, however, not yet clear.

Cyclosporine leads to activation of the renin-angiotensin system (RAS), by both direct effects of cyclosporine on juxtaglomerular cells (48) and indirect effects from the renal vasculature hemodynamic changes (arteriolar vasoconstriction) secondary to decreased vasodilator factors and increased endothelin (49). The effects of cyclosporine on the RAS have been reviewed in detail previously (50,51). Because RAS activation reduces renal blood flow through the action of angiotensin II (49), this mechanism represents a vicious circle, which further enlarges the renal hemodynamic changes associated with the use of CNIs. In addition, recruitment of renin-containing cells in the afferent arterioles has been observed in association with cyclosporine use, both in rats (52) and in humans (53). The increased renin secretion associated with calcineurin inhibition is sometimes seen as hyperplasia of the juxtaglomerular apparatus, which is typically observed in states of chronic renin stimulation (54). Moreover, cyclosporine not only leads to activation of the RAS, but also augments the vasoconstrictory effects of angiotensin II in smooth muscle cells by influences on intracellular calcium stores, smooth muscle cell phenotypic maintenance, and contractility (51). The molecular mechanisms by which cyclosporine stimulates renin synthesis in the juxta-

glomerular cells, induces recruitment of renin-containing cells in the afferent arterioles, and leads to alterations in the vascular smooth muscle cells are currently not known.

In addition, cyclosporine induces imbalances in the vasodilator/vasoconstrictor ratio of arachidonic acid metabolites (eicosanoids), which ultimately promotes renal vasoconstriction. An explanation could be found in the association of NFAT and cyclooxygenase-2 (COX-2). The COX-2 gene promoter contains binding sites for NFAT, and NFAT has been reported to be of major importance for the regulation of COX-2 gene expression *in vitro* (55). In this light, it could be expected that inhibition of calcineurin activity also attenuates COX-2 expression. This was shown by Höcherl *et al.* (56,57), who demonstrated the selective suppression of renal COX-2 expression by both cyclosporine and tacrolimus in rats. The inhibition of NFAT-mediated COX-2 expression has also been reported in nonrenal cells (58). Furthermore, the renal effects of CNIs and selective COX-2 inhibitors have many similarities, including vasoconstriction of afferent arterioles, reduction in GFR, potassium retention, and sodium retention (59–61). Taken together, although this was not studied directly, COX-2 inhibition and associated decreased formation of prostaglandin E2 by calcineurin/NFAT inhibition could well be a major mechanism by which CNIs lead to renal vasoconstriction and decreased GFR. In addition, there is convincing evidence that COX-derived prostanoids are involved in the regulation of renin synthesis and secretion in the juxtaglomerular apparatus, as well as in tubular salt and water handling (see below) (62,63). Therefore, the effects of CNIs on COX-2 inhibition could be very important for the development of CNI nephrotoxicity.

Much evidence points to endothelial dysfunction as an essential factor in the pathogenesis of acute CNI nephrotoxicity. Cyclosporine and tacrolimus inhibit NO synthesis (64) and endothelium-dependent NO-mediated renal vasodilatation. Cyclosporine is known to increase free radical formation and superoxide production, likely through vasoconstriction-associated hypoxia (see below). By forming peroxynitrite, superoxides decrease NO bioavailability (65–67). In addition, cyclosporine decreases eNOS-mediated NO production through various mechanisms (68–70). This decreased NO bioavailability and production could then lead to decreased vasodilation and unopposed vasoconstriction, which is a main mechanism of cyclosporine-induced hypertension and decreased GFR (71).

Finally, in native kidneys, stimulating effects of cyclosporine on sympathetic nerve activity could play a role in the acute effects of cyclosporine by increased renal vascular resistance and secondarily decreased GFR (72–74). In transplanted kidneys, which lack innervation, the sympathetic effects of cyclosporine were not observed (75). The effects of cyclosporine on sympathetic nerve activity could be explained by the fact that calcineurin is ubiquitously expressed in neural tissue (76,77). Recently, it was suggested that calcineurin inhibition stimulates excitatory renal afferents through effects on synapsins. Synapsins are components of presynaptic vesicles and microvesicles of sensory nerve endings, including renal sensory nerve endings (78,79). Whether these mechanisms contribute to the neurotoxic effects of the CNIs has not been studied to date.

Tubular Effects: “Toxic Tubulopathy.” Histologically, acute CNI nephrotoxicity has been associated with isometric vacuolization of the tubular cytoplasm, as a result of enlargement of the endoplasmic reticulum and increased lysosomes (80–84). However, it should be noted that acute nephrotoxicity of CNIs with renal (graft) dysfunction is often present in the absence of morphologic lesions (pure functional/vascular effects). In contrast, isometric tubular vacuolization associated with the use of cyclosporine and tacrolimus can be found in the absence of renal dysfunction, and a recent study has demonstrated that isometric tubular vacuolization is not associated with progression to chronic CNI nephrotoxicity (85).

Notwithstanding the observation that isometric tubular epithelial cell vacuolization is commonly seen in the context of acute CNI nephrotoxicity after kidney transplantation, this vacuolization is not a specific finding and is also seen in allograft biopsies of patients maintained only on steroids and azathioprine, as well as in other clinical settings such as renal ischemia or tubular epithelial injury caused by intravenous administration of hyperosmotic fluids (*e.g.*, including solutions of mannitol, inulin, glucose, sucrose, dextran, hydroxyethylstarch, urea, and radiocontrast agents) (34,86–89). This tubular vacuolization is sometimes called “osmotic nephrosis” and can be distinguished from cyclosporine-induced acute tubular toxicity by the varying size of the vacuoles in osmotic nephrosis, in contrast to cyclosporine-induced vacuolization, which is typically isometric. Finally, intravenous immunoglobulins can also cause vacuolization of tubular epithelial cells, with perhaps a rather macrovacuolar appearance (90–92).

The processes by which CNIs lead to isometric tubular vacuolization are currently not known. Isometric tubular vacuolization could be the consequence of relative ischemia caused by afferent arteriolar vasoconstriction, but the possibility that direct effects of calcineurin inhibition in tubular epithelial cells cause alterations in endoplasmic reticulum structure and functioning cannot be excluded (93).

Next to tubular vacuolization, inclusion bodies are also sometimes noted in the tubular cytoplasm in association with cyclosporine use. Ultrastructurally, these inclusion bodies represent giant mitochondria and autolysosomes (81,82). However, giant mitochondria are also nonspecific and occur in a variety of conditions, including ischemic injury, and are often found in preimplantation donor biopsies, which limits their diagnostic value (94). It is currently not known what triggers the formation of these giant mitochondria, but it is clear that cyclosporine has important effects on mitochondrial functioning (95,96).

Finally, experimental and clinical evidence points to more subtle tubular injury. Not only are there significant effects of calcineurin inhibition on tubular electrolyte handling (see below), but other signs of sublethal tubular toxicity are also observed. This toxicity has been related to inhibition of the cell cycle through cyclosporine-induced accumulation of p53 (97,98). In addition, CNI-induced tubular toxicity is also suggested by the finding that urinary retinol binding protein concentration is associated with an increased risk of developing progressive renal failure in heart transplant recipients treated with cyclosporine (99,100). Retinol binding protein is normally

reabsorbed in the proximal convoluted tubules and can be used as a marker for tubular dysfunction associated with CNI use. Other markers of tubular dysfunction like urinary N-acetyl β -D-glucosaminidase, which is a lysosomal enzyme that originates from proximal tubular microsomes, have also been associated with reduced GFR induced by cyclosporine A (101,102). Finally, it was shown that sublethal cyclosporine exposure induces heat shock protein expression (103–105), decreased NO production in cultured tubular epithelial cells (106), and alterations in calcium influx and free cytosolic calcium concentration (107,108), further illustrating the direct toxic effects of calcineurin inhibition on tubular function.

Thrombotic Microangiopathy. Finally, although *de novo* thrombotic microangiopathy (TMA)/hemolytic uremic syndrome after renal transplantation can occur as a result of many factors, including ischemia-reperfusion endothelial injury, renal infections, rejection, anticardiolipin antibodies, malignancies, and various drugs, the use of the CNIs cyclosporine and tacrolimus is clearly an important risk factor for post-transplant TMA (109–113). This is attributed to the endothelial injury secondary to vasoconstriction-associated ischemia, and in addition, it has been suggested that cyclosporine and tacrolimus can increase platelet aggregation and activate prothrombotic factors. The mechanisms underlying TMA after renal transplantation were recently reviewed by Ponticelli (113).

Chronic CNI Nephrotoxicity

Chronic CNI nephrotoxicity is the Achilles' heel of current immunosuppressive regimens (114). Myers *et al.* were the first to demonstrate that cyclosporine not only induces reversible alterations in renal vascular resistance, but is associated with irreversible damage of the renal architecture (22). In this study in native kidney biopsies from heart transplant recipients, interstitial fibrosis and tubular atrophy were described, accompanied by focal glomerular sclerosis. Later, more detailed histologic analyses demonstrated that all three compartments of the kidneys can be irreversibly affected by cyclosporine and tacrolimus treatment: vessels (arteriolar hyalinosis), tubulo-interstitium (tubular atrophy and interstitial fibrosis), and glomeruli (thickening and fibrosis of Bowman's capsule and focal segmental or global glomerular sclerosis) (Table 1) (34,83,84). In protocol biopsy studies, it was shown that lesions suggestive of chronic CNI nephrotoxicity progress with time after transplantation. By 10 yr after transplantation, lesions suggestive of chronic CNI nephrotoxicity were seen in virtually all cases (115). However, this study did not contain a control arm, and the relative contribution of chronic CNI use *versus* other injury processes like rejection, infections, other nephrotoxic drugs, hypertension, diabetes, hyperlipidemia, and aging processes is therefore not known.

The etiology of chronic CNI nephrotoxicity has been studied extensively. A combination of cyclosporine-induced hemodynamic changes and direct toxic effects of cyclosporine on tubular epithelial cells is thought to play a role (Figure 1).

Vascular Effects. Nodular hyaline deposits in the media of afferent arterioles (arteriolar hyalinosis) is regarded as a hallmark of CNI nephrotoxicity, and is characterized by the re-

placement of necrotic smooth muscle cells by focal or circular lumpy protein (hyaline) deposits at the periphery of the wall of afferent arterioles. Eventually, these nodular hyaline deposits become sufficiently large to cause narrowing of the vascular lumen (83). Arteriolar hyalinosis is commonly regarded as irreversible, although it has been reported that complete regression of severe cyclosporine-associated arteriolar pathology and remodeling of arterioles can occur after stopping or reducing the dose of cyclosporine (116,117).

The study on the etiology of arteriolar hyalinosis associated with the use of CNIs is complicated by the fact that it has been difficult to reproduce this process in animal studies. In salt-depleted rats, however, which appeared to be a valid model for chronic CNI nephrotoxicity (118), it was shown that arteriolar hyalinosis lesions begin with granular eosinophilic transformation of smooth muscle cells in afferent arterioles, followed by vacuolization of smooth muscle cells and discrete hyaline deposits in vessel walls (119). The underlying processes and molecular mechanisms leading to these smooth muscle cell changes are currently not well elucidated, but may be related to the important function of calcineurin-NFAT in smooth muscle cells (120–122). Furthermore, it could be hypothesized that there is an association between prolonged arteriolar vasoconstriction induced by imbalances in the release of vasoactive substances and cyclosporine and tacrolimus treatment (see above), but this has not been shown directly.

Tubular-Interstitial Effects. In the context of chronic cyclosporine and tacrolimus use, it is likely that the associated arteriolar pathology and narrowing of the arteriolar lumen is a major contributor to the development of (typically striped) interstitial fibrosis and tubular atrophy, as well as glomerular sclerosis. Local hypoxia or ischemia of the tubulo-interstitial compartment, resulting from renal vasoconstriction induced by cyclosporine or tacrolimus, lead to the formation of free radicals or reactive oxygen species (66,123,124). The importance of free radical formation in CNI nephrotoxicity was first suggested by the beneficial effects of vitamin E on lipid peroxidation and cyclosporine-induced renal damage (125,126). Like is the case with hypoxia in other organs, renal vasoconstriction can lead to renal hypoperfusion and hypoxia-reoxygenation injury and subsequently to the formation of reactive oxygen species or free radicals, which then causes cellular injury and promotes cellular death by apoptosis (66,127). This is further illustrated by the finding that catalase, an enzyme that specifically breaks down the reactive H_2O_2 into H_2O and O_2 and acts as a scavenger of reactive oxygen species, reduces cyclosporine-associated renal tubular epithelial cell senescence (128). This finding, however, does not exclude possible direct effects of cyclosporine in the generation of reactive oxygen species. This second hypothesis is supported by the finding that rat proximal tubular epithelial cells exposed to cyclosporine accumulate intracellular reactive oxygen species and lipid peroxidation products, along with an altered glutathione redox state (129).

In addition, upregulation of TGF- β is considered an important etiologic factor in chronic CNI nephrotoxicity. TGF- β expression of tubular epithelial cells is directly upregulated by cyclosporine and has also been observed after treatment with

tacrolimus (130–135), independent of the hemodynamic effects of calcineurin inhibition (136). TGF- β promotes interstitial fibrosis by decreasing the degradation and increasing the production of extracellular matrix proteins (137,138). In addition, TGF- β induces epithelial mesenchymal transition (EMT). EMT is a multistep process in which renal tubular epithelial cells lose their epithelial phenotype and acquire new characteristic features of mesenchyme, which includes disruption of polarized tubular epithelial cell morphology, *de novo* α -smooth muscle actin expression and actin reorganization, loss of cell–cell adhesions through downregulation of E-cadherin, destruction of basement membrane, and increased cell migration and invasion. EMT is recognized as a major mechanism contributing to renal interstitial fibrosis (139–143) and is related to chronic cyclosporine use (144).

Angiotensin II not only is important for its hemodynamic actions and acute CNI nephrotoxicity (see above), but also has pleiotropic effects including aldosterone release, stimulation of tubular transport, proinflammatory effects, and profibrogenic and growth stimulatory actions, which are mainly mediated by AT₁ receptors and induction of TGF- β (49,138). Activation of the RAS, therefore, not only is important for its hemodynamic contribution to CNI nephrotoxicity, but also directly promotes renal interstitial fibrosis and chronic CNI nephrotoxicity, as has been shown in rats (145,146). The observation that salt depletion (leading to RAS activation) is essential for achievement of chronic CNI nephrotoxicity in animal models supports the important role of RAS activation and angiotensin II generation (118,119,147). However, it should be noted that the mechanisms of CNI nephrotoxicity observed in salt-depleted rats do not fully overlap with the pathophysiology of chronic CNI nephrotoxicity in humans. An in-depth analysis of these differences is provided by Lee *et al.* (50). Increased aldosterone secondary to RAS activation could also in itself aggravate interstitial fibrosis through production of growth factors (like TGF- β) and reactive oxygen species, as well as inhibition of extracellular matrix degradation (see below), as was recently reviewed by Remuzzi *et al.* (148).

Calcineurin inhibition may also directly activate apoptosis genes and increase apoptosis in tubular and interstitial cells, thereby inducing tubular atrophy (96,127,149–151). In addition, inflammation with macrophage infiltration has been associated with chronic CNI nephrotoxicity (42,136,152,153). Finally, cyclosporine competitively inhibits P-glycoprotein (multidrug resistance protein 1 [MDR1] or ATP-binding cassette subfamily B, member 1 [ABCB1]) activity, which is a drug transporter expressed at the apical membrane of tubular cells. From this, it is possible that decreased excretion of endogenous toxic substances accumulate locally in the kidney when cyclosporine therapy is used (see below) (154).

Glomerular Effects. The chronic use of CNIs can induce many different types of glomerular injury. Most commonly, global glomerulosclerosis results from severe CNI-associated arteriolar hyalinosis and arteriolopathy and secondary glomerular ischemia (115,155,156), as was also seen in diabetes mellitus (157). Furthermore, tubular damage (see above) leads to the development of atubular glomeruli, which are perfused glo-

meruli that are disconnected from their proximal tubule. Atubular glomeruli appear smaller and often have periglomerular fibrosis (capsular fibrosis), or may become severely contracted within an enlarged glomerular cyst (155,158,159). In addition, calcineurin inhibition may cause focal segmental glomerulosclerosis lesions, possibly caused by hyperfiltration injury associated with either arteriolar hyalinosis or global glomerulosclerosis (84,155,160–163).

Electrolyte Disturbances

In addition to the impact of CNI use on renal hemodynamics and structure (see above), cyclosporine and tacrolimus also lead to tubular functional alterations and ion homeostasis disturbances like hyperkalemia, hypomagnesemia and magnesium wasting, hyperchloremic metabolic acidosis (distal tubular acidosis), and hyperuricemia (164–168).

Some of the effects of cyclosporine and tacrolimus on tubular function can be explained by reduced expression of the Na⁺-K⁺-2Cl⁻-cotransporter (NKCC2) at the apical membrane of tubular epithelial cells (169,170). As was shown in patients with type I Bartter's syndrome (NKCC2 mutations) and in patients treated with NKCC2 inhibitors (*e.g.*, furosemide), decreased expression of NKCC2 would lead to polyuria, nephrocalcinosis, magnesium wasting, hyperreninemic hyperaldosteronism, and juxtaglomerular apparatus hyperplasia (171), which are all features of CNI use. The finding that cyclosporine attenuates the natriuretic effects of loop diuretics, likely by the inhibition of COX-2-mediated renal prostanoid formation, fits well with this model (57). However, the only factors that do not fit with the Bartter-like syndrome are hyperkalemia and metabolic acidosis (172). The hyperkalemia seen with calcineurin inhibition is likely multifactorial and relates to inhibitory effects on Na⁺-K⁺-ATPase in collecting ducts (169,170,173,174) and possibly to distal tubular acidosis. In addition, there is evidence that decreased numbers of mineralocorticoid receptors, which are detected in 75% of patients who are treated with cyclosporine, lead to hyperkalemia and metabolic acidosis as a result of aldosterone resistance (175). Recently, it was demonstrated that cyclosporine reduces paracellin-1 expression in thick ascending limb cells (176). The resulting decrease in magnesium transport likely contributes to the magnesium wasting and hypomagnesemia induced by cyclosporine (176), which is in itself associated with chronic interstitial fibrosis, a faster rate of decline of kidney function, and increased rates of graft loss in renal transplant recipients with CNI nephrotoxicity (177,178). Finally, it was shown that cyclosporine indirectly opens ATP-sensitive K⁺ channels by inhibition of calcineurin, which could contribute to the CNI-associated hyperkalemia (179). The effects of cyclosporine on hyperuricemia are reviewed by Clive (167). These findings, however, do not explain the whole picture, and the effects of calcineurin inhibition on tubular transport are likely much more complex and vary between the different nephron segments (180), as well as between cyclosporine and tacrolimus (181–183). The molecular mechanisms underlying the inhibitory effects of CNIs on tubular transport function are currently not known, and further research is needed in this field.

Diagnosis, Differential Diagnosis, and Nonspecificity of the Histologic Lesions

In kidney transplantation, the differential diagnosis between cyclosporine/tacrolimus-related nephrotoxicity and other injury phenomena remains very difficult. Opposite phenomena share the same clinical picture of gradual decrease in renal graft function and lead to nonspecific interstitial fibrosis, tubular atrophy, and finally complete scarring of the renal allograft (32). Lesions considered more specific for CNI nephrotoxicity, such as tubular vacuolization and new onset or progressive arteriolar hyalinosis, are also seen secondary to other processes. Table 1 provides an overview of the histologic lesions associated with the use of CNIs, together with the differential diagnosis for each lesion. A combination of several histologic features associated with the use of CNIs and exclusion of other processes that lead to similar histologic findings would be mandatory for making a positive diagnosis of CNI nephrotoxicity. The CNI nephrotoxicity score recently proposed by Kamtham *et al.* represents a first step in the standardization of the composite histologic damage induced by CNIs (184), but further validation studies are necessary.

After nonrenal organ transplantation, the picture may be clearer, and the study of CNI nephrotoxicity in native kidneys may be regarded as less troublesome (22–24,185), although the underlying disease can be associated with or lead to renal problems. Also the frequent use of concomitant nephrotoxic agents may mask the true effects of the chronic use of CNIs on native kidneys. In addition, it should be kept in mind that an essential difference between native kidneys and transplanted kidneys is the absence of innervation of transplanted kidneys; this innervation plays a prominent role in the regulation of renal vascular resistance (see above). Therefore, the extrapolation of findings on CNI nephrotoxicity in native kidneys to transplanted kidneys should be interpreted cautiously.

Another concern for the histologic diagnosis of CNI nephrotoxicity is the sometimes poor reproducibility of the histologic grading of the lesions (186–191). It is important to take into account the inter- and intraobserver variability of the histologic scoring according to the evolving Banff classification of renal allograft pathology (192–196), especially when the histologic data are used for scientific purposes and for comparison between different centers. But also for routine clinical practice, the moderate to even poor reproducibility of histologic scoring of renal allograft biopsies is of concern. This can be partly overcome by intensive and continuous training of involved and dedicated pathologists, and by rescoring biopsies for studies in a blinded way by a “central” pathologist, which obviously avoids interobserver variability. In addition, the reproducibility of interstitial fibrosis can be improved by using computerized quantitative scoring methods (197,198), and the scoring of other lesions like arteriolar hyalinosis could be refined for improvement of reproducibility (191).

Comparison Between Cyclosporine and Tacrolimus

Although cyclosporine and tacrolimus are not structurally related (and despite initial optimism), it has been demonstrated

that tacrolimus has nephrotoxic properties similar to those of cyclosporine and induces similar histologic injury (83,199,200). In addition, in a study with 61 cyclosporine analogs, it was shown that the ability to induce nephrotoxicity *in vivo* correlates with the immunosuppression activity of these agents (201). This study very strongly suggested that the mechanisms of immunosuppression are the same as those of toxicity, that is, calcineurin inhibition (201). The association between the tissue-specific degree of calcineurin inhibition (greatest in kidney) and the tissue-specific side effects of cyclosporine further supported the direct role of calcineurin inhibition in the side effects of these drugs (18,19). Finally, for many aspects of CNI nephrotoxicity, there is now also increasing molecular evidence that the calcineurin-NFAT pathway could be pivotal in its pathogenesis (see above). Intriguingly however, a recently developed CNI with increased immunosuppressive potential (ISA247; voclosporin) induced renal dysfunction in only a very low number of patients treated for plaque psoriasis (202). In renal transplantation, preliminary results demonstrate that voclosporin is not inferior to tacrolimus with regard to biopsy-proven acute rejection, with a similar or slightly better safety profile: lower triglyceride and higher magnesium levels and less post-transplant diabetes mellitus. However, interim results did not show a benefit of voclosporin over tacrolimus with regard to kidney function (203,204). More detailed analyses of these data and longer term results are awaited.

There is substantial evidence that tacrolimus has a lower nephrotoxicity potential than cyclosporine. Animal studies have demonstrated that the vasoconstrictive effect of tacrolimus is weaker than of cyclosporine (205–207), and this was also confirmed in humans (208,209). Moreover, the fibrogenic potential of tacrolimus also appears to be lower (210), although these results could not be confirmed in a more recent study (135). In nonrenal solid organ transplantation, there are single- and multicenter studies as well as registry analyses demonstrating the benefit of tacrolimus over cyclosporine with regard to renal function (24,211–214), although other studies did not observe this benefit of tacrolimus over cyclosporine (215).

In renal transplantation, this analysis is much more difficult to perform, because of the interference with renal allograft rejection phenomena. However, when tacrolimus and cyclosporine efficacy were at similar level in renal transplantation (similar incidence of acute rejection), tacrolimus therapy was associated with significantly lower serum creatinine levels or higher GFR compared with cyclosporine (216–218), and even better graft survival (219). A change from cyclosporine to tacrolimus for renal allograft dysfunction was also associated with significant improvement in renal function (220,221). The SYMPHONY study, a recent large randomized multicenter trial comparing different immunosuppressive regimens, demonstrated the benefit of low-dose tacrolimus over cyclosporine in terms of both graft function and graft survival (222).

In addition, there are also marked differences in the nonrenal toxicity profile of cyclosporine *versus* tacrolimus. In a recent meta-analysis and a randomized trial in kidney transplantation, tacrolimus was associated more with diabetes mellitus, tremor, headache, dyspepsia, vomiting, diarrhea, and hypomag-

nesemia than was cyclosporine, which was associated more with hirsutism, gingival hyperplasia, higher LDL cholesterol, and higher triglyceride levels (218,219). The reasons for these differences between cyclosporine and tacrolimus are currently not known.

Risk Factors for CNI Nephrotoxicity

A brief summary of the clinical risk factors for CNI nephrotoxicity is provided in Table 2.

Systemic Levels of Cyclosporine and Tacrolimus

Concentration-Effect and Concentration-Toxicity Relationship. The main issue after renal transplantation is to maintain a reasonable balance between efficacy (avoid rejection) and toxicity (especially CNI nephrotoxicity) of the immunosuppressive agents used. In the first years of clinical use of cyclosporine, immunosuppressants were administered in fixed doses based on body weight. Dose adjustments were made only for apparent side effects, and a dose–effect relationship was already suggested by Calne in 1979 (2). In 1981, Keown *et al.* were the first to establish an association between serum concentrations of cyclosporine and the immunosuppressive capacity in renal allograft recipients (223), which was later confirmed (18). Klintmalm *et al.* were the first to demonstrate a relationship between cyclosporine doses, plasma levels, and renal allograft interstitial fibrosis (31,224). This association between cyclosporine

nephrotoxicity and high cyclosporine doses or levels was then confirmed by others (225).

In contrast to cyclosporine, for which no dose-finding studies were performed before its introduction into clinical practice, extensive studies were performed before the general introduction of tacrolimus. Similar to cyclosporine, significant concentration–rejection and concentration–toxicity correlations were found (226–228). It should be noted that the ranges used in these dose-finding studies for tacrolimus were very broad (pre-dose trough level range, 5 to 25 ng/ml). After it became clear that cyclosporine and tacrolimus are drugs with a narrow therapeutic window, great care was given to keep dosage within preset target ranges (228,229).

Pharmacokinetics–Pharmacogenetics. Maintaining the concentrations of the CNIs cyclosporine and tacrolimus within preset target ranges turned out to be complicated by their high inter- and intraindividual pharmacokinetic variability, resulting in very low dose–concentrations correlations (230,231). This unpredictable dose–concentration relationship results from a high variability in absorption, distribution, metabolism, and elimination of these compounds. The clinical pharmacokinetics of cyclosporine and tacrolimus are reviewed by Fahr (230) and Staatz and Tett (231). Intestinal absorption of both cyclosporine and tacrolimus is low and variable, and is influenced by concomitant ingestion of food, diabetes, uremia, ethnicity, gastrointestinal problems, and diarrhea. This can be largely attributed to variability in expression and function of the metabolizing cytochrome P450 3A isozymes (mainly CYP3A4 and CYP3A5) and of the multidrug efflux transporter P-glycoprotein (from permeability-glycoprotein; also MDR1 and ABCB1). In blood, cyclosporine and tacrolimus are extensively distributed in erythrocytes. In plasma, more than 90% of cyclosporine and tacrolimus is bound to plasma proteins like α 1-acid glycoprotein, albumin, globulines, and lipoproteins. Metabolism of cyclosporine and tacrolimus occurs mainly in the liver and the gastrointestinal tract through the hydroxylating and demethylating actions of CYP3A4 and CYP3A5 isozymes. Metabolism of these agents is virtually complete, with less than 1% of the parent drug appearing in urine or feces. After metabolization, cyclosporine and tacrolimus metabolites are eliminated in the bile; less than 5% is excreted in the urine.

Significant interindividual and intraindividual variability in the expression and function of CYP3A5, CYP3A4, and P-glycoprotein contribute to the highly variable pharmacokinetics of both CNIs. This can be partly attributed to interactions with other drugs, which cause either inhibition or stimulation of expression or activity of these enzymes and transporter (*e.g.*, corticosteroids, antibiotics, antihypertensive or antiarrhythmic agents, antimycotics, antiepileptics). In addition, interfering disease conditions like diarrhea likely affect expression or activity of these enzymes or transporters, as has been suggested for tacrolimus (232,233). The long-term maturation of tacrolimus pharmacokinetics, in both adult and pediatric patients (234,235), is likely also mediated through alterations in CYP3A4/5 activity or expression (234). Finally, in recent years, there has been extensive evidence that CNI pharmacokinetics can be influenced by genetic polymorphisms in CYP3A5 (236–

Table 2. Clinical risk factors for the development of calcineurin inhibitor nephrotoxicity

Risk Factors for Calcineurin Inhibitor Nephrotoxicity
Systemic overexposure to cyclosporine and tacrolimus
Local exposure to cyclosporine and tacrolimus
interactions with drugs interfering with ABCB1-mediated transport in the tubular epithelial cells (<i>e.g.</i> , mTOR inhibitors)
ABCB1 genotype of the kidney
ABCB1 expression in renal tubular epithelial cells
Exposure to metabolites of cyclosporine and tacrolimus
CYP3A4/5 genotype of the patient
CYP3A5 expression in renal tubular epithelial cells
interactions with other drugs which lead to altered exposure to calcineurin inhibitor metabolites (<i>e.g.</i> ketoconazole)
Older kidney age
Use of nonsteroidal anti-inflammatory drugs
Salt depletion and diuretic use
Genetic polymorphisms of other genes (<i>e.g.</i>, TGF-β, ACE)

ABCB1, ATP-binding cassette subfamily B, member 1; TGF- β , transforming growth factor β ; ACE, angiotensin converting enzyme.

241); the impact of single nucleotide polymorphisms in *ABCB1* is less clear. The largest cohort studies did not demonstrate an association of *ABCB1* polymorphisms with cyclosporine pharmacokinetics (238,242–244), and the effects of *ABCB1* polymorphisms on tacrolimus pharmacokinetics are not consistent (238–240,245,246). This demonstrates that *ABCB1* variants, at maximum, explain tacrolimus pharmacokinetics to a limited degree. The effects of *CYP3A4*, *CYP3A5*, and *ABCB1* single nucleotide polymorphisms on cyclosporine and tacrolimus pharmacokinetics have been reviewed in detail by Hesselink *et al.* (247) and more recently by Anglicheau *et al.* (248).

Therapeutic Drug Monitoring. The low therapeutic index of cyclosporine and tacrolimus, the high variability in the pharmacokinetics, and the concentration–effect and concentration–toxicity associations suggest that therapeutic drug monitoring of these agents is useful. Routine monitoring of cyclosporine and tacrolimus levels is now common practice. In most centers, CNI dose is adjusted according to the predose trough (C_0) concentrations (249,250). However, especially for cyclosporine, there is only a poor correlation between C_0 levels and total exposure (measured as area under the time–concentration curve; $AUC_{0\text{ to }12}$). Monitoring of cyclosporine levels 2 h after administration (C_2) appeared to be the single time point that correlates best with total exposure (251,252), and could offer additional benefit over C_0 (253). Application of C_2 monitoring is, however, more complicated than C_0 monitoring, and there are no conclusive direct studies showing a significant benefit of C_2 over C_0 monitoring, which is a main reason most centers still rely on C_0 monitoring for cyclosporine (249,254). For tacrolimus, the correlation between C_0 and total $AUC_{0\text{ to }12}$ is much better, and although it is obvious that measurement of abbreviated pharmacokinetic profiles or the use of Bayesian forecasting models would increase accuracy importantly (250,255,256), C_0 level monitoring is generally regarded as sufficient (249). This better correlation of tacrolimus C_0 with total $AUC_{0\text{ to }12}$ could be one of the reasons tacrolimus could be less nephrotoxic than cyclosporine.

Therapeutic Drug Monitoring to Avoid CNI Nephrotoxicity. Despite these major efforts in establishing accurate therapeutic strategies for CNI monitoring, the application of such monitoring in routine clinical practice has not prevented kidney transplant recipients from developing acute rejection, nor has it avoided CNI nephrotoxicity, which is virtually universal by 10 yr after transplantation (115). However, the important interindividual variability in the rate at which chronic CNI nephrotoxicity develops is yet unexplained.

Acute CNI nephrotoxicity has been associated with relatively higher systemic exposure to cyclosporine (156) and tacrolimus (85,91). A recent study could not confirm this association, although there was a clear trend for tacrolimus, without reaching statistical significance (257). The lack of an association between tacrolimus exposure and acute CNI nephrotoxicity in this last study could be due to low numbers of patients treated with tacrolimus.

Whether the rate of development of lesions suggestive of chronic CNI nephrotoxicity is associated with the interindividual, relatively small variation in systemic exposure to cyclo-

sporine and tacrolimus, if kept within preset target ranges, is still a matter of debate. For cyclosporine, although some studies suggested an association of chronic CNI nephrotoxicity lesions with higher cyclosporine exposure (156,258), other studies have found that relatively low levels are associated with higher chronic histologic damage (259,260). A similar association of lower levels of tacrolimus with higher increase in chronic tubulo-interstitial damage was reported recently (261). In addition, this association was seen only when no additional induction therapy with IL-2 receptor blockers was given (261). Taken together, these last studies suggest that the observed progression of chronic damage is more the result of subclinical alloimmune phenomena than of overexposure to CNIs. This seems counterintuitive and provocative in the light of recent trials demonstrating short-term benefit of CNI-minimization protocols, with even shorter time to biopsy-proven acute rejection in the standard-dose group compared with the low-dose group, and equal prevalences of adverse events and similar graft function at 12 mos after transplantation between low- and high-dose groups (222,262). However, these differences in rejection timing are possibly more related to differences in mycophenolic acid (MPA) exposure between these groups, or in the use of daclizumab in the low-dose group. Regarding CNI nephrotoxicity, in these two randomized trials (222,262), the high-exposure group did not have significantly lower graft function, but because no protocol biopsies were performed in these studies and no long-term results are available yet, it is not known whether there could be differences in the prevalence of subclinical CNI toxicity between patients treated with high-dose *versus* low-dose cyclosporine. From this, it can be concluded that to date, there is no hard evidence that systemic exposure to cyclosporine and tacrolimus represents the major determinant of the risk for chronic CNI nephrotoxicity. Whether lower CNI systemic exposure in *de novo* renal transplantation leads to lower incidence or slower progression of chronic CNI nephrotoxicity needs to be tested in prospective clinical trials, which should include subclinical histologic appearance of the allografts as a surrogate end-point. Finally, it should be noted that several studies have related the risk for “chronic rejection” and renal transplant outcome with a high intraindividual variability in cyclosporine exposure, but the underlying reasons remain unknown (263,264).

In nonrenal transplantation, the scene should be clearer and not complicated by rejection phenomena. However, because no routine kidney biopsies are performed in nonrenal transplant recipients, not much data are available on the evolution of histologic CNI nephrotoxicity. In a study in cardiac transplant recipients, patients treated with low doses of cyclosporine had slightly lower mean serum creatinine concentrations, but similar pathologic changes were seen on the renal biopsies when compared with biopsies from patients treated with higher cyclosporine doses (265). This again suggests that low *versus* high dosage of cyclosporine confers only marginal protection against CNI-mediated injury. With regard to the impact of systemic cyclosporine exposure on renal dysfunction after cardiac transplantation, two large studies demonstrated no relationship between cyclosporine concentration and the decline in

renal function measured as the slope of serum creatinine as a function of time or by serial GFR measurements (266,267), and a case-control study (268) showed that cyclosporine doses and trough levels in the 24 heart transplant patients who developed end-stage renal disease were not different from those of patients who maintained stable renal function. Likewise, in liver transplantation, no differences were found in renal function or the incidence of nephrotoxicity between high- and low-dose cyclosporine groups, at least in the short term (269). Furthermore, in the first randomized trial on CNI minimization in liver transplantation, delayed introduction of lower-dose tacrolimus has been associated with better renal function in the first 6 mo, but this effect disappeared by 1 yr after transplantation (270).

Taken together, these studies in both renal and nonrenal solid organ transplantation suggest that interindividual susceptibility to chronic CNI nephrotoxicity is not directly related to interindividual variability in systemic exposure, if CNI levels are kept within relatively narrow target ranges using therapeutic drug monitoring.

Local Renal Exposure to Cyclosporine and Tacrolimus

A main reason close monitoring of systemic levels of cyclosporine and tacrolimus has not prevented development of chronic CNI nephrotoxicity could be that local levels in the kidney allograft are not directly related to the systemic levels, and that other factors could determine the local levels of these drugs in kidney tissue. Both for cyclosporine and tacrolimus, it was demonstrated that levels in renal tissue are much higher than in blood (18,271), but the degree of interindividual variability and the determinants of local cyclosporine or tacrolimus levels are currently not known.

There is, however, accumulating indication that the interindividual variability in local renal cyclosporine or tacrolimus exposure in kidney tissue contributes significantly to chronic CNI nephrotoxicity. First, there is the observation that both sirolimus and everolimus increase chronic nephrotoxicity of CNIs, both in rats (272) and in humans (273–275). This is explained by robust evidence that these mTOR inhibitors interact with cyclosporine excretion in tubular epithelial cells through competition for P-glycoprotein (276,277), and hence lead to accumulation of cyclosporine in these cells. Local renal accumulation of cyclosporine was demonstrated *in vitro* by Anglicheau *et al.* (277) and *in vivo* by Napoli *et al.* (278) and Podder *et al.* (279). These latter studies measured increased local renal cyclosporine concentrations in kidney tissue after concomitant treatment with sirolimus in rats. In addition, the study by Podder *et al.* (279) demonstrated that higher local renal concentrations of cyclosporine correlated significantly with decreased renal function and increased histologic damage (279). Although the first report on the combination of tacrolimus with sirolimus was encouraging (280), later studies also observed increased nephrotoxic potential of this combination of tacrolimus with sirolimus (281,282). It should, however, be noted that the synergistic effects of tacrolimus/sirolimus appears to be less important compared with the combination cyclosporine/sirolimus, both in animal studies and in a clinical setting (282,283).

Further evidence for the importance of local renal CNI concentrations is found in studies associating P-glycoprotein expression or function with CNI nephrotoxicity. P-glycoprotein is expressed at the apical side of tubular epithelial cells and is the main protein responsible for the excretion of the CNIs tacrolimus and cyclosporine (284,285), although the renal excretion of these CNIs is only limited (see above). In 1997, del Moral *et al.* suggested that variability in local P-glycoprotein expression is important for chronic CNI nephrotoxicity by using immunohistochemistry and Western blotting in a rat model of chronic CNI nephrotoxicity (286). Another study in rats showed that exposure of cultured renal tubular cells to cyclosporine induces P-glycoprotein overexpression (287). In addition, del Moral *et al.* demonstrated that the up-regulation of P-glycoprotein was inversely related to the incidence of arteriolar hyalinosis, interstitial fibrosis, and periglomerular fibrosis (286). Human studies confirmed this upregulation of tubular epithelial P-glycoprotein expression with cyclosporine treatment, as well as lower expression of P-glycoprotein in renal allograft biopsies with CNI nephrotoxicity (288,289). These human studies confirm the animal data and suggest that interindividual variability in local renal P-glycoprotein expression contributes to the local susceptibility to CNI nephrotoxicity. However, these case-control studies were not prospective and need to be validated in larger and, more importantly, prospective studies, as the association found does not prove any causal role of local P-glycoprotein expression variability. Whether a similar upregulation of P-glycoprotein expression is also present with tacrolimus treatment is currently not known.

In addition to this suggestion that the variability in tubular P-glycoprotein expression contributes to the susceptibility to CNI nephrotoxicity, a recent study has also demonstrated that variability in P-glycoprotein function could be important in the development of CNI nephrotoxicity. A single nucleotide polymorphism in *ABCB1*, the *TT* genotype at position 3435 of *ABCB1* of the donor was associated with chronic CNI nephrotoxicity (290). This frequent polymorphism has been associated with altered conformation and function of P-glycoprotein (291–293). However, another case-control study in liver transplant recipients found the opposite effect: an association of the *TT* genotype at position 2677 (which is in linkage disequilibrium with the *TT* genotype at position 3435) with lower risk for kidney dysfunction (294). The reason for this discrepancy is not clear but likely relates to differences in the effects on CNI pharmacokinetics, which was not assessed in these studies but could have played a role (see above). In addition, it should be emphasized that the definition of CNI nephrotoxicity was not very stringent in these studies and was not based on histologic criteria.

The most plausible hypothesis to explain these studies associating P-glycoprotein expression or activity with CNI nephrotoxicity is local accumulation of cyclosporine when apical P-glycoprotein expression or function are lower. This was shown for the interaction between cyclosporine and sirolimus (see above). In humans, no studies directly associating renal P-glycoprotein with local renal CNI concentrations are available. However, a study in mice found no differences in local renal

cyclosporine levels between MDR1 knock-out and wild-type mice, but a strong effect on cyclosporine concentrations in brain (295). This needs to be validated in humans, but if the absence of influence of P-glycoprotein expression on local renal exposure to CNIs is also true in humans, alternative hypotheses need to be formulated. It has been shown previously that decreased expression or altered function of P-glycoprotein plays a role in the apoptotic response to nephrotoxic agents and protects proximal tubular cells against apoptotic stress (296). Because cyclosporine use is associated with apoptotic mechanisms (see above), defective P-glycoprotein activity or decreased expression could increase the direct nephrotoxic effects of cyclosporine, which would explain the association of *ABCB1* genotype or expression with CNI nephrotoxicity in previous human studies. This hypothesis would also be an explanation for the apparent discrepancy between the minor importance of the kidney in cyclosporine and tacrolimus pharmacokinetics, and for the suggested contribution of local renal P-glycoprotein to CNI nephrotoxicity.

From this, it is clear that well-controlled prospective studies including pharmacokinetic data and robust histologic analyses are necessary to elucidate the exact role of renal P-glycoprotein expression and function and *ABCB1* polymorphisms on local CNI concentrations, apoptosis, and the development of CNI nephrotoxicity.

Exposure to Metabolites of Cyclosporine and Tacrolimus

Another yet-unanswered question is whether there is any contribution of cyclosporine and tacrolimus metabolites to CNI nephrotoxicity. It has been demonstrated previously that cyclosporine metabolite levels in patient blood often greatly exceed levels of the parent drug (297), and the same could be expected in renal tissue. Although most of these cyclosporine metabolites are considerably less immunosuppressive than the parent molecule (298,299), some were shown to reduce GFR of isolated perfused rat kidneys (297) and to cause tubular vacuolization (300) comparable to that of the parent drug. This could represent a causal explanation for the positive association between cyclosporine metabolite levels in blood and renal dysfunction in humans (301). Although some of these metabolites share immunosuppressive characteristics with cyclosporine, the effects of these metabolites on tubular function, TGF- β expression, and other aspects of CNI nephrotoxicity are currently not known. In contrast to cyclosporine, only the tacrolimus metabolite MII appears to have immunosuppressive capacity; the other metabolites do not (302,303). The nephrotoxic potential of tacrolimus metabolites has not been studied to date.

However, because CNI-metabolite levels can greatly exceed cyclosporine levels in patient blood, especially in patients with abnormal liver function (304), these metabolites maybe represent an important and unrecognized factor in the development of clinical CNI nephrotoxicity. *In vivo* evidence supporting this hypothesis is found in combination therapy of ketoconazole with CNIs. The combination of ketoconazole with cyclosporine and tacrolimus appeared to reduce the levels of the CNI metabolites by inhibition of the cytochrome P450 system (304–306). Coadministration of these agents leads to dramatic in-

creases in total exposure to cyclosporine and tacrolimus and could lead to acute toxicity (307). However, when systemic levels of cyclosporine and tacrolimus were maintained at a similar level compared with the control arms, renal function was significantly better in the ketoconazole groups, both for cyclosporine (308) and for tacrolimus (309). Also, in the tacrolimus-ketoconazole group, there was a significant improvement in graft function over the first years after transplantation, whereas this was not seen in the tacrolimus control group. In heart transplant recipients, however, although the combination of cyclosporine with ketoconazole appeared to be safe, no benefit of combining cyclosporine with ketoconazole on renal function was observed, although it should be noted that in this study only short-term results were available; the effect of ketoconazole coadministration on chronic CNI nephrotoxicity was not reported (305).

Recently, common polymorphisms in *CYP3A4* and *CYP3A5* genes have been associated with the development of chronic CNI nephrotoxicity late after renal transplantation in patients treated with tacrolimus (234). Although no tacrolimus levels were measured in renal tissue, the finding that carriers of the *CYP3A5*1* polymorphism (expressors of *CYP3A5*; 236) had a higher risk of developing chronic CNI nephrotoxicity compared with nonexpressors (*CYP3A5*3/*3*) (234), supports the hypothesis that tacrolimus metabolites play a role in the development of CNI nephrotoxicity. Because this association was found with recipient's *CYP3A5* genotype (which will affect intestinal and hepatic but not renal allograft *CYP3A5*), hepatic and intestinal *CYP3A5* should be responsible for this and would affect the levels of tacrolimus metabolites in the blood. Indeed, the generation of tacrolimus metabolites was higher in liver microsomes expressing *CYP3A5* compared with nonexpressing microsomes, and kidneys contribute only marginally to tacrolimus metabolism (310). The *CYP3A5* genotype of the kidney donors was not assessed in this study (234). Another study in liver transplantation has assessed the impact of the recipient *CYP3A5* genotype on the cumulative incidence of renal dysfunction over the first year after transplantation and reported completely opposite results, with the *CYP3A5* expressors (*CYP3A5*1* carriers) having significantly less renal dysfunction compared with nonexpressors (311). The reason for this apparent discrepancy in the impact of the *CYP3A5*1* carrier status on renal (allograft) function remains to be clarified, but likely relates to the fact that renal transplant recipients expressing *CYP3A5* in liver (related to the recipients' *CYP3A5* genotype) have higher systemic exposure to tacrolimus and its nephrotoxic metabolites, whereas *CYP3A5* expression in kidney tissue (related to the recipients' genotype in liver transplantation) could be protective in the sense that liver transplant recipients expressing *CYP3A5* have less local renal tacrolimus accumulation. This last hypothesis was suggested previously by Joy *et al.* in an immunohistochemical case-control study relating decreased local renal *CYP3A5* expression with CNI nephrotoxicity (312). It should also be noted that a recent study in heart transplant recipients did not observe any association between *CYP3A5*1* polymorphism and renal function (313).

Taken together, there is some suggestion that cyclosporine

and tacrolimus metabolites could contribute to clinical CNI nephrotoxicity. However, these human studies do not provide robust proof that cyclosporine and tacrolimus metabolites contribute to CNI nephrotoxicity because no CNI metabolites were measured in blood or renal tissue. These studies certainly need to be reproduced in controlled *in vitro* and animal studies and should be validated in larger prospective human studies.

Local Renal Susceptibility Factors for CNI Nephrotoxicity

In addition to the degree of exposure of kidneys to cyclosporine and tacrolimus or their metabolites, there is also evidence that interindividual variability in susceptibility to CNI nephrotoxicity is determined by local renal factors, independent of local cyclosporine or tacrolimus levels.

First, there is some evidence that the age of a kidney is a major determinant of its susceptibility to CNI nephrotoxicity. It is obvious that higher age of a kidney is independently associated with chronic histologic damage, as was described in autopsy studies (314). From this, it is no surprise that higher kidney age is an independent predictor of tubular atrophy, interstitial fibrosis, and functional impairment after renal transplantation, which was shown in protocol biopsy studies in adult and pediatric renal allograft recipients (85,261,315,316). However, these studies not only showed an association with nonspecific chronic histologic damage, but also demonstrated an association of higher donor age with medial arteriolar hyaline sclerosis suggestive of CNI nephrotoxicity (85,261). A similar and independent association of higher age with lesions suggestive of CNI nephrotoxicity was described in patients treated with cyclosporine for other reasons (317). In animal studies, older animals with pre-existing age-related renal dysfunction developed significantly worse nephrotoxicity compared with younger animals (318), although another study described similar increase in collagen mRNA in older *versus* younger animals (319). In a human setting, it is difficult to prove that higher kidney age is associated with increased susceptibility to CNI nephrotoxicity because the relative contribution of age *versus* CNI effect is hard to assess, because no lesion is really specific for any process. Some indirect evidence could be found in the interaction between kidney age and CNI cessation and its impact on the evolution of renal allograft function, with older donor kidneys having significantly less improvement of GFR after cyclosporine cessation compared with younger donor kidneys (320). Although this is no definite proof, this finding suggests that CNIs induce more irreversible damage to the kidneys from older donors compared with those from younger donors, that is, increased susceptibility of older donor kidneys to CNI nephrotoxicity. Whether pre-existing renal disease, hypertension, and diabetes mellitus increase local susceptibility to nephrotoxicity, independently of kidney age, remains to be elucidated.

The reason older kidneys could be more susceptible to chronic CNI nephrotoxicity likely relates to age-related changes in renal autoregulation, with excessive vasoconstriction and anatomical changes of the preglomerular vasculature and subsequent ischemia of the glomeruli (321–323). It could be expected that the CNI-associated alterations in vasodilator and

vasoconstrictory responses (see above) have more impact when these anatomical lesions and dysfunction are pre-existing. In addition, senescence of renal tissue is associated with impaired cellular repair mechanisms (324), which is particularly unfortunate for allografts from older donors in the context of increased susceptibility to CNI nephrotoxicity. Finally, there is some suggestion that CNIs accelerate cell senescence, possibly through the formation of reactive oxygen species (see above) (128). If this is true, renal cells from older kidneys exposed to a CNI will reach their cycling limit much sooner than similar kidneys not exposed to CNIs, and develop nonspecific molecular and morphologic changes associated with renal aging at a much faster pace (325–327).

Second, as was mentioned above, local renal P-glycoprotein not only could play a role in renal accumulation of cyclosporine and tacrolimus, but could also be important for tubular epithelial cell detoxification and protection against apoptotic stress.

Third, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to increase renal susceptibility to acute CNI nephrotoxicity. In healthy volunteers, indomethacin catalyzes cyclosporine-induced decrease in GFR (328), and in clinical studies, it was shown that NSAIDs and cyclosporine or tacrolimus have a synergistic effect on acute CNI nephrotoxicity, with decreases in renal plasma flow and GFR (329–331). This effect can be explained by the role of cyclo-oxygenase in the vascular effects of calcineurin inhibition (see above). However, although these studies demonstrate an additive pharmacodynamic effect, certain NSAIDs like diclofenac also interact with CNI pharmacokinetics and augment cyclosporine but decrease tacrolimus blood levels (330,331). Whether chronic use of NSAIDs in combination with CNIs would also lead to more rapid progression of chronic CNI nephrotoxicity is currently not known.

Fourth, studies in rats have clearly demonstrated the importance of salt depletion and RAS activation in the development of CNI nephrotoxicity (see above). It could be hypothesized that underlying RAS activation associated with relative salt depletion augments renal susceptibility to CNI nephrotoxicity also in a human setting (332), although this remains to be proven. The potential success of RAS inhibition in preventing CNI nephrotoxicity supports this hypothesis (see below).

Finally, genetic polymorphisms in genes involved in the pathogenesis of CNI nephrotoxicity have been associated with the risk for chronic CNI nephrotoxicity. It was demonstrated that a *TGF- β* polymorphism in codon 10 was associated with renal function in both adult and pediatric heart transplant recipients (333–335), although a study in liver transplant recipients could not confirm this association (336). In renal transplantation, *TGF- β* variants in the recipient were not associated with renal allograft outcome in a very large study (337), but this could be explained by the possibility that local *TGF- β* production could depend more on donor genotype than on recipient genotype. Angiotensin converting enzyme (*ACE*) gene polymorphisms have been associated with serum *ACE* levels (338). Because this enzyme appears to be pivotal in the development of CNI nephrotoxicity (see above), *ACE* variants could partly determine the individual risk for CNI nephrotoxicity associated

with the use of cyclosporine or tacrolimus. In a first study, no association between the *D* allele in the *ACE* gene and renal allograft function was found (339). However, in high-risk patients and pediatric kidney allograft recipients, a significant association between this *ACE* variant in recipients and renal allograft outcome was demonstrated (340,341). In nonrenal transplantation, this *ACE* variant has also been associated with renal dysfunction after bone marrow and heart transplantation (342,343).

Given the above evidence, it could be anticipated that older patients with native kidneys, or transplanted kidneys from older donors, could benefit most from a CNI-free immunosuppressive regimen. In addition, determining a patient's or donor's genotype of drug-metabolizing genes like *CYP3A5* and *ABCB1* (see above), or of molecules involved in CNI nephrotoxicity like TGF- β and *ACE*, could provide a reasonable tool to determine which patients are most susceptible for CNI nephrotoxicity.

Prevention and Treatment of CNI Nephrotoxicity

In this section, we will address the prevention and treatment of acute and chronic CNI nephrotoxicity. A summary of the available evidence is given in Table 3.

CNI Avoidance, Withdrawal, and Minimization

An in-depth overview of the clinical trials addressing this topic is beyond the scope of this article. Briefly, it is obvious that complete avoidance of CNIs in immunosuppressive protocols will fully prevent the development of CNI nephrotoxicity. However, the exclusion of CNIs from the immunosuppressive regimens falls short of preserving allograft function due to inadequate acute rejection prophylaxis with the other immunosuppressive regimens (222,262,344,345). Therefore, CNI withdrawal may be a better option, through delivery of CNIs during the early high rejection risk period after transplantation followed by secondary conversion to less nephrotoxic agents, before significant irreversible renal damage occurs (344–346). Finally, novel non-nephrotoxic immunosuppressive regimens, for example, the combination of mycophenolate mofetil and costimulatory blockade (belatacept), offer excellent short-term results (347), but longer term follow-up in more patients is needed to validate these first positive results with complete CNI avoidance.

There is increasing interest in CNI minimization protocols in which the doses of cyclosporine or tacrolimus are adjusted to lower target levels, both for *de novo* immunosuppressive protocols from time of transplantation and for rescue therapy after detection of histologic damage or renal dysfunction attributed to CNI nephrotoxicity (recently reviewed in 344). These approaches appear to be relatively safe (262,344,345), especially when mycophenolate mofetil is combined with tacrolimus, as was recently demonstrated in a large multicenter randomized trial in renal transplantation (222). By minimizing CNI levels, CNI nephrotoxicity might be partially avoided, but it has become clear that the increased risk of allograft rejection could annihilate these positive effects and even explain why the ta-

colimus group has a better outcome than the other groups. To date, no randomized studies are available assessing the impact of CNI minimization protocols on the histologic evolution of CNI nephrotoxicity, and the relative contribution of rejection phenomena *versus* CNI nephrotoxicity is not known from minimization protocols. Given the finding that chronic CNI nephrotoxicity also occurs in psoriasis patients treated with low-dose cyclosporine (348), it can be expected that persistent nephrotoxicity will occur as long as the CNIs are continued.

Other Approaches

An alternative approach could be to keep CNIs in the immunosuppressive regimens for their excellent rejection prevention effects, and add more targeted therapy to prevent or treat CNI nephrotoxicity. Given the gradual elucidation of the molecular and physiologic mechanisms underlying CNI nephrotoxicity (see above), more targeted therapy is becoming available.

Calcium Antagonists. Because vasoconstriction of the afferent arterioles appears to play a pivotal role in acute and chronic CNI nephrotoxicity (see above), many groups have studied the potential role of vasodilatory agents for the avoidance of CNI nephrotoxicity. Calcium antagonists like nifedipine appear to improve GFR in rats (349). In human studies after renal transplantation, the calcium antagonists nitrendipine and later lacidipine were shown to prevent the fall in renal plasma flow and GFR associated with cyclosporine administration (209,350,351). In a first randomized trial in renal allograft recipients, it was shown that patients treated with the combination of cyclosporine and nifedipine had better renal function with the same degree of BP control (352). In another randomized trial, a similar effect was demonstrated for lacidipine: the renal allograft recipients treated with concomitant lacidipine had significantly better renal allograft function from 1 yr onward, independent of the effects on BP (353).

Likewise, in nonrenal solid organ transplantation, treatment for 1 wk with the dihydropyridine nifedipine normalized BP and improved renal function, for example, in cardiac allograft recipients with mild hypertension and renal dysfunction (354). More recently, these positive effects of calcium antagonist use on CNI nephrotoxicity have been confirmed by a randomized study with dihydropyridine amlodipine after heart transplantation (355). Also a randomized trial with verapamil in heart and lung transplantation demonstrated beneficial effects of verapamil on renal function (356). These last results should however be interpreted cautiously, as cyclosporine dose requirements also decreased significantly with cyclosporine treatment, and alterations in cyclosporine metabolite levels could have contributed to the observed effects (see above). In apparent contrast with these positive studies, a long-term follow-up study in heart transplant recipients did not find a protective effect of calcium channel blockers in the prevention of CNI nephrotoxicity (267). In this last study, it should, however, be emphasized that the type of calcium antagonists used is not specified, and different calcium antagonists have different effects on renal vascular resistance, and perhaps even more importantly, on CNI pharmacokinetics (306).

Table 3. Therapeutic options to prevent or treat calcineurin inhibitor nephrotoxicity

Option	Rationale	Effect in Animal Studies	Effect in Human Studies	Comment
Decrease exposure to calcineurin inhibitors				
CNI avoidance	Completely avoid exposure to CNIs	NA	+/-	Increased rejection risk, maybe not with costimulatory blockers (e.g., belatacept)
CNI withdrawal	Exposure to CNIs for only a limited time, to bridge the high rejection risk period early after transplantation	NA	+/-	Increased rejection risk
CNI minimization	Lower but continuous exposure to CNIs	NA	+	Safe on short-term; long-term results not known
Decrease exposure to calcineurin inhibitor metabolites				
Inhibitors of CYP3A (e.g., ketoconazole)	Lower exposure to potentially toxic cyclosporine or tacrolimus metabolites	?	+	More frequent monitoring because of risk for overdosing/cost saving, but only very few studies
Decrease local renal susceptibility to CNI nephrotoxicity				
Dihydropyridine calcium antagonists (nifedipine, lacidipine, amlodipine)	Counteract the vasoconstrictory mechanisms of CNI use	+	+	Positive effects both on GFR and on long-term outcome
ACE inhibitors and angiotensin II receptor blockers	Counteract the pivotal role of the RAS in the development of CNI nephrotoxicity	+	+/-	RAS inhibition in itself causes renovascular changes, GFR is unaltered but chronic damage may be avoided
Spirolactone	Counteract the pivotal role of aldosterone in the development of CNI nephrotoxicity	+	?	No human studies available
Vasodilatory prostanoids	Counteract the vasoconstrictory mechanisms of CNI use	+	No effect	Only very few human studies available
NO donors	Counteract the vasoconstrictory mechanisms of CNI use	+	No effect	Only very few human studies available
Anti-oxidants	Counteract the pivotal role of oxidative stress in the development of CNI nephrotoxicity	+	?	No human studies available
Anti-TGF- β antibodies	Counteract the pivotal role of TGF- β in the development of CNI nephrotoxicity	+	?	No human studies available
Statins	Effects on vascular smooth-muscle cells and on the progression of kidney diseases	+	?	No human studies available
Magnesium supplementation	Counteract the potential influence of hypomagnesemia on acute and chronic CNI nephrotoxicity	+	?	No human studies available

References are provided in the text. NA, not appropriate; CNI, calcineurin inhibitor.

RAS Inhibition. The pivotal role of RAS activation in the pathophysiology of CNI nephrotoxicity could suggest that RAS inhibition will prevent its development. The effects of ACE inhibitors (ACEIs) and angiotensin II receptor blockers (ARBs) on CNI nephrotoxicity have been studied extensively. In rats, it was shown that ACEIs and ARBs can prevent cyclosporine-induced interstitial fibrosis and improve renal function (145,357–359). Also, in humans, ACE inhibition reduced CNI nephrotoxicity associated with cyclosporine use (360) and improved alterations of the cardiovascular system generally observed in renal transplant patients (361). Again in humans, the ARB losartan was shown to significantly decrease the plasma levels of TGF- β and endothelin (362,363). Creatinine clearance, however, tended to be lower with the addition of losartan, likely through the hemodynamic effects of angiotensin II receptor blockade (362,363). It is, therefore, not clear whether cotreatment with ARBs is able to slow the progression of CNI nephrotoxicity in a human setting. Finally, spironolactone treatment could also abrogate many RAS effects and aldosterone-mediated effects of CNI treatment, at least in rats (364,365). For an extensive review of the potential role of spironolactone therapy for CNI nephrotoxicity, we refer to the review by Bobadilla *et al.* (41). No human studies with spironolactone to prevent the development of CNI nephrotoxicity are available.

In a randomized trial comparing the ACEI lisinopril with the calcium antagonist nifedipine, Mourad *et al.* found that each drug was associated with a similar degree of renal protection in long-term cyclosporine treated patients (366). However, another randomized trial comparing nifedipine to lisinopril for the prevention of CNI nephrotoxicity found that in the first 2 yr after transplantation, graft function increased only with nifedipine (367).

Vasodilatory Prostanoids. Only a limited number of studies have evaluated the use of vasodilatory prostanoids on CNI nephrotoxicity. In rats, it was shown that prostaglandin E2 reduces nephrotoxicity of cyclosporine, but this was likely mediated by an interaction between prostaglandin E2 and intestinal cyclosporine absorption (368). Misoprostol, a prostaglandin E1 analog, reduced CNI nephrotoxicity in vitro and in rats treated with chronic cyclosporine (369,370), and in a human study, misoprostol was shown to improve renal function (371). This effect in humans was, however, associated with a lower incidence of acute rejection with misoprostol treatment, misoprostol in itself did not prevent the development of CNI nephrotoxicity in humans (371). Likewise, another study in patients with rheumatoid arthritis did not observe any effect of misoprostol treatment on acute CNI nephrotoxicity (372).

NO Donors. Another approach has been to use L-arginine or molsidomine for the prevention of CNI nephrotoxicity. In rats, L-arginine greatly ameliorated kidney dysfunction induced by cyclosporine (373,374), but not in all studies (375). In humans, no effect of L-arginine was observed (376). Molsidomine was not assessed in human studies for CNI nephrotoxicity.

Other. Finally, other therapeutic approaches are promising for the prevention of CNI nephrotoxicity, such as anti-TGF- β antibodies (133,377), antioxidants (129,378–381), statins (358),

and magnesium supplementation (42,382,383). However, no human studies with these approaches are available.

Conclusion

The use of the CNIs cyclosporine and tacrolimus has led to major advances in the field of transplantation, with excellent short-term outcome. However, the chronic nephrotoxicity of these drugs is the Achilles' heel of current immunosuppressive regimens. Chronic CNI nephrotoxicity is associated with mostly irreversible histologic damage to all compartments of the kidneys, including glomeruli, arterioles, and tubulo-interstitium, but the nonspecificity of most lesions makes the differential diagnosis with other injurious processes cumbersome. The pathophysiologic mechanisms underlying CNI nephrotoxicity are partly elucidated, although the main question whether nephrotoxicity is secondary to the actions on the calcineurin-NFAT pathway remains largely unanswered. It becomes clear that local renal factors are more important for susceptibility to CNI nephrotoxicity than systemic exposure to cyclosporine and tacrolimus. These factors include variability in P-glycoprotein and CYP3A4/5 expression or activity, older kidney age, salt depletion, the use of NSAIDs, and genetic polymorphisms in genes like TGF- β and ACE. Prevention and eventually therapy for CNI nephrotoxicity is aimed at lowering total systemic blood levels and decreasing local renal exposure to the CNIs or their metabolites. In addition, better insight into the mechanisms underlying CNI nephrotoxicity might pave the way toward more targeted therapy or prevention of CNI nephrotoxicity.

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None.

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