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Review

Pathophysiology of Post Transplant Hypertension in Kidney Transplant: Focus on Calcineurin Inhibitors Induced Oxidative Stress and Renal Sodium Retention and Implications with RhoA/Rho Kinase Pathway

Lorenzo A Calò[a] Verdiana Ravarotto[a] Francesca Simioni[a] Elena Naso[a] Francesco Marchini[a] Luciana Bonfante[a] Lucrezia Furian[b] Paolo Rigotti[b]

[a]Department of Medicine, Nephrology and [b]Department of Surgery, Renal and Pancreas Transplant Unit, University of Padova, Italy

Key Words
Post transplant hypertension • Calcineurin inhibitors • Angiotensin II • Rho kinase • Oxidative stress • Gitelman's syndrome • Sodium retention

Abstract
Post-transplant hypertension is a common occurrence during treatment with calcineurin inhibitors (CNIs) in kidney transplant population. The pathogenesis of vasoconstriction induced by CNIs involves vascular tone alterations and kidney sodium transport regulation.
Among the factors involved a key role is played by the activation of intrarenal renin-angiotensin system with enhanced release of Angiotensin II (Ang II) and increase of oxidative stress. A common pathway between oxidative stress and hypertension induced by CNIs may be identified in the involvement of the activation of RhoA/Rho kinase pathway, key for the induction of hypertension and cardiovascular-renal remodeling, of the oxidative stress mediated increased nitric oxide (NO) metabolism and increased renal sodium retention via increased activity of thiazide-sensitive sodium chloride cotransporter (NCC) in the distal tubule.
We examined literature data including those coming from our group regarding the role of oxidative stress and sodium retention in CNIs induced hypertension and their involvement in cardiovascular-renal remodeling.
Based on the available data, we have provided support to the activation of RhoA/Rho kinase pathway as an important effector in the pathophysiology of CNIs induced post-transplant

Lorenzo A Calò MD, PhD
Department of Medicine, Nephrology, University of Padova,
Via Giustiniani, 2, 35128 Padova (Italy)
Tel. +39/049/8213071, Fax +39/049/8217921, E-Mail renzcalo@unipd.it

hypertension via activation of oxidative stress and sodium retention. Clarification of how the biochemical and molecular mechanisms that regulate the processes involved in CNIs induced post transplant hypertension work and interact, would provide further insights not only into the comprehension of the pathophysiology of CNIs induced post transplant hypertension but could also have a positive impact on the clinical ground through the identification of significant targets. Their specific pharmacologic targeting might have multiple beneficial effects on the whole cardiovascular-renal function. The demonstration that in kidney transplanted patients with CNIs induced post-transplanted hypertension, the treatment of hypertension with different antihypertensive drugs inducing a comparable blood pressure reduction but different effects for example on oxidative stress and oxidative stress related proteins and/or Rho kinase and sodium retention, could be helpful for the choice of the antihypertensive treatment in these patients which takes advantage from effects of these drugs beyond blood pressure reduction.

Introduction

Cardiovascular mortality is the main cause of mortality in kidney transplant population [1-3]. Post transplant hypertension is frequently observed in kidney transplant recipients and it has a recognized prognostic impact due to the deleterious effects on the kidney graft function and for the contribution to the development of cardiovascular diseases, which significantly impact on the long term outcomes [1, 2]. The prevalence of arterial hypertension after kidney transplantation has been reported to be as high as 85% [1, 2]. The use of calcineurin inhibitors, such as tacrolimus and cyclosporin, has increased the prevalence of post transplant hypertension to 60- 85% in transplant patients treated with these drugs [4].

The pathogenesis of hypertension after kidney's transplant is multifactorial, but one of the most important factors is the introduction in immunosuppresive therapy of calcineurin inhibitors (CNIs), cyclosporine (CsA) and tacrolimus, that are considered the cornerstone of the immunosuppressive regimen after transplantation. The pathogenesis of vasoconstriction induced by CNIs and cyclosporine in particular involves vascular tone alterations and kidney sodium transport regulation [3]. Among the factors involved, a key role is played by the activation of intrarenal renin-angiotensin system. The enhanced release of Angiotensin II (Ang II) leads not only to increased oxidative stress and free oxygen radicals production by the NADPH oxidase [5], but also to consequent increase of nitric oxide (NO) metabolism. The demonstration that Ang II increases production of reactive oxygen species (ROS) by vascular smooth muscle cells [6-8] and the fact that ROS induced endothelial dysfunction and hypertension [6-9], have established a common pathway between oxidative stress and hypertension, which may be identified in the involvement of the induction of RhoA/Rho kinase pathway, key for the induction of hypertension and cardiovascular-renal remodeling [8-10]. As a matter of fact, post transplant hypertension adversely affects cardiovascular mortality in kidney transplant population, hence the knowledge and comprehension of the pathophysiological associations between kidney transplant and post transplant hypertension are fundamental in order to improve both graft and patient survivals.

We summarize here what is known on the pathophysiology and the biochemical and molecular mechanisms involved in the CNIs-induced post transplant hypertension from the contributions of our and others groups' studies, giving particular emphasis on those related with Ang II and oxidative stress signaling, renal sodium retention and the links between them.

Angiotensin II and Oxidative Stress

The demonstration that angiotensin II (Ang II) increases production of reactive oxygen species (ROS) by vascular smooth muscle cells and the fact that ROS induce the development of endothelial dysfunction and hypertension [6-9], have established a link between oxidative stress and hypertension. Angiotensin II (Ang II) induces NADPH oxidase, with increased production of reactive oxygen species (ROS) [6-9]. In rats, CsA led to higher Ang II concentrations in plasma and kidney, leading to vasoconstriction and hypertension, which represent the short term signaling of Ang II, and production of ROS, which is essentially the mediator of the long term signaling of the hormone, leading to cardiovascular-renal remodeling [6-9]. These signaling pathways include the activation of a wide spectrum of signaling mechanisms mediated via specific intracellular pathways [6, 9, 11, 12], which include the Ca^{++}-dependent and RhoA/Rho kinase pathways [6, 9]. The Ca^{++}-dependent pathway causes smooth muscle contraction and hypertension through phosphoinositide specific PLC-generated second messengers [6], increased PKC activity and phosphorylation of the regulatory chain of myosin II [13]. The RhoA-Rho-kinase pathway leads to both vasoconstriction and cardiovascular-renal remodeling [8, 10, 14] via modulation of the phosphorylation state of the regulatory chain of myosin II, mainly through the inhibition of myosin phosphatase target protein-1 (MYPT-1). By this mechanism, RhoA-Rho-kinase contributes to agonist induced Ca^{++} sensitization of smooth muscle [14, 15], ultimately resulting in smooth muscle cell contraction [8, 14]. Of note, the activation of these signaling pathways is counterbalanced by the vasodilatory and antiproliferative activity of nitric oxide [6, 16, 17]. The activation of Rho kinase pathway in posttransplant hypertension has been recently demonstrated in humans [18], confirming the role of Ang II and oxidative stress in CNIs induced hypertension as both have been shown to activate Rho kinase [19-21].

Endothelial nitric oxide (NO) is known to be an important vasorelaxing factor and inhibitor of vascular proliferation, hypertrophy and remodeling. It is crucial in the maintenance of a state of basal vasodilation. One of the most important effects of ROS is the reduction of NO bioavailability: superoxide anion radical (O_2^-) reacts with NO, destroying it via its conversion to peroxynitrites. In addition, given that the activation of Rho kinase has been shown to downregulate the endothelial NO synthase [8, 22], the increased Rho kinase activity may also contribute to the reduced bioavailability of NO shown in post transplant hypertension.

The involvement of oxidative stress and oxidative stress signaling in post transplant endothelial dysfunction and hypertension has been shown in in animals [23] and humans,[24] through the demonstration of increased NADPH activity. In humans we showed increased mononuclear cell expression of $p22^{phox}$, a 22-kDa α subunit of cytochrome b558 included in the NADPH oxidase, which plays a key role in O_2^- production [24]. It functions as an integral subunit of the final electron transport from NADPH to haeme and molecular oxygen in generating O_2^-, and is stimulated by Ang II [5]. Mononuclear cell $p22^{phox}$ gene expression was significantly higher in transplanted patients with hypertension compared with normotensive patients. In contrast, the RNA production of heme oxygenase (HO)-1, which is induced and protective from oxidative stress [25], and total plasma antioxidant power was higher in normotensive kidney-transplanted patients compared with the group of hypertensive patients suggesting the existence of a correlation between leucocyte intracellular oxidative stress and hypertension [24].

A NO-mediated counterregulatory mechanism protective from CsA-induced vasoconstriction has also been shown. In hypertensive kidney transplant patients under chronic CsA treatment, in fact, quantification of mononuclear cell endothelial NO synthase mRNA and NO metabolites plasma levels showed, compared to normotensive controls, an upregulation of NO system notwithstanding the presence of hypertension, in addition to increased hydroperoxides and peroxynitrite plasma levels, which were also present in

patients compared to control subjects [26]. This suggests that CsA-induced vasoconstriction and hypertension cannot be a consequence of decreased levels of ecNOS as both ecNOS mRNA as well as NOS enzymatic activity were increased, as indicated by increased levels of plasma NO metabolites [26], making rationale the case of the induction of a NO mediated counterregulatory mechanism protective from CsA-induced vasoconstriction. This counterregulatory mechanism was also shown in normal human volunteers in whom acute infusion of CsA produced vasoconstriction and a simultaneous increase in endothelial NO release, and in cultured endothelial cells where the incubation with CsA increased ecNOS gene expression [27]. The apparent contradiction between NO system upregulation and CsA-induced vasoconstriction is explained by the CsA-mediated induction of superoxide anions and reactive oxygen species production, which, reacting with NO, produce peroxynitrites, reducing the NO bioavailability and, therefore, its vasodilatory action. The demonstration that chronic CsA treatment leads to increased plasma levels of NO metabolites therefore, indicates that CsA is not a direct inhibitor of ecNOS, but rather that the effects of CsA in transplanted patients must occur downstream from the generation of NO. One potential mechanism is that CsA increases the turnover of NO, as shown by the increased plasma levels of peroxides. CsA induces O_2^- production, which reacts with NO, destroying it via its conversion to peroxynitrites. This destruction of NO induced by CsA would explain the presence of vasoconstriction despite increased NO production: the level of NO present is insufficient to maintain the vasodilatation. Another possible mechanism, which could link the CsA to the overexpression of ecNOS mRNA could be the result of the effect of CsA on the activity of calcineurin [28, 29]. CsA reduces the phosphatase activity of calcineurin, therefore removing the inhibitory effect led by the phosphatase activity of calcineurin on ecNOS gene expression, thereby stimulating ecNOS transcription. Calcineurin inhibitors induced oxidative stress could, therefore, represent an attractive link between NO increased metabolism and reduced activity and CsA-induced vasoconstriction and hypertension in transplant patients chronically treated with CsA (Fig. 1).

TGFβ Signaling

CNIs-mediated oxidative stress induces hypertension not only modifying NO metabolism, but also through other mechanisms. Stimulation of TGFβ signaling system mediated by immunosuppressive agents induced ROS production is another of their most important side effects that may lead to graft related long-term complications, such as fibrogenesis and chronic rejection. We have shown that CsA and tacrolimus in kidney transplant patients with post transplant hypertension in addition to increase the expression of $p22^{phox}$, HO-1 and endothelial NO synthase, induced an increased expression of TGFβ [30, 31], established oxidative stress related effector, which activates oxidative stress related kinases such as MAPK/ERK [32], finally leading to cardiovascular-renal remodelling and atherogenesis. The increased expression of TGFβ is consistent with an increased oxidative stress-related response in post transplant hypertensive kidney transplant patients. TGFβ is, in fact, one of the effector signals of oxidative stress [33]. *In vitro*, oxidative stress enhances TGFβ gene expression [33] and, *in vivo*, rats placed on antioxidant-deficient diets demonstrate increased TGFβ expression, renal hypertrophy, proteinuria, tubulointerstitial thickening and loss of glomerular filtration rate, associated with increased lipid peroxidation of the renal membranes [32]. Therefore, the increase of TGFβ, a major pro-fibrotic cytokine [34], could accelerate the progression of renal disease associated with hypertension. Furthermore, our study revealed that the treatment with an ACE-inhibitor leads to the decline of $p22^{phox}$ and TGFβ expression [30], further underlining the role of renin-angiotensin system in the induction of oxidative stress and the role of both oxidative stress and TGFβ in the development of kidney post-transplant hypertension and its long term consequences.

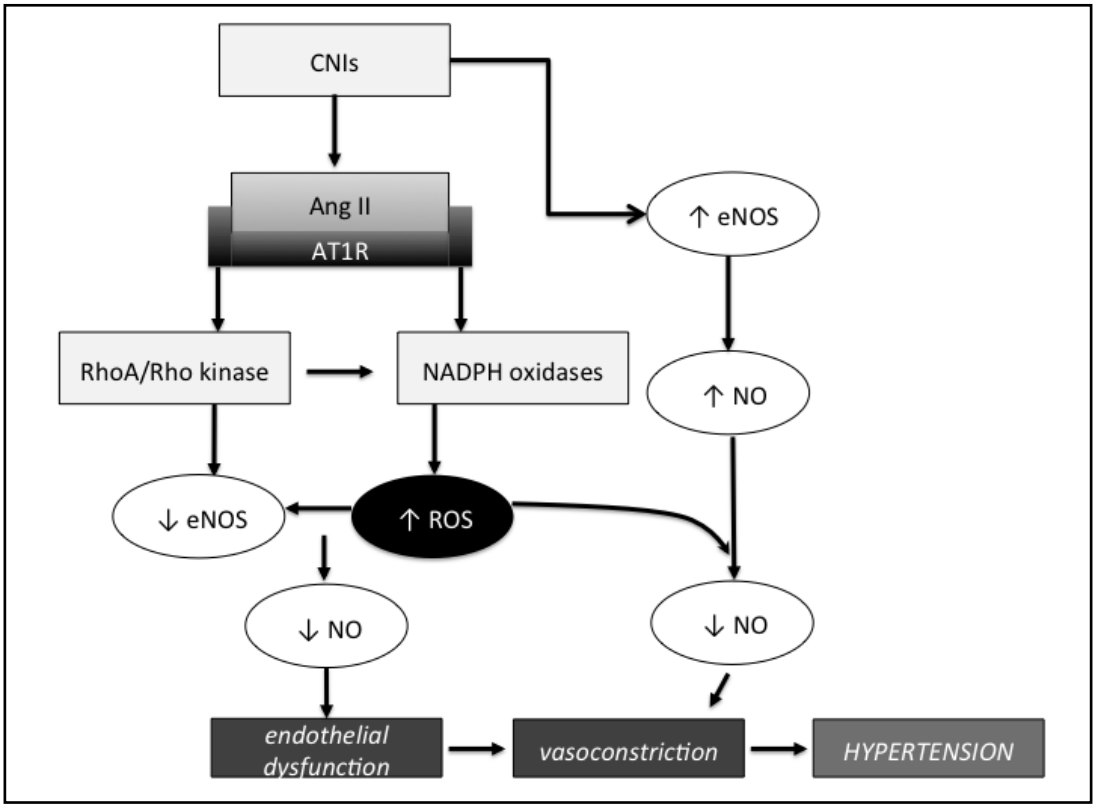

Fig. 1. Pathophysiologic mechanism for CNIs induced-Ang II/Rho kinase/Oxidative stress mediated vasoconstriction and hypertension.

Renal Sodium Retention

CsA-induced hypertension was shown to be sodium-dependent [35], although the renal site of the sodium retaining effect of CNIs has still to be clearly identified [36]. However, studies have shown that CNIs cause an increased sodium reabsorption via the increased activity of the thiazide-sensitive sodium chloride cotransporter (NCC) and Na-K-2Cl cotransporter (NKCC2), which is made possible through the prevention of the inhibitory effect of calcineurin on 'with-no-lysine' kinases (WNK), glucocorticoid-regulated kinase 1, STE20/SPS1-related proline alanine-rich kinases (SPAK) and oxidative stress-responsive protein type 1 kinase (OSR1) that activate NCC [37]. Blankenstein et al [38]., have shown that rats treated with CsA had increased phosphorylation of NCC and NKCC2, both deputed to sodium reabsorption. NCC and NKCC2 are specifically found in different portions of the nephron. The NKCC2 is expressed in the apical membrane of the thick ascending limb of the loop of Henle and in the macula densa, while NCC is expressed in the late portion of the distal convoluted tubule (DCT). However, in stimulated arginine vasopressin (AVP)-deficient Battleboro rats the treatment with CsA induced activation of NCC but not of NKCC2, suggesting that NCC and NKCC2 are activated by different signaling and that the direct epithelial action of calcineurin inhibition is sufficient for the activation of NCC mediated by WNK-SPAK/OSR1, while NKCC2 stimulation requires additional stimulation by AVP [38]. Therefore inhibiting calcineurin, CNIs prevent its inhibitory effect on these kinases, with consequent increased activation of NCC [37, 39].

This CNIs mediated increased activity of NCC induces sodium reabsorption and causes hypertension. The involvement of calcineurin was also demonstrated through the

dephosphorylation of NCC after acute oral K^+ administration. CNIs induce, as major side-effect, hyperkalemia in addition to hypertension. The acute K^+ administration, resembling this condition, induces cell depolarization with Cl^- entrance determining NCC phosphorylation by both Cl^- dependent and independent ways that involve ORS1/SPAK activation [40, 41].

The mechanisms of sodium reabsorption are under control of Ang II, which occurs in multiple ways along the nephron. Beside the stimulation of aldosterone release, which in turns induces Na^+/K^+-ATPase and epithelial sodium channels (ENaCs) upregulation in the DCT and collecting duct, Ang II also stimulates the production of AVP. In addition, AVP itself modulates NCC function through SPAK activation [42]. Moreover, Ang II was reported to be an upstream regulator of WNK-OSR1/SPAK kinases as in mice its infusion showed a significantly increased phosphorylation of these kinases [43]. *In vitro* studies highlighted the direct effect of Ang II on NCC activation, which seems to be time dependent and to require WNK4. In some experimental models, the co-expression of WNK4 and NCC results in reduced activity of NCC, but this effect is reversed in presence of Ang II. Furthermore, the activation of NCC by Ang II occurs only in presence of WNK4 and this mechanism is SPAK phosphorylation dependent. These findings are supported by knock-out models for SPAK phosphorylation site, which induces a Gitelman syndrome-like phenotype, which is caracterized by non functional NCC [44].

Considering all these studies, the influence of Ang II on the activation of NCC is also relevant for understanding the pathophysiology of CNIs-induced hypertension.

Calcineurin was found to colocalize with NCC in the distal tubule and tacrolimus was not able to cause hypertension in NCC knockout mice, while in transgenic mice overexpressing NCC it increased the hypertensive response [37, 39]. A higher expression of NCC and phosphorylated NCC were found in transplant kidney biopsies of patients with CNI-induced hypertension and in kidney homogenates of mice treated with tacrolimus were found increased WNK and SPAK kinases and increased phosphorylated form of NCC [37, 39]. Tutakhel et al. recently confirmed a significantly higher abundance of total NCC and phosphorylated NCC in urinary extracellular vesicles of renal transplanted subjects compared to healthy volunteers or transplanted recipients treated with CNI-free immunosuppressive regimens [45]. Similar results were also shown with CsA in rats [46]. These animals developed salt-sensitive hypertension, hyperkalemia, renal tubular acidosis and hypercalciuria [39], a clinical picture similar to the Gordon syndrome a rare hereditary form of hypertension [47], which is caused by mutations in WNK kinases that activate NCC [48]. Of note, normo/hypothension, hypokalemia, sodium wasting, metabolic alkalosis and hypocalciuria, clearly the opposite clinical picture of CNIs induced hypertension and Gordon syndrome, is presented by the Gitelman's syndrome, rare genetic tubulopathy caused by inactivating mutations in the gene coding for NCC, which lead to sodium and potassium wasting [49], further indirectly underlining the role of sodium retention via activation of NCC as contributing to CNI induced hypertension. Moreover, patients in whom NCC is genetically activated, such as Gordon syndrome, or inactivated, such as Gitelman's syndrome, show opposite changes in vascular reactivity with severe hypertension in the former and hypotension and reduced oxidative stress in the latter [8, 48]. In addition patients with Gitelman's syndrome showing blunted short and long term Ang II signaling via AT1R, reduced oxidative stress, lack of cardiovascular remodeling, upregulation of NO system, increased NO mediated vasodilation and downregulation of RhoA/Rho kinase pathway [8, 10], also represent in terms of biochemical and molecular mechanisms the opposite of the condition present in CNIs induced hypertension, therefore further indirectly supporting the role of Ang II, oxidative stress signaling and NCC activation mediated sodium retention as key processes in the induction of CNI mediated hypertension. Furthermore, in untreated essential hypertensive patients we have shown that Rho kinase activity, in terms of protein level of p63 RhoGEF, a specific mediator transducing the Ang II message from activated AT1R to RhoA/Rho kinase activation via Gq protein, leading to vascular contraction, proliferation, and cardiovascular remodeling [8, 10], and in terms of MYPT-1 phosphorylation state, marker of Rho kinase

activation, was increased [50] and declined after 6 months treatment with the Ang II type 1 receptor blocker olmesartan [51]. Also these data, although not directly coming from CNIs induced posttransplant hypertension, further indirectly support the role of Ang II/RhoA/Rho kinase/oxidative stress/NCC activation interrelated signaling as key processes in the induction of CNIs mediated hypertension.

The role of sodium retention by the kidney in the pathophysiology of CNI induced hypertension may also be linked to the activation of sympathetic nervous system and oxidative stress signaling by CNI.

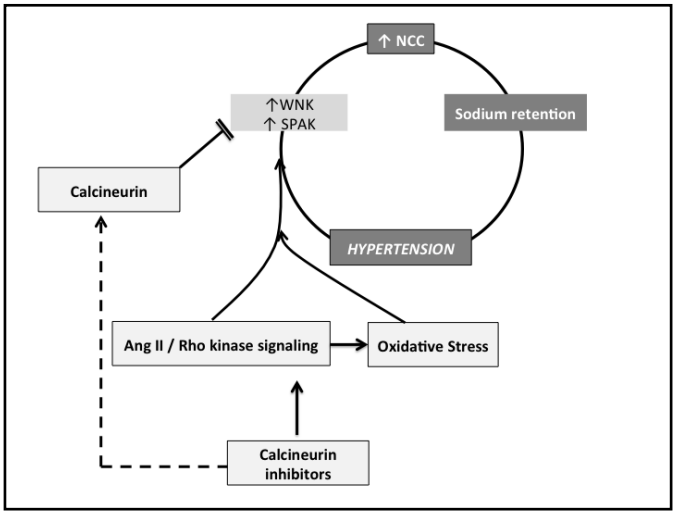

Fig. 2. Pathophysiologic mechanism for CNIs induced-Ang II/Rho kinase/Oxidative stress mediated sodium retention and hypertension.

CsA-induced renal sodium retention was shown in rats to be caused by the activation of sympathetic nervous system, because denervation abrogated the sodium-retaining effect [52]. Furthermore, norepinephrine has been reported to increase the abundance of phosphorylated NCC due, at least in part, via oxidative stress-response kinases, demonstrating the role of sympathetic stimulation of NCC in the generation of salt sensitive hypertension [53] and confirming the interactions between renal sodium handling and vascular tone. The association of all these evidence, therefore, supports in humans the presence of a link between increased renal sodium reabsorption, Ang II, RhoA/Rho kinase pathway, oxidative stress, their signaling systems and increased vascular tone in the generation of hypertension and provides a unifying mechanism linking renal sodium retention via increased activity of NCC, sympathetic stimulation, Ang II, RhoA/Rho kinase pathway, oxidative stress and vascular reactivity to be considered of pathophysiologic relevance in CNIs induced hypertension [3] (Fig. 2).

Conclusion

The knowledge and comprehension of the pathophysiological associations between kidney transplant and post transplant hypertension induced by CNIs are fundamental in order to improve both graft and patient survivals due to the weight of post transplant hypertension on cardiovascular mortality in kidney transplant population.

Ang II and oxidative stress signaling, renal sodium retention and sympathetic stimulation are deeply involved in CNIs-induced post transplant hypertension. Their biochemical and molecular mechanisms and the links between these mechanisms, as provided by studies in transplanted patients chronically treated with CNIs and by those derived from studies in rare genetic diseases such as Gordon syndrome and Gitelman's syndrome, both directly and indirectly confirm the importance of their pathophysiologic role in CNIs induced post transplant hypertension in kidney transplant patients. Clarification of how these biochemical/molecular mechanisms work/interact would provide further insights into the pathophysiology of CNIs induced hypertension but could also have a secure positive impact on a clinical ground identifying significant targets whose pharmacologic targeting might have multiple beneficial effects on the whole cardiovascular-renal function of transplanted

patients.

The demonstration that in kidney transplanted patients with CNIs induced post-transplant hypertension, the treatment of hypertension with different antihypertensive drugs inducing a comparable blood pressure reduction but different effects for example on oxidative stress and oxidative stress related proteins and/or sodium retention [30, 31, 37], could be helpful for the choice of the antihypertensive treatment in these patients, which takes advantage from effects of these drugs beyond blood pressure reduction.

Disclosure Statement

The authors have nothing to disclose.

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