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# Mechanisms of Calcineurin Inhibitor Nephrotoxicity in Chronic Allograft Injury

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## 1. Introduction

The first successful transplantation of a human kidney was performed more than 50 years ago by Murray and colleagues in 1954 between identical twins. The success of this transplantation was due to the fact that no significant rejection occurs between genetically identical twins and therefore immunosuppression was not necessary in this particular case (Merrill *et al.*, 1956). However, solid-organ transplantation could not be considered truly successful until the 1970's after significant technical and pharmacological advances. In particular, the discovery and development of the calcineurin inhibitors (CNIs) has made allograft transplantation routinely successful with greatly reduced risk of acute rejection. In the absence of pharmacological agents to address the primary pathological mechanisms involved, renal transplantation has now been the standard management of end stage renal failure for the past four decades (Wolfe *et al.*, 1999). Short-term renal allograft and allograft recipient survival rates have increased significantly during the last decade largely due to improved patient monitoring. However, allograft half-life beyond 1 year post-transplant remains largely unchanged. While rates of early allograft failure have significantly reduced, late renal allograft dysfunction remains a significant problem in the transplant population (de Fijter). Chronic allograft injury (CAI) is the most prevalent cause of allograft dysfunction in the first decade after transplantation. The term CAI is used to describe deterioration of renal allograft function and structure due to immunological processes (i.e. chronic rejection) and/or a range of simultaneous non-immunological factors such as CNI-induced nephrotoxicity, hypertension and infection. This chapter will outline the pathophysiology and etiology of CAI and the role that CNI nephrotoxicity plays in this disease process. It will also review experimental studies that have identified important molecular mechanisms involved and discuss strategies utilised to minimise the development and progression of CAI.

## 2. Chronic allograft injury

### 2.1 Definition and terminology

In 2006, a study analysed the US Transplantation Data United Network for Organ Sharing (UCLA and UNOS) Renal Transplant Registry database which contained more than 138,000

cases. The analysis showed that despite remarkable improvements in short-term graft outcomes, the allograft loss rate after the first year post-transplant had not significantly changed over the previous 10 years (Kaneku and Terasaki, 2006). Therefore, prolonging the survival of kidney allografts in the long term is the major focus of modern transplant medicine. The majority of renal allografts fail after a period of progressive functional decline which is associated with glomerulosclerosis, tubular atrophy, interstitial fibrosis, and arteriosclerosis. This process is referred to as chronic allograft injury (CAI). The terminology associated with CAI has been revised significantly during the last decade and so, for clarity, some time will be devoted to clearly defining the current nomenclature and its evolution. In the late 1990's, allograft failure more than 3 months after transplantation came to be designated as chronic allograft nephropathy. This term came into existence through use of the Banff classification scheme of kidney-transplant diseases which organised biopsy specimens into those with acute injuries versus those with chronic injuries (Racusen *et al.*, 1999). Chronic allograft nephropathy included at least four distinct entities that could not always be differentiated on biopsy (chronic rejection, chronic toxic effects of calcineurin inhibitors (CNIs); hypertensive vascular disease; and chronic infection, reflux, or both). The rationale for the term was that chronic allograft nephropathy was preferable to "chronic rejection," since this would imply that immunological mechanisms of injury were the primary pathological mechanism. Therefore, chronic allograft nephropathy was utilised as a cover-all term for cases where fibrosis was detected in the interstitium and where tubular atrophy was evident. However, CAI is a multifactorial process in which both immunological (e.g. antibody-mediated rejection) and non-immunological factors (e.g. ischemia-reperfusion injury, hypertension and CNI nephrotoxicity) play a role. These factors are not mutually exclusive and CAI most likely results from the combination of these multiple factors. Over time, all of these factors became synonymous with 'chronic allograft nephropathy' and disease entities with different etiologies were classified together leading to loss of important stratifying elements which would allow physicians to determine more specific diagnosis and treatments. Therefore, the terminology of 'chronic allograft nephropathy' was discouraged in the 8<sup>th</sup> Banff Conference in 2005 because it was felt that the term encouraged the misconception that chronic allograft injury is one specific disease rather than a collective term for non-specific scarring from all potential causes of chronic allograft dysfunction with fibrosis (Racusen and Regele, 2010). The new classification system replaced the term 'chronic allograft nephropathy' with '*interstitial fibrosis and tubular atrophy*' (IF/TA) with no evidence of any more specific etiology, and requires the recognition and recording of other specific morphological features such as polyomavirus nephropathy (immunostaining for SV40 antigen) or CNI nephrotoxicity (arteriolar hyalinosis with peripheral hyaline nodules in the absence of hypertension or diabetes). However, this new classification also has limitations and the usage of 'chronic allograft nephropathy' has persisted in leading academic journals and conference proceedings. For the purposes of this chapter, the most recent terminology conventions will be utilised.

## 2.2 Aetiology and pathophysiology of chronic allograft injury

CAI leads to chronic allograft dysfunction manifesting clinically as a decline in renal function and the development of hypertension and proteinuria (Sijpkens *et al.*, 2003). The half-life of cadaveric renal transplants is 12-14 years, with longer survival in living-donor grafts. Progressive deterioration of function with fibrotic changes accounts for about 35-40%

of all late allograft loss (Sijpkens *et al.*, 2003). The functional and structural changes observed during CAI share striking similarities with those observed in other forms of chronic progressive kidney disease. Histologically, CAI is characterised by atherosclerosis, glomerulosclerosis, interstitial fibrosis, and tubular atrophy (Paul, 1995; Paul, 1995). Ultimately, progressive decrease in the functional nephron mass is the major pathophysiological process. While multiple mechanisms contribute to the reduction in functional nephron mass, renal tubulointerstitial fibrosis (TIF) is considered the final common mechanism leading to end-stage renal disease regardless of the initiating insult (Iwano and Neilson, 2004; Guarino *et al.*, 2009; Liu, 2009). It has been demonstrated in both experimental and clinical studies that TIF correlates more consistently with renal functional impairment than glomerular damage (Nath, 1998). TIF is characterised by the gradual loss of the tubular epithelium, progressive accumulation of fibroblasts and alpha smooth muscle actin ( $\alpha$ -SMA)-positive myofibroblasts and accumulation of extracellular matrix (ECM) components in the tubular interstitium (Masszi *et al.*, 2004). This accumulation occurs because of a pathological imbalance in the production and degradation of ECM. The exact mechanism by which tubulointerstitial fibrosis results in renal functional decline is not fully clear, however a number of factors are thought to contribute including obliteration of postglomerular capillaries (Kang *et al.*, 2002), formation of atubular glomeruli (Marcussen, 1995) and tubular atrophy (Strutz *et al.*, 2002). Concomitant with the development of interstitial fibrosis is the fate of cells of the tubulointerstitium. As fibrosis progresses, tubular cells and peritubular capillaries decrease in number and ultimately disappear. Interstitial fibroblasts become activated and increase in number, and there is notable infiltration of inflammatory cells into the interstitial compartment (Eddy, 1996; Eddy, 2005).

### 3. Calcineurin inhibitors – Immunosuppressive agents

Prior to the discovery of calcineurin inhibitors (CNIs) in the 1970s, attempts at solid organ transplantation had consistently failed due to rejection of the allograft by the recipient's immune system. Over the past 40 years, immunosuppressive agents have revolutionised solid organ transplantation. In their absence, progressive immune-mediated injury occurs in transplanted organs. The general mechanisms of action of all current and established immunosuppressive therapies operate based on their primary site of action. The immunosuppressants agents can be classified as inhibitors of transcription (cyclosporine, tacrolimus), inhibitors of nucleotide synthesis (azothioprine, mycophenolate mofetil, leflunomide) and inhibitors of growth factor signal transduction (sirolimus, leflunomide (Suthanthiran and Strom, 1994; Suthanthiran *et al.*, 1996). The initial and maintenance therapy of such immunosuppressive agents prevents allograft rejection.

In the United States, there was a marked improvement in both short and long-term kidney graft survival from cadaveric and living donors between 1988 and 1996 (Hariharan *et al.*, 2000). During this period also the use of tacrolimus steadily increased as cyclosporine use decreased. In 1993, 95% of patients undergoing kidney transplant were using cyclosporine while only 2% used tacrolimus. This was followed by a gradual decline in the use of cyclosporine, down to 30% in 2002 in the United States according to the US Department of Health Organ Procurement and Transplantation Network 2003. The reasons for this conversion are most likely related to multi-centre trial data which has suggested lower acute rejection rates with tacrolimus (Pirsch *et al.*, 1997). Today, both CNIs are pivotal in the

prevention of allograft rejection and are part of more than 90% of immunosuppressive protocols used in organ transplantation.

### 3.1 Cyclosporine A

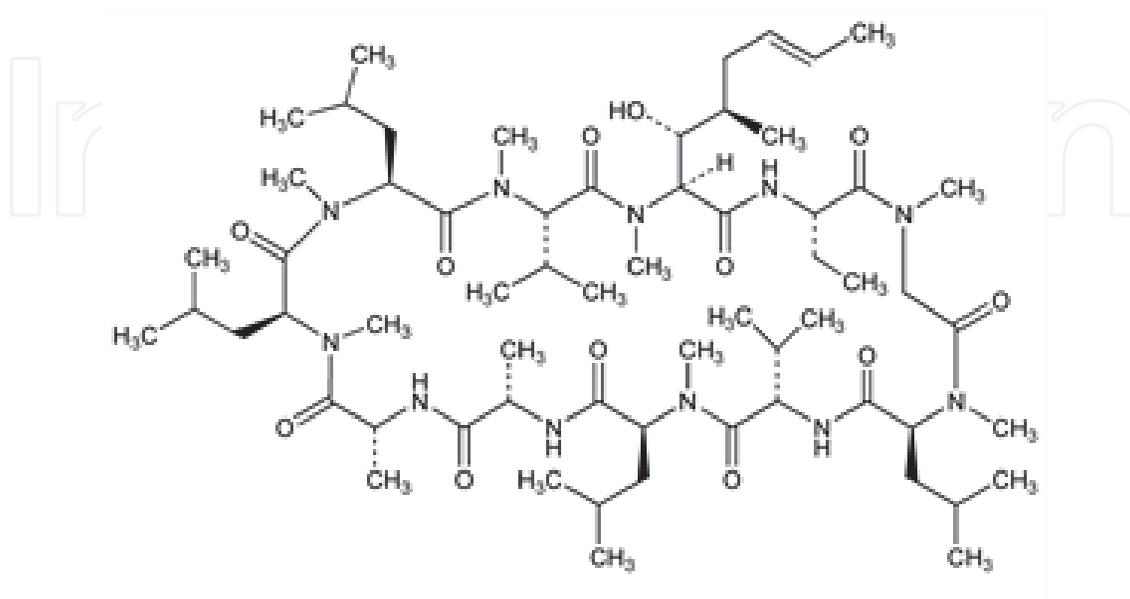


Fig. 1. Chemical Structures of CsA (Adapted from [www.medicinescomplete.com](http://www.medicinescomplete.com))

#### 3.1.1 Discovery

Members of the cyclosporine family were first isolated in 1970 from mycelia of two strains of the soil fungus, Fungi Imperfecti of the species *Tolypocladium infatum* (Ruegger *et al.*, 1976). Cyclosporine A (CsA) was first identified by a group in Sandoz Ltd., Basel, Switzerland, who were conducting routine screening for novel agents with fungal antibiotic activity. On investigation, it was observed that while CsA completely blocked T-cell activation, the same concentrations of CsA were not cytotoxic to cells and did not block the proliferation of other cell types suggesting that CsA could make a useful immunosuppressive agent (Borel, 1976; Borel *et al.*, 1976; Borel *et al.*, 1977). Further work by Borel *et al.* led to the purification of CsA and the initiation of *in vitro* and *in vivo* testing of the immunosuppressive and toxic effects of CsA. The first clinical uses of CsA took place in 1978 following a kidney transplantation (Calne *et al.*, 1978), and bone marrow transplantation (Powles *et al.*, 1978). In 1983, CsA was registered for organ transplantation in Switzerland, under the registered trademark of Sandimmune. Later that same year, the drug was registered in the United States. By 1985, the first clinical trials of CsA in the treatment of autoimmune diseases had begun (Borel, 1986; Borel and Gunn, 1986). Since its clinical introduction in 1983, CsA revolutionised the field of organ transplantation (Calne, 1986).

#### 3.1.2 Pharmacokinetics

CsA is a neutral, lipophilic cyclic undecapeptide consisting of 11 amino acids, with a molecular weight of 1203 daltons. The peptide is essentially insoluble in water and contains four amide groups, all of which are intra-molecularly hydrogen bonded in both the tetragonal and orthorhombic crystal structures (Stevenson *et al.*, 2003). CsA is soluble in

organic solvents and stable in solution at temperatures below 30°C. It is however sensitive to light, cold, and oxidation (IARC International Agency for Research on Cancer 1990). CsA is not compatible with alkali metals, aluminium and heat. Carbon monoxide, carbon dioxide, nitrogen oxides, hydrogen chloride gas and phosgene constitute the hazardous combustion or decomposition products associated with CsA (Sigma Chemical Co. 2002). CsA is readily absorbed because it is a highly lipophilic molecule, despite being a peptide. While oral administration is the preferred route of CsA prophylaxis, its absorption may be incomplete, slow and erratic thus suggesting a major drawback of oral formulation. The bioavailability of oral CsA averages about 30% (Holt *et al.*, 1994) and large inter- and inpatient variations in CsA absorption have been observed partly due to the unpredictable bioavailability of CsA following oral administration. These variations most likely reflect differences in the separation of CsA from its vehicle in the intestine. Genetic variations in cytochrome P450 and P-glycoprotein may also be involved (Rivory *et al.*, 2000). Many factors can affect CsA absorption, however the presence of normal serum low-density lipoprotein levels, co-administration of CsA with food and prolonged therapy have been shown to improve the drugs absorption (Bennett *et al.*, 1996). Obesity may also be an influential factor due to CsA being a hydrophobic compound with preferential distribution to adipose tissue and lipoproteins (Cather *et al.*, 2001).

CsA is significantly metabolised in the liver by the cytochrome P450 (CYP) 3A4 system, largely by hydroxylation and N-demethylation (Whalen *et al.*, 1999). It is extensively biotransformed to approximately 15 metabolites, which are almost completely eliminated via the biliary system. Less than 3% of the parent compound is excreted in urine and thus higher blood levels of CsA do not occur in patients with renal dysfunction (Lim *et al.*, 1996). CsA plasma levels peak in 2 to 4 hours and the serum half life ranges from 6.3 hours to 20.4 hours. The therapeutic concentration in whole blood is between 40 and 200 ng/mL.

### 3.1.3 Clinical use and mechanism of action

CsA is primarily renowned as a powerful immunosuppressant for use in organ transplantation to prevent graft rejection in kidney, liver, heart, lung and combined heart-lung transplants (Li *et al.*, 2003). In addition, it is used to prevent rejection following bone marrow transplantation and in the prophylaxis of host-versus-graft disease. It is also used in the treatment of psoriasis, atopic dermatitis, encephalomyelitis, rheumatoid arthritis, nephrotic syndrome, and uveoretinitis (Cather *et al.*, 2001; Gordon and Ruderman, 2006).

CsA is a potent inhibitor of cell-mediated immunity, and a less potent inhibitor of humoral immunity. While the precise molecular mechanism of CsA action is incompletely understood, the major pathway involves inhibition of calcineurin and subsequent inhibition of the expression of the interleukin-2 (IL-2) gene in activated T-cells (Kronke *et al.*, 1984). CsA binds to cyclophilin, a cytoplasmic prolyl peptidyl isomerase and undergoes conformational changes converting CsA to an active formulation. The CsA-cyclophilin complex then binds with high affinity to calcineurin, a Ca<sup>2+</sup>-dependent serine-threonine phosphatase (Liu, 1993). In T-lymphocytes, calcineurin is activated by a rise in cytoplasmic Ca<sup>2+</sup> which occurs after T-cell receptor activation by antigen recognition. Calcineurin then dephosphorylates the cytoplasmic form of the transcription factor NFAT (nuclear factor of activated T-cells), allowing NFAT to translocate to the nucleus where, in combination with its nuclear form, fos or jun, it initiates transcription of the T-cell growth factor IL-2 (Jain *et al.*, 1993; Jain *et al.*, 1993; Wesselborg *et al.*, 1996). The CsA-cyclophilin complex binds to and

inhibits calcineurin, thereby blocking NFAT dephosphorylation, IL-2 transcription and T-cell growth resulting in immunosuppression (Fruman *et al.*, 1992). In addition, CsA has recently been found to inhibit the JNK and p38 signalling pathway activity triggered by antigen recognition in T-cells (Matsuda and Koyasu, 2000). The presence of all three target pathways for CsA in T-cells may explain the high specificity of its immunosuppressive effects. The mechanism of action of CsA is summarised in Figure 2.

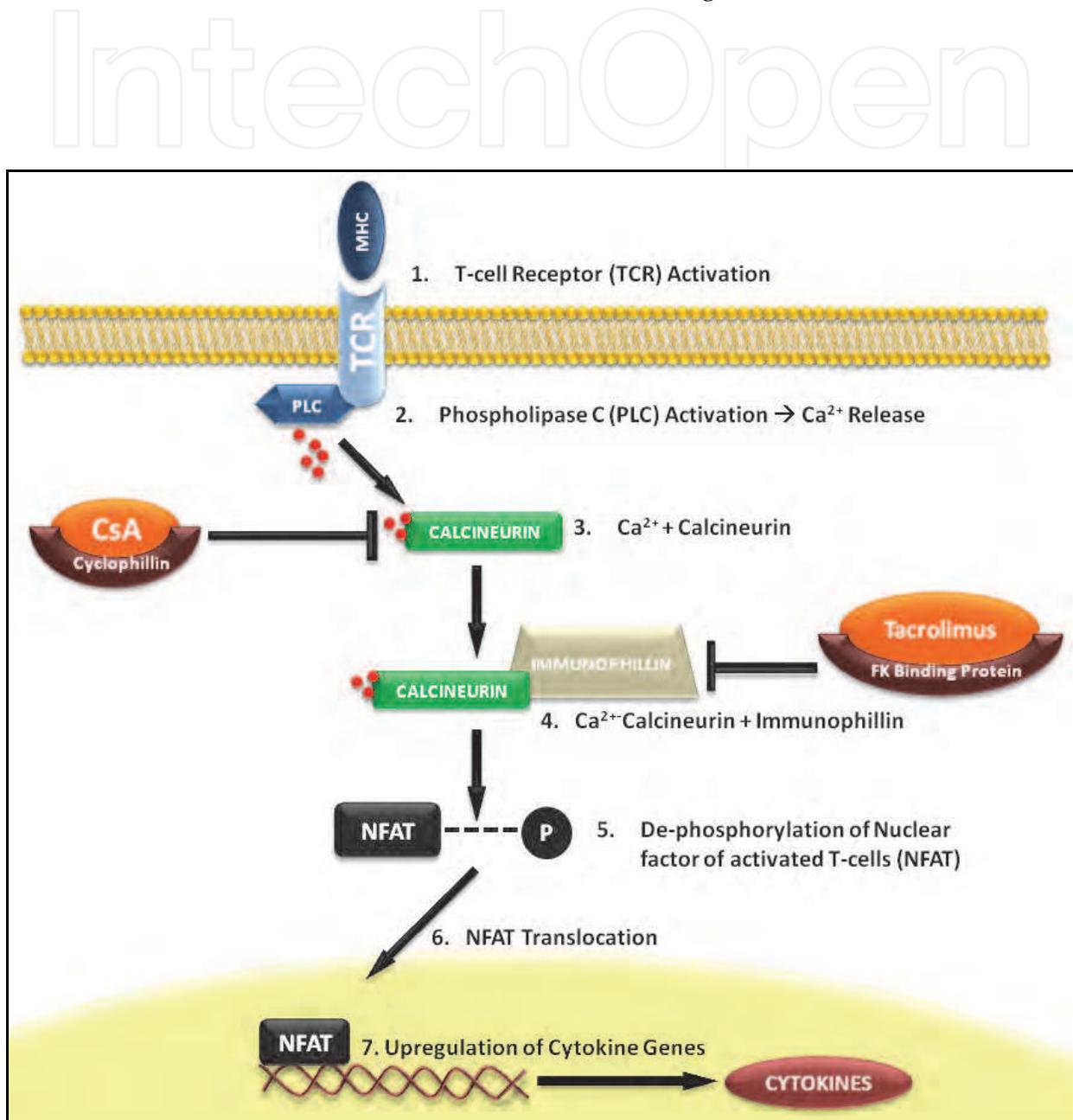
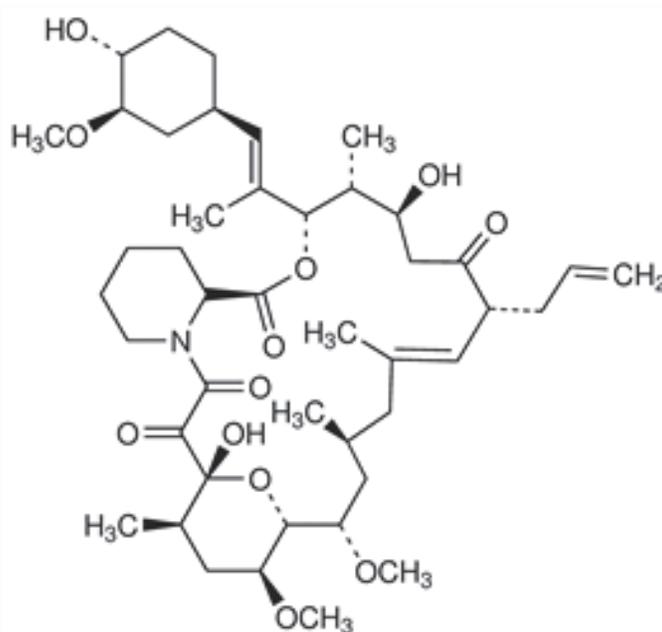


Fig. 2. Mechanism of Action of Calcineurin Inhibitors CsA and Tacrolimus

### 3.2 Tacrolimus (FK506)



(Adapted from [www.medicinescomplete.com](http://www.medicinescomplete.com))

Fig. 3. Chemical Structure of tacrolimus (FK506)

#### 3.2.1 Discovery

Tacrolimus, also known as FK506 or Prograf®, is a macrolide compound that was isolated by Fujisawa researchers from the culture broth of a soil microorganism *Streptomyces tsukubaensis* near Mount Tsukuba, Japan in 1984 (Kino and Goto, 1993). By 1987, the marked immunosuppressive properties of tacrolimus were confirmed following extensive *in vitro* and *in vivo* testing (Kino and Goto, 1993), thereby identifying what would become a cornerstone of immunosuppressive prophylaxis after solid organ transplantation. Tacrolimus was first launched in Japan in 1993, for prevention of allograft rejection in liver or kidney transplant patients. In the US, tacrolimus (Prograf®) was approved for prevention of rejection in liver transplant recipients in 1994, and in kidney transplant recipients in 1997. In 2003, tacrolimus was used as initial immunosuppression in 67% of kidney recipients and 89% of liver recipients (UNOS United Network for Organ Sharing 2004). It wasn't until the mid-1990s that tacrolimus became available in Europe (Pratschke *et al.*, 1998).

Tacrolimus is a potent immunosuppressive agent that is effective in allograft prophylaxis after organ transplantation. While it has been shown to be 10 to 100 times more potent than CsA (Goto *et al.*, 1991), tacrolimus has significantly reduced the incidence and severity of acute rejection rates in organ transplantation (Kaplan *et al.*, 2003; Toz *et al.*, 2004). In addition, patients receiving tacrolimus therapy require less concomitant corticosteroid therapy thus reducing the risk of adverse corticosteroid-associated side effects (Busuttil and Holt, 1997).

#### 3.2.2 Pharmacokinetics

Tacrolimus is a 23-membered macrolide structure ( $C_{44}H_{69}NO_{12}$ ) with a molecular weight of 822 Da (Christians *et al.*, 1992). As a result of cis-trans isomerism of the C-N amide bonds,

tacrolimus forms two rotamers in a ratio 2:1. It is soluble in alcohols, halogenated hydrocarbons, and ether but just minimally soluble in aliphatic hydrocarbons and water (Christians *et al.*, 1992).

Tacrolimus is available for use in liquid, oral or topical formulations. Following intravenous administration, tacrolimus undergoes extensive tissue distribution and binds tightly to erythrocytes (Yura *et al.*, 1999). It is metabolised by the intestinal and hepatic cytochrome P450 system to at least nine metabolites (Christians *et al.*, 1992). A review by Kelly and Kahan reported that CYP3A4 is primarily responsible for the metabolism of tacrolimus (Kelly and Kahan, 2002). The demethylated metabolites exhibit an immunosuppressive activity up to 70% of that of the parent compound (Iwasaki *et al.*, 1993). Excretion of tacrolimus is mainly via the biliary system in the form of several metabolites.

The oral formulation is composed of capsules of a solid dispersion of tacrolimus in hydroxypropyl methylcellulose (Lake *et al.*, 1995). Following oral administration of this highly lipophilic drug, absorption is not complete, with its bioavailability ranging from 10-60%. It reaches maximum blood levels after 1-2 hours after dosing and its half-life ranges from 8-24 hours. It has been postulated that interactions with the proximal small intestinal CYP enzymes may affect pharmacokinetics and absorption of the drug, thus attributable for the low and highly variable oral bioavailability (Lampen *et al.*, 1995). Drug level monitoring is critical as tacrolimus has a high inter-individual variability and a narrow therapeutic index (Jusko, 1995).

### 3.2.3 Clinical use and mechanism of action

Tacrolimus is a potent immunosuppressive agent that is effective in allograft prophylaxis after organ transplantation, for therapy of acute rejection and in treatment of different immune diseases (Lampen *et al.*, 1995). In addition to its powerful immunosuppressive properties, tacrolimus ointment (tacrolimus ointment) has been utilised in the treatment of moderate to severe atopic eczema (atopic dermatitis) which is unresponsive to conventional therapy. During atopic eczema, the skin's immune system is, to some degree, overactive in its protection. Tacrolimus works by suppressing such over-activity i.e., its therapeutic efficacy is attributed to its immunomodulatory effects on different immune cell types (Lan *et al.*, 2004).

The mechanism of action of tacrolimus is similar to that of CsA but involves binding of tacrolimus to the cytoplasmic immunophilin, FK binding protein 12 (FKBP12). The tacrolimus-FKBP complex then mediates inhibition of calcineurin's phosphatase activity and the subsequent, NFAT-dependent production of IL-2 and other cytokines. While tacrolimus is extremely effective in allograft prophylaxis after organ transplantation, the onset of nephrotoxicity is a major drawback that limits its use clinically (Katari *et al.*, 1997). A significant number of additional toxicities have been reported following administration of tacrolimus. Such toxicities include neurotoxicity (Veroux *et al.*, 2002), which has been suggested to be linked to the inhibition of calcineurin phosphatase, cardiomyopathy (Seino *et al.*, 2003), anaemia, chronic diarrhoea (Webster *et al.*, 2005), post-transplant diabetes mellitus (Araki *et al.*, 2006), and lymphoproliferative disease (Caillard *et al.*, 2006) and infections (Griffith *et al.*, 1994). The mechanism of action of tacrolimus is summarised in Figure 2.

### 3.3 Calcineurin inhibitor toxicity

While CsA and tacrolimus differ in their molecular structure and intracellular binding characteristics, both compounds ultimately inhibit calcineurin. Inhibition of the calcineurin-

NFAT pathway by CNIs is not specific to immune cells and it can lead to toxic changes in addition to immunosuppressive effects. Furthermore, there is evidence to suggest that the molecular effects of CNIs are not limited to NFAT dependent mechanisms.

Several adverse effects have been reported following CNI administration. These include acute and chronic renal dysfunction, haemolytic-uraemic syndrome, electrolyte disturbances (hyperkalaemia, hypomagnesaemia, and hypocalcaemia), tubular acidosis, defects in urinary concentrating ability, hepatotoxicity, neurotoxicity, dyslipidaemia, gingival hyperplasia, hypertrichosis, malignancies, and increased risk of cardiovascular events (Burdmann *et al.*, 1995; Li *et al.*, 2004). Hypertension is another common adverse reaction to CNIs and is thought to be associated with the decrease in renal function (Ponticelli, 2005). CNI-induced hypertension is however reversible upon decrease or cessation of dose. Female transplant recipients taking CsA during pregnancy tend to have an increased number of premature deliveries in addition to low birth weight offspring, thus suggesting that CsA crosses the placental barrier (Cather *et al.*, 2001).

The most significant toxic side effect of CNI administration is nephrotoxicity (Burdmann *et al.*, 1995; Li *et al.*, 2004; Ponticelli, 2005). This includes both acute and chronic nephrotoxic effects. Acute CNI nephrotoxicity is largely characterised by hemodynamically induced renal dysfunction which is generally fully reversible by cessation or decrease of the drug (Cattaneo *et al.*, 2004). While the majority of available data on acute toxicity relates to CsA, tacrolimus effects are believed to be similar (Lloberas *et al.*, 2008). Evidence suggests that the hemodynamic disruption observed in acute CNI nephrotoxicity is mediated by inappropriate activation of the renin-angiotensin system (RAS). RAS activation stimulates production of potent vasoconstricting factors such as angiotensin II, endothelin and thromboxane, while also suppressing the synthesis of vasodilating prostacyclins, prostaglandin E2, and nitric oxide (NO) (Wissmann *et al.*, 1996; Lamas, 2005). RAS appears to be activated by the direct action of CsA on juxtaglomerular cells and by indirectly by induced renal arteriolar vasoconstriction (Kurtz *et al.*, 1988). A relatively rare complication related to acute CNI nephrotoxicity is hemolytic uremic syndrome (HUS). Since 1980, the number of case reports describing transplant patients who developed acute arteriopathy and severe renal impairment consistent with HUS while on CsA or tacrolimus, has steadily increased (Cattaneo *et al.*, 2004). The primary pathology is an extensive thrombotic process in the renal microcirculation with several glomerular capillaries occluded by thrombi extending from the afferent arterioles and containing platelet aggregates (Medina *et al.*, 2001). The mechanism by which CNIs induce this acute obliterating arteriopathy remains ill-defined. However, the renal vascular endothelium is likely the primary target. Endothelial cell injury disrupts the synthesis of vasodilatory and antithrombotic substances, while promoting the generation of vasoconstrictive and aggregating mediators promoting the development of HUS and renal failure (Cattaneo *et al.*, 2004).

While the basis of acute CNI nephrotoxicity is primarily hemodynamic, features of tubular damage are also often observed. Acute CNI nephrotoxicity is often accompanied by significant vacuolisation of tubular epithelial cells likely induced by enlargement of the endoplasmic reticulum and increased lysosomal volume (Morozumi *et al.*, 1986; Morozumi *et al.*, 2004). These effects may be the result of localised ischemia downstream of CNI-induced intrarenal vasoconstriction, however evidence suggests that CNIs, and CsA in particular has direct cytotoxic effects on tubular epithelial cells. CsA induces endoplasmic reticulum stress and cell death by induction of pro-apoptotic proteins (Healy *et al.*, 1998;

Pallet *et al.*, 2008; Pallet *et al.*, 2008). Similar effects on tubular cells were observed with tacrolimus (Du *et al.*, 2009; Du *et al.*, 2009).

While acute CNI nephrotoxicity is generally reversible, long-term exposure to CNIs can lead to irreversible damage to renal structure and function. Chronic CNI nephrotoxicity is characterised by histological lesions that are associated with irreversible and progressive interstitial renal fibrosis which correlates with diminishing renal function (Cattaneo *et al.*, 2004). Typical histopathological changes include arteriolar hyalinosis, tubular atrophy, interstitial fibrosis, thickening and fibrosis of the Bowman's capsule, and focal, segmental or global glomerular sclerosis (Williams and Haragsim, 2006). The development of these chronic alterations most likely involves a combination of CNI-induced hemodynamic changes and direct toxic effect of CNIs on tubular epithelial cells. Arteriolar hyalinosis (hyaline deposits around afferent arterioles) is a characteristic sign of chronic CNI toxicity. Hyaline deposits progressively replace necrotic vascular smooth muscle cells. The cause of smooth muscle cell necrosis is unclear but may be a consequence of calcineurin-NFAT inhibition (Nieves-Cintrón *et al.*, 2007). If the hyaline deposits are large they can cause significant narrowing of vascular lumen and may promote the progression of chronic CNI injury (Mihatsch *et al.*, 1998).

Arteriopathy and narrowing of the renal vasculature lumen can be considered major contributors to the development of chronic CNI nephrotoxicity but it is increasingly evident that the direct effects of CNIs on the tubular epithelial cells of the nephron play a major role in the progression and development of interstitial fibrosis during CAI. Free radicals and reactive oxygen species are produced in response to local ischemia and cell death in the tubulointerstitium. Another significant factor involved in the progression of chronic interstitial changes is the profibrotic cytokine transforming growth factor beta 1 (TGF- $\beta$ 1). TGF- $\beta$ 1 promotes the development of interstitial fibrosis by inhibiting ECM degradation, stimulating production of ECM proteins by modulating the normal function of tubular epithelial cells and resident interstitial fibroblasts (McMorrow *et al.*, 2005; Slattery *et al.*, 2005; Feldman *et al.*, 2007; Hertig *et al.*, 2008). These mechanisms will be discussed in detail below. However, the events that likely contribute to fibrosis in CAI are summarised in Figure 4.

They can be arbitrarily divided into three phases (Mannon, 2006; Mannon and Kirk, 2006). In the initiation phase, there is tissue injury, which may occur by either antigen dependent or independent insults. Regardless of the aetiology of the initiating event, the result is the fibrogenesis phase, consisting of inflammatory and proliferative responses, regulated by cytokines, chemokines and growth factors. This cascade of events results in the matrix accumulation phase. This is due to either increased production and/or reduced degradation of matrix, ultimately resulting in fibrosis.

#### **4. Molecular mechanisms of CNI-induced chronic allograft injury**

As described earlier, during CAI the decline in renal function correlates most closely with the degree of TIF. Therefore the molecular mechanisms contributing to the development of TIF have been a major research focus. Over the last decade, the molecular mechanisms underlying CNI nephrotoxicity have been extensively studied in our laboratory and others utilising a variety of *in vitro* and *in vivo* model systems, and clinical cohort studies. Particular focus has been on the direct effects of CsA and tacrolimus on the tubular interstitium and the principal cells that reside there; the tubular epithelial cells and interstitial fibroblasts.

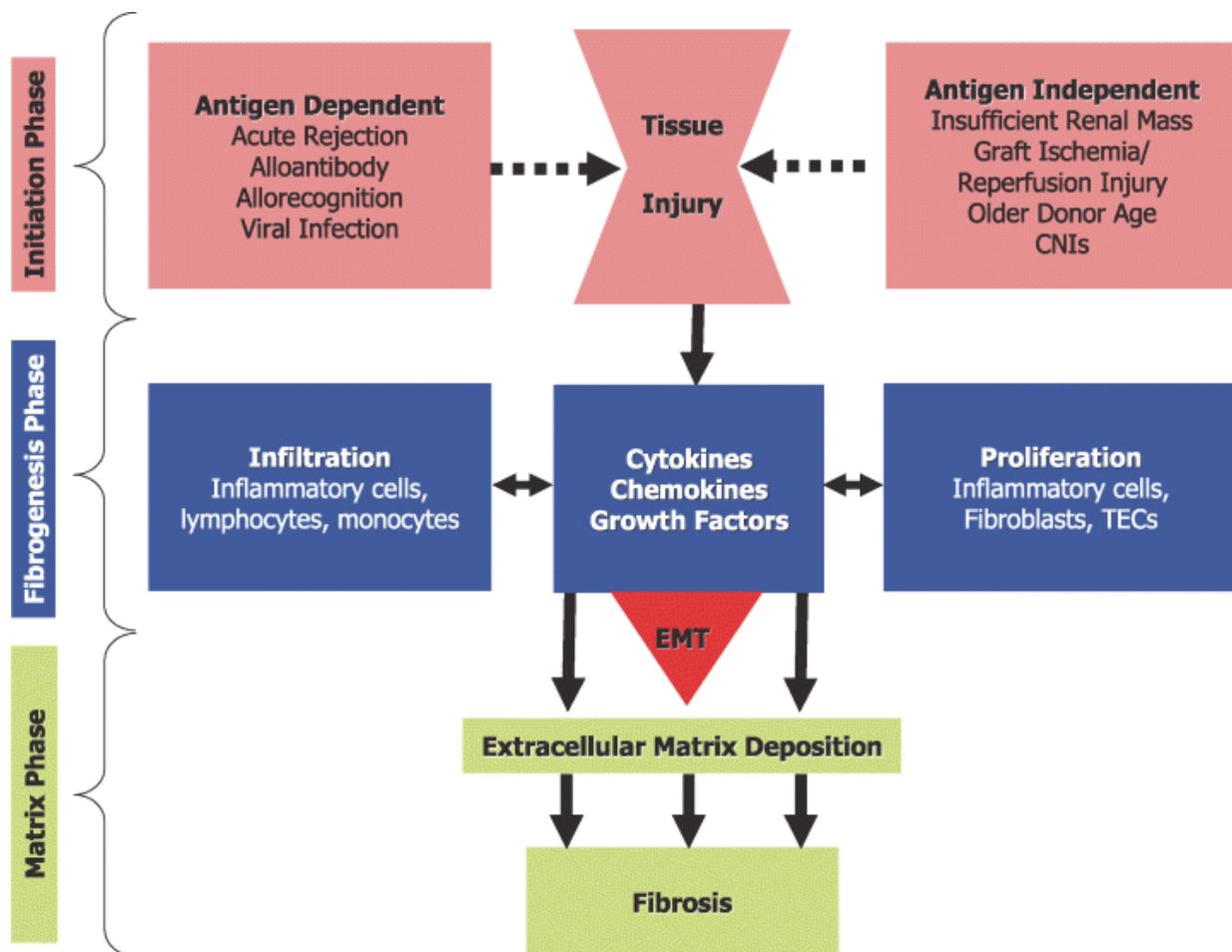


Fig. 4. Schema of the development of fibrosis in chronic allograft nephropathy (Adapted from Mannon, 2006)

#### 4.1 *In vitro* data

Work from our laboratory has demonstrated that both CsA and tacrolimus have direct effects on proximal and distal tubular epithelial cells ranging from cell death and disruption of normal barrier function, to pro-fibrotic and phenotype altering effects. For the purposes of this discussion, the focus will be on effects relating to fibrosis and TIF in CNI nephrotoxicity. The majority of this work has focused on the effects of CsA on proximal tubular epithelial cells (PTECs).

The primary pathological features observed in the tubulointerstitium during TIF are interstitial ECM accumulation, decreased numbers of functioning tubular epithelial cells and increased numbers of activated fibroblasts (termed myofibroblasts). Accumulated evidence suggests that these events do not occur in isolation but are functionally linked. It is accepted that the main source of the ECM that accumulates during TIF is the myofibroblasts. However, the source of these myofibroblasts has been, and remains a point of debate. Previously, it was held that the increase in myofibroblasts was due to infiltrating circulatory fibroblasts which became active in the interstitium. However, significant *in vitro* evidence has demonstrated that a major source of myofibroblasts is the tubular epithelium itself through a process termed epithelial-mesenchymal transition (EMT). Under normal physiological conditions, epithelial to mesenchymal transitions are important events in

embryonic morphogenesis (Hay and Zuk, 1995). Embryonic mesenchymal cells emerge from epithelial cell populations following loss of epithelial cell polarity, the disappearance of differentiated epithelial cell-cell junctions, reorganisation of the actin cytoskeleton, and the redistribution of organelles. Conversely, embryonic mesenchymal cells have the ability to completely regain epithelial phenotypes by the reverse process, mesenchymal-to-epithelium transition (MET) (Thiery, 2003). From a disease perspective, many of the molecular and phenotypic modifications occurring during developmental EMT are also observed in the most aggressive metastatic cancer cells and in fibrotic diseases (Thiery, 2003). Therefore EMT has emerged as a potentially central element in the development of metastatic tumours and tissue fibrosis. EMT is a stepwise progression in which numerous phenotypic changes occur leading to the loss of epithelial markers and function and the acquisition of a more (myo)fibroblast-like phenotype. The myofibroblast is a morphological intermediate between fibroblasts and smooth muscle cells. Like fibroblasts, they have the ability to produce and secrete large quantities of ECM components such as collagen I and III and fibronectin, and like smooth muscle cells they express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) conferring the ability to contract and equipping the myofibroblast with enhanced locomotive ability. Thus, EMT may contribute significantly to renal failure through the accumulation of ECM and perhaps more significantly, through loss of epithelial cells leading to reduced tubular integrity and function. The PTEC may no longer be regarded as passive victims in renal disease, but as active contributors to renal fibrosis (Becker and Hewitson, 2000).

Yang and Liu (Yang and Liu, 2001) proposed that at the cellular level, EMT involves a number of coordinated events that are both necessary and sufficient for the completion of the EMT process. Using an optimised, sub-cytotoxic dose of CsA (4.2 $\mu$ M) a number of cell characteristics were examined in our laboratory including morphology and cytoskeletal arrangement, epithelial junctional integrity, expression of epithelial markers (e.g. E-cadherin), expression of myofibroblast markers (e.g.  $\alpha$ -smooth muscle actin) and cell motility (Selection depicted in Figure 5). Human proximal tubular epithelial cells exposed to CsA exhibited major morphological changes associated with mesenchymal phenotypes. CsA treatment also resulted in significant cytoskeletal rearrangement, stress fibre formation and the development of filopodia. A number of important phenotypic markers were affected by CsA treatment. ZO-1 and E-cadherin expression were downregulated, while expression of the myofibroblast marker  $\alpha$ -SMA was strongly induced. Treatment with CsA significantly enhanced the migratory ability of tubular epithelial cells. This effect was of great importance in relation to renal fibrosis as the ability of the myofibroblast to migrate into the interstitium contributes significantly to disease progression. *In vivo*, CsA has been observed to have significant effects on renal tubular ultrastructure causing a characteristic, stripped fibrosis (Morales *et al.*, 2001). However, the link between these morphological changes and EMT had not been previously made. This model of CsA-induced EMT was the first published example of drug-induced EMT in the literature (McMorrow *et al.*, 2005; Slattery *et al.*, 2005).

Having established that CsA did induce EMT in proximal tubular epithelial cells, the mechanisms underlying the effects were examined. Using microarray generated gene expression profiles and specific inhibitors, the major mediators of CsA-EMT were elucidated. Unsurprisingly, TGF- $\beta$ 1 was found to figure prominently in the induction of EMT. However, it was the mechanism of TGF- $\beta$ 1 induction by CsA which proved most interesting. CsA-induced TGF- $\beta$ 1 production appeared to be dependent on protein kinase C

beta (PKC- $\beta$ ) activity (Slattery *et al.*, 2008). This was of interest as PKC- $\beta$  is currently under investigation as a significant contributor to the development of diabetic nephropathy. These results suggested that the mechanisms underlying the development of diabetic nephropathy and CsA nephropathy may be common and so similar therapeutic strategies could be employed in both disease situations. Further experimental work identified the E2A transcription factors (E12 and E47) as downstream mediators of the effects of CsA on E-cadherin expression (Slattery *et al.*, 2006; Slattery *et al.*, 2008).

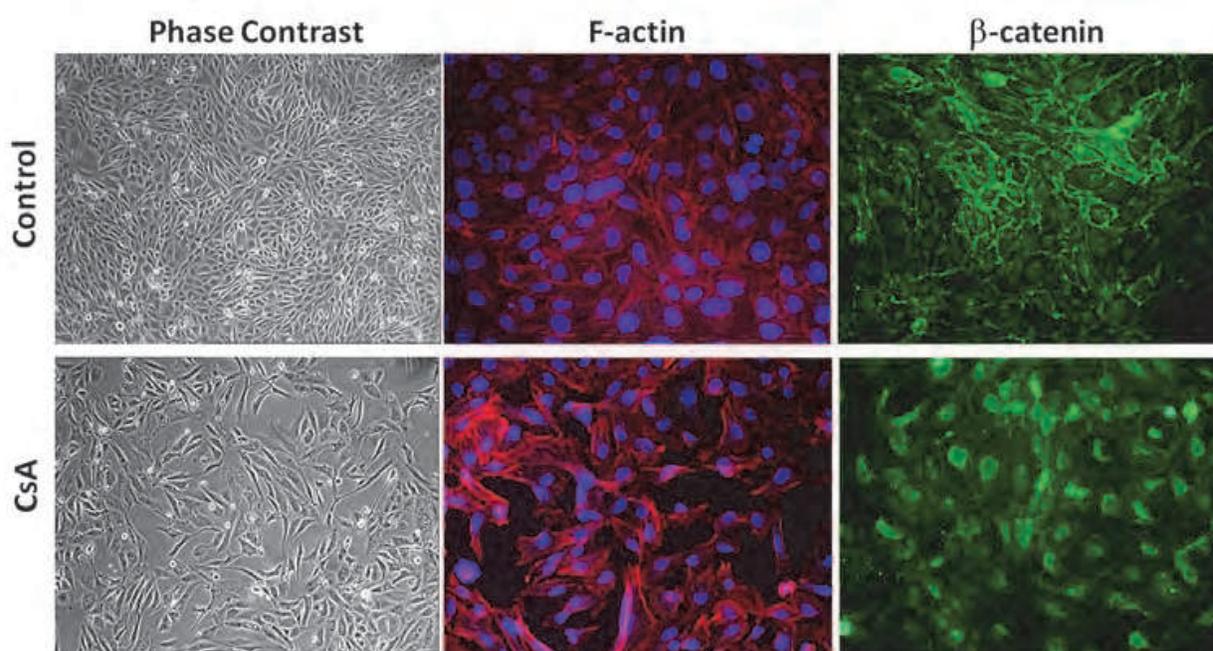


Fig. 5. Effects of CsA on normal PTEC morphology, F-actin distribution and  $\beta$ -catenin distribution in PTECs. (Adapted from Slattery *et al.* 2005)

#### 4.2 *In vivo* data - Animal models and clinical studies

In parallel with *in vitro* studies, an *in vivo* rodent model of CsA-induced CAI was also established to investigate the mechanisms involved (O'Connell *et al.*). CsA nephrotoxicity was induced in CD-1 mice by daily CsA administration for 4 weeks. Mirroring *in vitro* findings, decreased E-cadherin and increased  $\alpha$ -SMA expression was observed. In addition, TGF- $\beta$ 1 was significantly increased by CsA treatment in this *in vivo* model. In addition to investigating the molecular mechanism of CsA-induced nephrotoxicity, we have also focused on identifying novel diagnostic techniques to identify patients at risk of developing CAI in advance of currently used techniques such as proteinuria and creatinine monitoring. At present, a histological diagnosis with a renal transplant biopsy is the 'gold standard' for determining CAI. Therefore the development of novel, predictive indicators of CAI would be highly desirable. One strategy utilised to identify novel biomarkers has been urinary proteomic analysis in both the CsA mouse model (O'Connell *et al.* 2011) and in a clinical patient cohort (Johnston *et al.*, 2011) (Depicted in Figures 6 and 7 respectively). In the animal studies significant alterations in urinary podocin and uromodulin were observed which may be indicative of damage to the glomeruli and tubules after CsA treatment. Furthermore, E-cadherin, superoxide dismutase and vinculin levels in the urine may be early indicators of CsA nephrotoxicity.

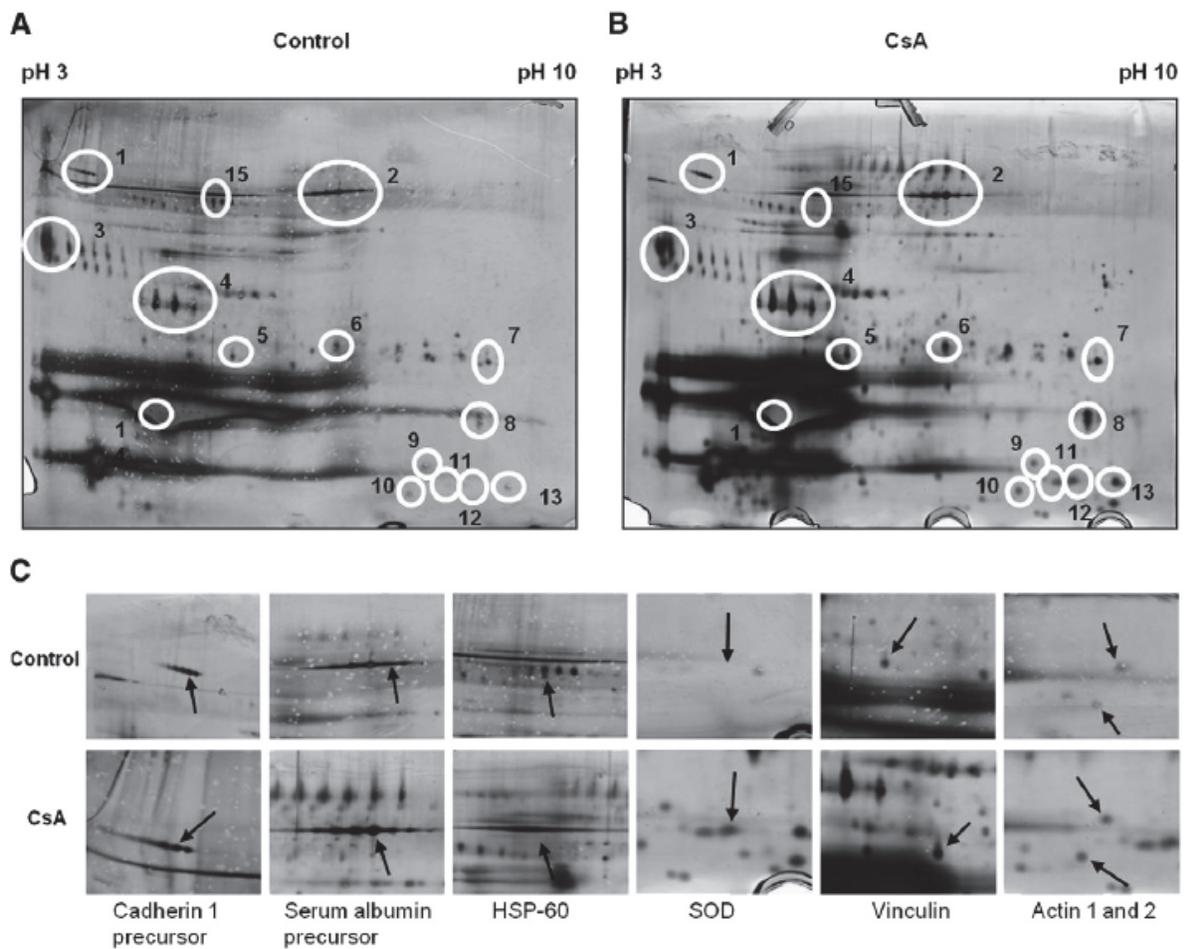


Fig. 6. Analysis of urinary proteins in a CsA-induced mouse model of CNI nephrotoxicity by 2D gel electrophoresis (Adapted from O'Connell *et al.* 2011).

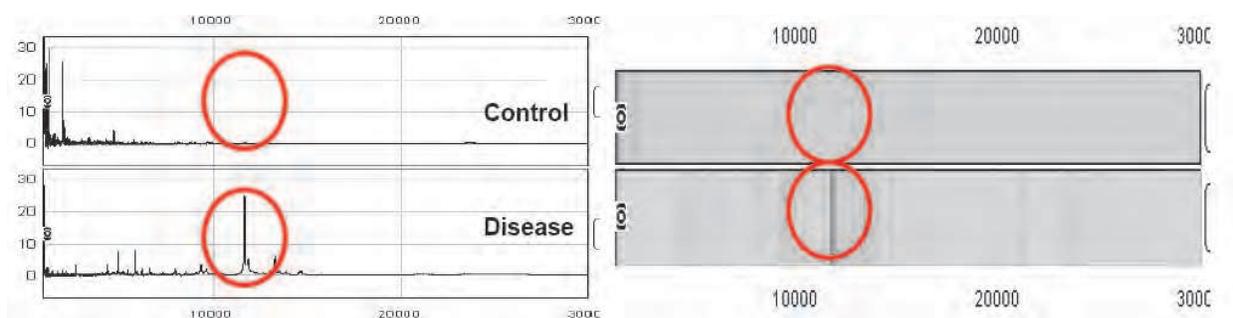


Fig. 7. Analysis of urinary proteins in clinical patient urine samples using ProteinChip and SELDI-TOF analysis (Adapted from Johnston *et al.* 2011).

In the human clinical studies, 34 renal transplant recipients with histologically proven CAI and 36 patients with normal renal transplant function were compared. High-throughput urinary proteomic profiles were generated using ProteinChip arrays and surface-enhanced laser-desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS). Following SELDI, biomarker pattern software analysis was performed which led to the identification of

a novel biomarker pattern that could distinguish patients with CAN from those with normal renal function. One of the identified proteins was  $\beta 2$  microglobulin which was a powerful distinguishing factor between CAI patients and control patients. Further validation is required to determine if this  $\beta 2$  microglobulin protein biomarker will allow for diagnosis of CAN by non-invasive methods in a clinical setting but this study clearly shows the potential of urinary proteomics to identify patients at risk of developing CAI.

## 5. Conclusion

In the continued absence of therapeutic strategies to prevent and/or reverse fibrosis which is the primary pathological driving force in chronic kidney disease, renal transplantation remains the primary treatment for end stage renal failure. It follows that calcineurin inhibitors will continue to be heavily utilised in anti-allograft rejection therapies. Therefore, furthering our understanding of the molecular basis of CNI nephrotoxicity must remain a major research topic with the goal of developing therapeutic strategies to reduce or eliminate the development of chronic allograft injury.

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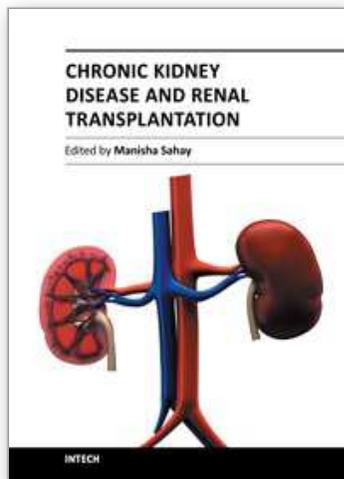
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This valuable resource covers inpatient and outpatient approaches to chronic renal disease and renal transplant with clinical practicality. This first section of the book discusses chronic disease under distinct topics, each providing the readers with state-of-the-art information about the disease and its management. It discusses the fresh perspectives on the current state of chronic kidney disease. The text highlights not just the medical aspects but also the psychosocial issues associated with chronic kidney disease. The latest approaches are reviewed through line diagrams that clearly depict recent advances. The second section of the book deals with issues related to transplant. It provides effective and up-to-date insight into caring for your transplant patients.

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