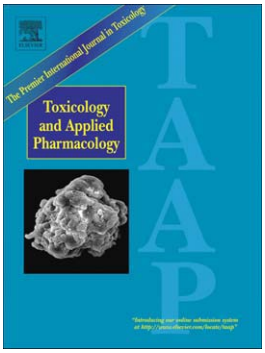

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Identification of novel indicators of cyclosporine A nephrotoxicity in a CD-1 mouse model

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Short title: Indicators of CsA nephrotoxicity *in vivo.*

Abstract

The calcineurin inhibitor cyclosporine A (CsA) is a widely used immunosuppressive agent. However, nephrotoxicity is a serious side effect observed in patients which limits clinical use of CsA. CsA nephrotoxicity is associated with tubulointerstitial injury progressing to nephropathy. This is typically diagnosed by invasive renal biopsy and is often only detected when the disease process is well advanced. Therefore identification of novel, early indicators of CsA nephrotoxicity could be clinically advantageous. This study aimed to establish a murine model of CsA nephrotoxicity and to identify urinary proteins that may indicate the onset of CsA-induced nephropathy using 2-D gel electrophoresis. CsA nephrotoxicity was induced in CD-1 mice by daily CsA administration for 4 weeks. By week 4, elevated serum creatinine and proteinuria were observed after CsA treatment indicating significant renal dysfunction. Decreased cadherin-1, increased α-smooth muscle actin and fibroblast specific protein 1 in kidney tissue indicated disruption of normal tubular architecture. Alterations in podocin and uromodulin were also observed which may indicate damage to other segments of the nephron. Proteomic analysis of urine identified a number of differentially regulated proteins that may be involved in early CsA nephropathy including cadherin 1, superoxide dismutase and vinculin. These findings suggest novel mechanisms of CsA nephrotoxicity and identify novel potential markers of the disease.

Keywords: Cadherin 1 (CDH-1), Connective tissue growth factor, fibroblast specific protein 1, transforming growth factor beta 1 and 2-DE.

INTRODUCTION

The use of calcineurin inhibitors (CNIs), cyclosporine A (CsA) and tacrolimus has revolutionized solid organ transplantation over the last 30 years (Gaston 2009). However, clinical use of CsA is associated with both acute and chronic nephrotoxicity which is a major limiting factor in its use. While alternative therapeutics have been sought, CNIs remain our most effective and widely used immunosuppressants (Gaston, 2009). While acute CsA nephrotoxicity is managed clinically through careful monitoring of renal function and appropriate regimen adjustment, the balance between preventing immunologic allograft loss and the management of chronic CNI nephrotoxicity (particularly CsA nephropathy) is still a major issue in renal transplantation (Bestard *et al.*, 2005). Early diagnosis of nephropathy can greatly improve patient prognosis. However the initial stages of CsA nephropathy are largely asymptomatic making early diagnosis difficult. Therefore identification of novel, early disease indicators is currently a major research focus.

Current diagnostic techniques employed to detect CsA nephropathy are inadequate. The primary method is estimation of glomerular filtration rate (eGFR) (Cockcroft and Gault, 1976). However this technique is limited since eGFR varies greatly both between patients, and over time within a patient (Kwong *et al.*, 2010). eGFR is the net result of the complex interaction of multiple factors including age, blood pressure and other diseases. Many of these factors are variable and so compensation can often occur, leading to stabilisation of eGFR, effectively masking early renal functional decline. These factors mean that eGFR can be a very insensitive indicator of renal damage. Determination of serum creatinine and blood urea nitrogen (BUN) are also used to estimate renal function although these tests can be insensitive and have poor diagnostic value (Dieterle *et al.*, 2010). Measurement of albumin and/or protein in the urine to detect renal damage may be more sensitive than the determination of eGFR on an individual basis, especially in early disease states. However biopsy studies have clearly shown that intra-renal pathology often occurs well in advance of microalbuminuria (Rastaldi *et al.*, 2002). Furthermore, the relationship between proteinuria and CsA nephropathy is complex (Li and Yang, 2009) limiting its power as an early indicator of CsA nephrotoxicity.

CsA nephropathy is characterized by tubulointerstitial fibrosis (TIF), tubular vacuolization, glomerulosclerosis, and arteriolopathy (Hara *et al.*, 2009). Of these, TIF is thought to be the primary mechanism driving the progression of CsA nephropathy (Bobadilla and Gamba, 2007). TIF is characterized by the gradual loss of tubular epithelial cells, and progressive accumulation of fibroblasts and myofibroblasts (α-smooth muscle actin (α-SMA) and fibroblast specific protein-1 (FSP-1) positive cells). The accumulation of myofibroblasts results in excessive production and deposition of extracellular matrix (ECM) in the tubulointerstitium (Lan, 2003). Previous studies from this research group have demonstrated the direct toxic effects of CsA on renal tubular epithelial cells *in vitro* (Kiely *et al.*, 2003; McMorrow *et al.*, 2005; Slattery *et al.*, 2005; Martin-Martin *et al.*, 2010). It is clear that some of the downstream pathogenic effects of CsA are mediated by transforming growth factor beta 1 (TGF-β1) and it's downstream effector connective tissue growth factor (CTGF) ,(Grotendorst, 1997; Gupta *et al.*, 2000; Shihab *et al.*, 2003; Slattery *et al.*, 2006). Conversely, the TGF-β1 antagonist bone morphogenetic protein 7 (BMP-7) is downregulated in rat models of CsA nephrotoxicity (Tuglular *et al.*, 2004). However the precise mechanism of CsA-induced nephrotoxicity remains to be fully elucidated.

The aims of this study were to establish an *in vivo* mouse model of CsA nephropathy and evaluate a number of putative nephrotoxicity markers. These markers included TGF-β, CTGF and BMP-7 which have been proposed in published studies, across a number of other models of nephrotoxicity, as being significantly involved in disease progression and may be suitable as indicators of toxicity or therapeutic targets (El Chaar *et al.*, 2007; Dudas *et al.*, 2009; Phanish *et al.*, 2010). TGF-β, CTGF and BMP-7 were therefore evaluated in a CsA nephrotoxicity model, and a high-throughput proteomic screening methodology was utilized to identify novel, early indicators of CsA nephropathy. 2-DE has been widely used to identify potential urinary markers of disease in a range of settings including hepatocellular carcinoma (Jia *et al.*, 2010) and juvenile idiopathic arthritis (Rosenkranz *et al.*, 2010). Identification of discriminating urinary proteins in

the current model may lead to novel markers of CsA nephrotoxicity and may also help to further elucidate the mechanisms underlying CsA nephrotoxicity.

METHODS

Animal Treatment

Male CD-1 mice weighing 25-35g (6-8 week) were housed in the UCD biomedical facility according to ethical and legal guidelines in a temperature and light controlled environment. All experiments were approved by the UCD Animal Research Ethics Committee (P04-07). Government approval (B100/3539) was also granted by the Irish Department of Health under section 11 of the Cruelty to Animals Act. For the duration of the experiment mice were maintained on a low sodium diet (Harlan, UK Ltd.). CsA (Sigma-Aldrich, Cat no. C1832) was made up as a 1 mg/ml stock solution in olive oil (Sigma-Aldrich). CsA was administered by intraperitoneal injection (15mg/kg/day), daily for 1 week or 4 weeks, as indicated. Control mice received 1ml/kg of vehicle (olive oil) by intraperitoneal injection, daily for 1 week or 4 weeks, as indicated. These doses and time points were based upon data from other relevant *in vivo* studies (Thomas *et al.*, 1998; Clarke and Ryan, 1999; Yang *et al.*, 2002; Ling *et al.*, 2003). Using this protocol ensured that by 4 weeks of CsA treatment significant nephropathy had developed. The 1 week CsA group was utilised to examine early toxic effects of CsA before overt histological alterations had manifested. (Ling *et al.*, 2003; Chaaya *et al.*, 2011). Mice had free access to food and water throughout the experiments.

At the end of each treatment period mice were housed in group metabolism cages for 24 hours for urine collection which was then frozen at -80℃. After this 24 hour period mice were euthanized and blood samples obtained by cardiac puncture. Kidneys were collected for analysis of histology, gene and protein expression. Half a kidney was used for RNA isolation (Trizol method, T9424 Sigma-Alrdich) and half for protein isolation (RIPA buffer method Sigma-Aldrich, R0278). Half of the other kidney was snap frozen in liquid N_2 and the other half was fixed in neutral buffered formalin. Each treatment group i.e. 1 week control; 1 week CsA; 4 week control and 4 week CsA contained 6 mice. Urine was collected from each group of 6 mice and processed for analysis as one pooled sample for each group.

Renal function and histology

Renal function was assessed by determination of serum creatinine and urinary protein (proteinuria). Serum creatinine was measured using the Quantichrom™ Creatinine Assay Kit (Cat no. DICT-500, Bioassay systems assay kit), according to the manufacturers protocol. This colorimetric assay is based on the improved Jaffe method. Proteinuria was measured using the Bradford Assay for assessing total protein quantities in a biological sample (Bradford, 1976). Urine samples were normalised for urinary output. Half a kidney was fixed in neutral buffered formalin, paraffin embedded and sectioned at 5µm. After de-waxing, gross renal histology was examined using standard haematoxylin and eosin (H&E) staining (Sigma-Aldrich). Collagen staining of sections was performed using Masson's Trichrome stain (Sigma-Aldrich). Sections were stained using an automated slide stainer (Leica autostainer XL).

Quantitative Polymerase Chain Reaction (PCR)

Total RNA was isolated using the trizol method from half a kidney stored at -80℃ in RNAlater™ (Ambion Cat no. AM7020) according to the manufacturers protocol. 1µg of total RNA was used to synthesis cDNA. A Real-Time PCR TaqMan assay was used to quantify the relative expression levels of genes of interest and has been described previously (Feighery *et al.*, 2008). Briefly, cDNA was amplified on the ABI 7900HT Sequence Detection System at default thermal cycling conditions: 2 min @ 50˚C, 10 min @ 95˚C for enzyme activation and then 40 cycles of 15 sec @ 95˚C for denaturation and 1min @ 60˚C for annealing and extension. Results were analysed using the delta Ct method of analysis. Primer sequences for murine TGF-β1 were designed in the Conway Institute genomics core facility and synthesized by Applied Biosystems. Primer specificity was assessed by nBLAST in the NCBI database.

Name: Mouse TGF-β1 NM_011577.tx-529 Forward

Sequence: AATTCCTGGCGTTACCTTGGT

Name: Mouse TGF-β1 NM_011577.tx-600 Reverse

Sequence: GACGTCAAAAGACAGCCACTCA

Commercially available gene expression assays were used for mouse CTGF (Mm00515790_g1), Cadherin 1 (Mm00486906_m1), α-SMA

(Mm00426835_g1) and FSP-1 (Mm 00803372_g1) and human TGF-β (Hs99999918_m1) and CTGF (Hs00170014_m1) all from Applied Biosystems.

Quantitative Enzyme-linked Immunosorbant Assay (ELISA)

A TGF-β1 ELISA was used to determine the effect CsA had on urinary TGF-β1 protein levels. This was done according to the manufacturing company's (Cat no. DB100B, R&D systems) protocol. The specificity and sensitivity of the assay was assessed using 5ng of TGF-β1 as a positive control and sterile water as a negative control.

Western blot analysis

Total kidney protein was isolated using the RIPA buffer method (Sigma-Alridch, R0278) from renal homogenates according to the manufacturers protocol. The SDS-PAGE procedure used was that of Laemmli (Laemmli, 1970). Expression levels of renal proteins following CsA treatment was determined by Western blot and has been described previously (McMorrow *et al.*, 2005; Slattery *et al.*, 2005; Feighery *et al.*, 2008). Proteins of interest were detected using the following antibodies according to the manufacturers protocol, mouse monoclonal anti-α-SMA antibody (A2547 Sigma-Aldrich), mouse monoclonal anti-Cadherin 1 (CDH-1) antibody (61081, BD biosciences) or rabbit anti-CTGF polyclonal antibody (A gift from Dr. John Crean, UCD Conway Institute). In urinary western blots colloidal coomassie blue stain (Sigma-Aldrich, B8522) which stains all proteins within a polyacrylamide gel was used as a loading control to ensure equal loading of proteins.

2-dimensional gel electrophoresis (2-DE)

Identification of novel proteins in urine that may be involved in CsA induced renal fibrosis was assessed using 2DE and mass spectrometry which has been described previously (Feighery *et al.*, 2008). Briefly, urine samples were prepared for 2-DE by centrifugation at 12000g for 5 min to remove any potential contaminants. The samples were then resuspended in a 1:1 volume of lysis buffer (9.5 M Urea; 2% CHAPS; 0.8% Pharmalyte pH 3-10; 1% DTT).

Protein quantitation was measured using the Bradford assay prior to first dimension isoelectric focusing (IEF) (Bradford, 1976). The pooled urine samples were divided into 500µg aliquots for subsequent 2-DE, mass spectrometry and database analysis. This analysis was repeated at least five times.

Confocal microscopy

CDH1 localization was detected by indirect immune-fluorescence using an Alexa-546 conjugated secondary antibody (Invitrogen) (excitation 543nm/ emission 570nm). CDH-1 localization was detected by confocal microscopy (Zeiss LSM 510 Meta confocal microscope using 63x objective lens).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 4.0. Data was analysed by one-way analysis of variance (ANOVA) and multiple comparisons between control and treatment groups were made using the Bonferroni post-test. A student t-test was used for assessing statistical differences between two groups. A probability of 0.05 or less was deemed statistically significant. Results were expressed as the mean ± SEM. The following scheme was used throughout the work; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to time matched control.

RESULTS

Renal functional alterations in CD-1 mice following CsA treatment

Serum creatinine and proteinuria were assessed to determine the effects of CsA on renal function (Table 1). After 1 week CsA treatment, no significant change in either parameter was detected. After 4 weeks CsA treatment, significant increases in both serum creatinine levels and in urinary protein levels were observed compared to time-matched controls. Taken together these results suggested significant decline in renal function of CsA treated mice after 4 weeks.

Histopathological and gene expression alterations in CD-1 mice following CsA treatment

H&E and Masson trichrome staining of kidney tissue was performed to assess the effect of CsA treatment on renal histology (Figure 1A). After 1 week CsA treatment no significant changes to gross renal histology were detectable. However, after 4 weeks CsA treatment, significant histopathological alterations were observed compared to time-matched controls. These effects were particularly marked in glomerular and tubular compartments. Some renal tubules appeared disorganized, irregular in shape and separated from neighbouring tubules. Masson trichrome staining indicated marked accumulation of collagen within the tubulointerstitium after 4 weeks of CsA administration. Increased interstitial collagen has been observed in other models of TIF (Murphy *et al.*, 1999; Kattla *et al.*, 2008). The expression of fibroblast markers FSP-1 and α-SMA were assessed (Figure 1C and D). Significant increases in FSP-1 mRNA levels were detected at both 1 week and 4 weeks in CsA treated mice compared to time-matched controls. α-SMA protein levels were significantly increased in CsA treated mice at both 1 week and 4 weeks compared to time-matched controls. However, increased α-SMA mRNA levels were only detected after 4 weeks CsA treatment. This may be due to altered protein processing and/or post-translational modifications that may have resulted in increased protein without a detectable change in mRNA.

Taken together these results provide strong evidence of TIF in CsA treated mice by week 4.

The effect of CsA on mediators of TIF in CD-1 mice

The effects of CsA on TGF-β1, CTGF and BMP-7 in CD-1 mice were assessed as they are known mediators of TIF in other models (Ling *et al.*, 2003; Shihab *et al.*, 2003; Kattla *et al.*, 2008; Veerasamy *et al.*, 2009) (Figure 2). CsA treatment caused significant increases in TGF-β1 mRNA levels in whole kidney RNA after 1 week and 4 weeks treatment (Figure 2Ai). TGF-β1 protein was also significantly upregulated in both whole kidney and urine (Figure 2Aii, iii). However, in whole kidney protein, increased TGF-β1 was only detected after 4 weeks, whereas urinary TGF-β1 levels were significantly elevated in both 1 and 4 weeks CsA treated groups. The effect of CsA on CTGF mRNA levels in whole kidney RNA were similar to those on TGF-β1 with significant increases detected at both 1 and 4 weeks in the CsA groups (Figure 2B i). In contrast however, increased CTGF protein levels were detected in whole kidney at both 1 and 4 weeks but urinary CTGF protein levels were not significantly elevated until week 4 of CsA treatment (Figure 2B ii and iii). BMP-7 is a negative regulator of fibrosis. A significant reduction in BMP-7 gene expression in whole kidney RNA was detected following 4 week CsA treatment (Figure 2C). Together, these findings provide further evidence that TIF is well established in CsA treated CD-1 mice by 4 weeks. Furthermore, these results indicate that the fibrotic process has initiated by the 1 week time point. Considering these results, it was decided that urine analysis would focus on the one week treatment group since identification of significantly altered proteins at this stage would be more attractive as early nephrotoxicity indicators.

Evaluation of podocin and uromodulin as markers of CsA nephrotoxicity

Previous studies suggest that increased urinary levels of podocin, a slit-diaphragm protein, may be an early marker of glomerular damage *in vivo* (Sato *et al.*, 2009). Therefore we examined podocin in the present model. Urinary podocin was significantly increased following 1 week CsA treatment

(Figure 3A i). There was a corresponding decrease in podocin expression in whole kidney lysates after 1 week of CsA treatment (Figure 3A ii). Uromodulin (also known as Tamm-Horsfall protein) is normally detected at high levels in urine and has been proposed to play a protective role in the kidney (Prajczer *et al.*, 2010). Urinary uromodulin levels were significantly decreased following 1 week CsA treatment (Figure 3B i). Coomassie blue stain (Sigma-Aldrich, B8522) was used as a loading control to ensure equal loading of urinary proteins.

High throughput identification of novel urinary indicators of CsA nephropathy

Urine collected from the 1 week CsA treatment group and time-match controls were analysed by 2-D gel electrophoresis, mass spectrometry and database analysis to identify discriminating proteins in the urine of CsA treated mice. This method allows for the rapid identification of multiple proteins within a biological sample. Representative micropreparative gels are shown in Figure 4 A and B. Circles indicate protein spots that were excised and analysed by mass spectrometry (MS). Fifteen distinct proteins were identified by MS. A number of the peptides identified included, cadherin 1 (CDH-1) and serum albumin precursor (highlighted in Figure 4 C). All of the protein spots identified and the associated database analysis is shown in Table 2.

CsA-induced alterations in CDH-1 expression in CD-1 mice

Following proteomic investigation CDH-1 was chosen for further analysis. CDH-1 was of interest as it was the only renal tubule specific marker identified from the proteomic analysis. Since tubular dysfunction is a major mechanistic feature of CsA nephrotoxicity, urinary CDH-1 was of particular interest as a potential early indicator of tubular dysfunction. Since urinary CDH-1 levels were significantly increased, the effect of CsA on CDH-1 whole kidney protein levels was examined to determine whether the alterations in urinary CDH-1 correlated with CDH-1 levels in the kidney. Significant down-regulation of CDH-1 was observed by Western blotting at both 1 and 4 weeks (Figure 5A). This effect was also observed by

immunofluorescent microscopy where a marked reduction in the number of tubular epithelial cells expressing CDH-1 was observed following CsA treatment (4 weeks) (Figure 5B). CDH-1 staining on a 5μM kidney cross-section appeared as punctuate red staining located at the junctions, on the apical sides of the epithelial cells in the tubules. This tubular staining of CDH-1 was markedly decreased following CsA treatment and is indicated by the white arrows.

4 Discussion

Despite its nephrotoxic side-effects, CsA avoidance regimens result in increased acute rejection and these avoidance strategies have been discarded (Reis, 2010). Therefore, further elucidation of the mechanisms underlying CsA nephrotoxicity and identification of novel, early indicators of CsA nephropathy are of clinical importance. The complex and multi-factorial nature of CsA-induced nephrotoxicity makes early detection of this disease particularly challenging. In an effort to address this critical issue we established a murine model of CsA nephrotoxicity in CD-1 mice. Initially, we verified that CsA did in fact induce renal dysfunction in CD-1 mice. We examined a range of well established patho-physiological features associated with CsA nephropathy. Dieterle *et al.* have demonstrated that total proteinuria is a specific marker of glomerular injury and that in conjunction with serum creatinine measurements are excellent indicators of renal dysfunction (Dieterle *et al.*, 2010). In the current study, significantly elevated proteinuria and serum creatinine levels were evident by 4 weeks CsA treatment. Histopathological analysis revealed significant tubular atrophy and interstitial collagen accumulation in CsA treated mice by week 4. Fibroblast accumulation is also a feature of TIF (Qi *et al.*, 2006) and this was reflected by increased levels of FSP-1 and α-SMA protein in whole kidney lysates.

For the purposes of this study putative nephrotoxicity markers were designated as those identified in the literature as having a significant role in nephrotoxicity in a number of *in vitro* and *in vivo* models of renal disease. These included TGF-β1, CTGF and BMP-7 (Zeisberg *et al.*, 2003; Xu *et al.*, 2009). The profibrotic cytokine TGF-β1 is a major contributor to TIF and nephropathy (Ling *et al.*, 2003). Increased TGF-β1 mRNA and protein expression after CsA treatment suggests a role for TGF-β1 in the current model, and this is in agreement with previous studies (Shihab *et al.*, 2003; Shihab *et al.*, 2006; Lloberas *et al.*, 2008). However, despite some initially promising results in experimental models of CsA nephropathy (Ling *et al.*, 2003; El Chaar *et al.*, 2007), TGF-β1 blockade has not yet translated into an effective therapeutic strategy in human patients. Both CTGF and BMP-7 are downstream modulators of TGF-β1 signalling (Veerasamy *et al.*, 2009;

Phanish *et al.*, 2010). CTGF is pro-fibrotic and is upregulated in many models of TIF (Ito *et al.*, 1998; Gupta *et al.*, 2000; Yokoi *et al.*, 2002; Wang and Hirschberg, 2003). In contrast, BMP-7 is anti-fibrotic and is decreased in models of renal fibrosis (Turk *et al.*, 2009). The effects of CsA on CTGF and BMP-7 in the current study would likely favour the pro-fibrotic response and further underline the establishment of a fibrotic model after 4 weeks CsA treatment.

Combining the functional and histological observations with the results of gene expression studies, we concluded that 4 weeks of CsA treatment was sufficient to induce alterations consistent with CsA nephropathy. Critically, a subset of these effects were detectable by 1 week of CsA treatment suggesting that the mechanisms driving the pathophysiological response were initiating at that early time point. It was therefore determined that the 1 week time point would by the focus of further proteomic analysis of urine to identify potential markers of CsA nephrotoxicity.

In this study significant changes in urinary podocin were observed even after a short CsA treatment period, prior to gross morphological changes and severe nephrotoxicity. This is a significant and promising finding as there is emerging evidence from several experimental models and human diseases that podocyte damage and loss, can contribute to the initiation and progression of renal disease (Macconi *et al.*, 2009; Sato *et al.*, 2009; Wang *et al.*, 2009). Podocytes are specialized epithelial cells covering the basement membrane of the glomerulus and form the final barrier to serum protein loss. Podocin, a member of the stomatin family of membrane proteins, is exclusively expressed in the slit diaphragm that connects neighbouring podocytes in the glomerulus (Huber *et al.*, 2001). The slit diaphragm is a key structure involved in maintaining podocyte integrity. Measurement of urinary podocin along with other podocyte proteins has been proposed as a useful tool for detecting glomerular damage and renal disease development in other settings (Sakairi *et al.*, 2010), but this study is the first report associating podocin loss with CsA nephrotoxicity.

Uromodulin (Tamm-Horsfall protein) is the most abundant protein in normal urine (Hession *et al.*, 1987). In the present study decreases urinary Tamm-Horsfall protein levels were detected following 1 week of CsA

treatment. The biological role of Tamm-Horsfall protein is unclear, however decreased urinary Tamm-Horsfall protein is considered an indicator of tubular damage and has been proposed as a biomarker in other forms of renal disease (Kistler *et al.*, 2009; Prajczer *et al.*, 2010). Urinary Tamm-Horsfall protein levels have previously been proposed as an indicator of renal function in transplant recipients (Kaden *et al.*, 1994) and the results of this study suggest that urinary uromodulin levels may also be an indicator of CsA nephrotoxicity.

2-DE combined with mass spectrometry is a reliable high-throughput method which can be used to identify potential markers of toxicity. Using this technique, 15 urinary proteins that were significantly altered by 1 week of CsA treatment were identified (Table 2). These included Heat shock protein 60 (Hsp60), superoxide dismutase (SOD), apolipoprotein A1, mouse transthyretin and serum albumin precursor. Several isoforms of actin were also detected along with vinculin, another cytoskeletal protein. Further analysis is required to determine the specific roles of these proteins and their usefulness as indicators and/or therapeutic targets in CsA-nephropathy. One protein, CDH-1, was selected for further analysis. CDH-1 was increased in the urine of CsA treated mice with a corresponding decrease in whole kidney protein expression by 1 week of CsA treatment. CDH-1 is a critical epithelial adhesion molecule that is highly expressed along the tubular epithelium. Urinary loss of CDH-1 suggests breakdown of the tubular junctions likely leading to loss of tubular integrity and function (Slattery *et al.*, 2005). Increased urinary CDH-1 has been detected in patients with diabetic nephropathy has been suggested to have clinical diagnostic value in that setting (Jiang *et al.*, 2009). We propose that urinary CDH-1 may also be an early indicator of CsA nephropathy.

The results from this current study identify a number of novel aspects of CsA nephrotoxicity, highlighting roles for both the tubular epithelium and glomerular cells in establishing the pro-fibrotic environment. This research has identified a number of novel potential markers of CsA nephropathy. Further investigation will determine if these indicators translate to a clinical setting, and to what extent they may be useful as therapeutic targets.

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Conflict of Interest

The authors have no conflict of interest. The results presented in this manuscript have not been published previously in whole or part, except in abstract form.

FIGURE LEGENDS

Table 1: Functional alterations in CD-1 mice following CsA treatment.
CD-1 mice were treated intraperitoneally with 15 mg/kg/day cyclosporine A or 1ml/kg vehicle control for one week or four weeks. (A) Serum creatinine and proteinuria were used as indicators of renal function. Results are expressed as mean ± S.E.M. N = 6 for each treatment group. * indicates statistically significant difference to time matched control (* $p < 0.05$, *** $p < 0.001$).

Table 2: Proteins Identified by mass spectrometry and database analysis
The above table contains the protein spots identified by mass spectrometry and uniprot database analysis. CD-1 mice were treated with either cyclosporine A or vehicle control for one week. Mouse urine was collected and subjected to 2D PAGE and subsequent gels visualized and analysed using Progenesis software. Protein spots were then identified following mass spectrometry on a Finnigan LTQ mass spectrometer connected to a Surveyor chromatography system. Raw mass spectrometry data was analysed using Proline, a proteomics analysis platform and the International Protein Index (IPI) for mouse version 3.16 used as the FASTA search database. A second platform was also used in which MS/MS spectra were analysed using TurboSEAQUEST. A full description of this process is described by Feighery et al. (2008).

Figure 1 Histopathological alterations in CD-1 mice following CsA treatment. CD-1 mice were treated with either CsA (15mg/kg/day) or vehicle (1ml/kg/day) control for one week or four weeks. (A) Kidney tissue was fixed, sectioned and stained with H&E (1A; top panels) or with Masson's Trichrome stain (1A, bottom panels). Representative images are shown (n= 6 for each group) (Magnification 200X). Renal tubules appeared disorganized, irregular in shape and separated from neighbouring tubules (top row indicated by black arrows). Masson trichrome staining indicated marked accumulation of collagen (blue colour) within the tubulointerstitium after 4 weeks of CsA administration (bottom row indicated by black arrows). RNA and protein were extracted from each kidney. (B) FSP-1 and α-SMA gene expression levels

were examined by quantitative PCR. (C) α-SMA protein expression was examined by Western blot analysis. Results are expressed as mean ± S.E.M (n=6). * indicates statistically significant difference to time matched control (* p< 0.05, ** p< 0.01, *** p< 0.001).

Figure 2: Effect of CsA on mediators of TIF in CD-1 mice. CD-1 mice were treated with CsA or vehicle alone for one week or four weeks. Kidneys from each group were harvested, and RNA and protein were extracted. TGF-β1 gene and protein expression was examined by quantitative PCR and ELISA (2A i and ii). TGF-β1 protein expression in urine was examined by ELISA (2A iii). CTGF gene and protein expression was examined by quantitative PCR and Western blotting (B i and ii respectively). CTGF protein expression in urine was examined by Western blot analysis (B iii). Coomassie blue stain was performed to ensure equal protein loading. BMP-7 gene expression was examined by quantitative PCR (C). Results are expressed as mean ± S.E.M of a sample size of six mice per treatment group. * indicates statistically significant difference to time matched control (* p< 0.05, ** p< 0.01).

Figure 3: Evaluation of podocin and uromodulin as markers of CsA nephrotoxicity. CD-1 mice were treated with CsA or vehicle alone for one week. Urine collected from mice at the end of the treatment period. Kidneys from each group were harvested, and RNA and protein were extracted. Podocin protein levels were examined by Western blot analysis in urine (3A i), and in whole kidney protein lysates (3A ii). Uromodulin protein levels was examined by Western blot analysis in urine (3B i). Results are expressed as mean ± S.E.M of a sample size of six mice per treatment group. * indicates statistically significant difference to time matched control (* p< 0.05, ** p< 0.01).

Figure 4: High throughput identification of novel urinary indicators of CsA nephropathy. CD-1 mice were treated with either cyclosporine A or vehicle control for one week at which time urine was collected. Urinary proteins were separated by 2DE using a pH 3-10 gradient (4A - control, 4B –

CsA). Circles indicate expression and locations of spots identified by mass spectrometry and UniProt database analysis (see supplementary table for details). Images are representative of five analytical gels performed in duplicate.

2-DE images of selected up-regulated and down-regulated proteins following CsA treatment (C). Spots of interest are indicated by the arrows. The images on the top represent urine samples from control mice subjected to 2-DE. The images on the bottom represent the corresponding spot location in urine from mice treated with CsA.

Figure 5: CsA-induced alterations in CDH-1 expression in CD-1 mice. CD-1 mice were treated with CsA or vehicle alone for one week or four weeks. Kidneys from each group were harvested and total kidney protein extracted. CDH-1 protein expression was examined by Western blot analysis (A) and in kidney sections by immunofluorescent microscopy (B) (Magnification 400X). Results are expressed as mean ± S.E.M of a sample size of six mice per treatment group. * indicates statistically significant difference to time matched control (* $p < 0.05$, ** $p < 0.01$).

Table 1: Functional alterations in CD-1 mice following CsA treatment.

	1 week Control	1 week CsA	4 week Control	4 week CsA
Proteinuria (mg/24hour)	3.24 ± 0.19	3.94 ± 0.41	2.86 ± 0.04	5.34 ± 0.26***
Serum Cr (mg/dl)	0.43 ± 0.02	0.34 ± 0.04	0.56 ± 0.04	0.74 ± 0.03*

Table 2: Proteins identified by MS.

Protein Identification	accession no.	M/W (kDa)	% Sequence coverage	ID scores	Spot Intensity	Fold change
Up-regulated proteins						
1. Cadherin 1 precursor	P09803	98.2	12.3	1.6e-004	46.1 ± 2.4	2.00
2. Serum albumin precursor	P07724	68.6	24.5	3.6e-005	71.5 ± 3.3	2.47
3. Actin 11	P53496	41.6	14.0	1.4e-009	61.6 ± 3.9	2.75
4. Kallikrein K1 precursor	P15947	28.7	31.6	7.7e-008	63.9 ±5.9	2.11
5. Vinculin	Q64727	116.6	1.8	2.4e-004	13.1± 1.8	2.86
6. Apolipoprotein A1 precursor	Q00623	30.5	4.4	3.4e-007	9.9 ± 0.4	3.69
7. Voltage dependent anion selective channel protein 1	Q60932	32.3	14.7	3.7e-009	9.4 ± 1.3	3.74
8. Major urinary protein 1	P11588	20.6	69.7	2.5e-010	9.8 ± 2.5	4.75
9. Actin 1	P60710	41.7	6.0	3.8e-009	3.1 ± 0.5	8.49
10. Actin 2	Q96292	41.9	6.22	4.0e-007	1.5 ± 0.05	16.81
11. Mouse transthyretin	P07309	15.7	5.9	1.7e-004	0.7 ± 0.1	65.49
12. Mouse superoxide dismutase	P08228	15.9	14.6	2.4e-006	0.4 ± 0.07	70.88
13. Actin 4	P53494	41.8	13.9	1.8e-005	1.7 ± 0.2	43.72
14. Major urinary protein 11	P09438	17.5	82.0	1.5e-009	48.5 ± 3.1	0.99
Down-regulated proteins						
15. Heat shock protein 60	P63038	60.9	1.6	9.4e-004	44.7 ± 3.0	35.35

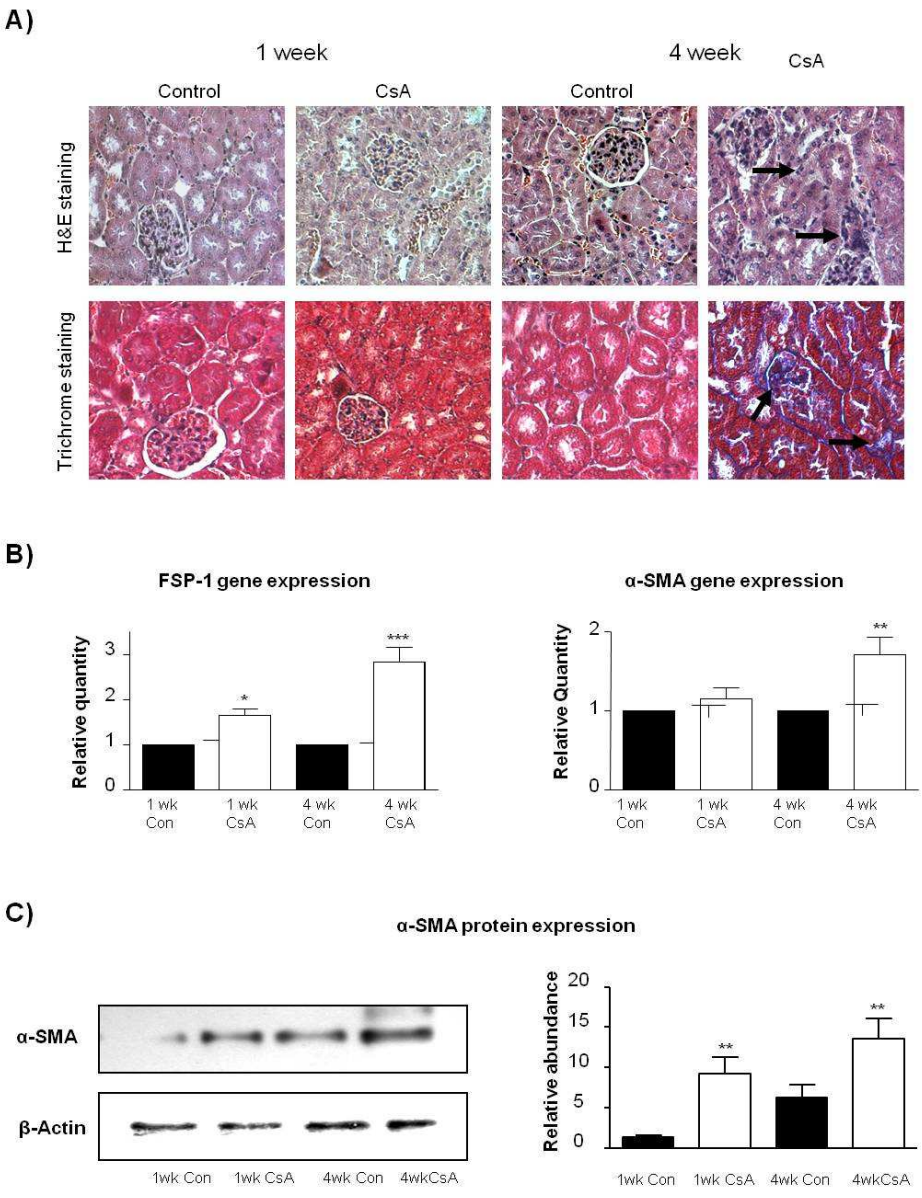

Figure 1: Histopathological alterations in CD-1 mice following CsA treatment.

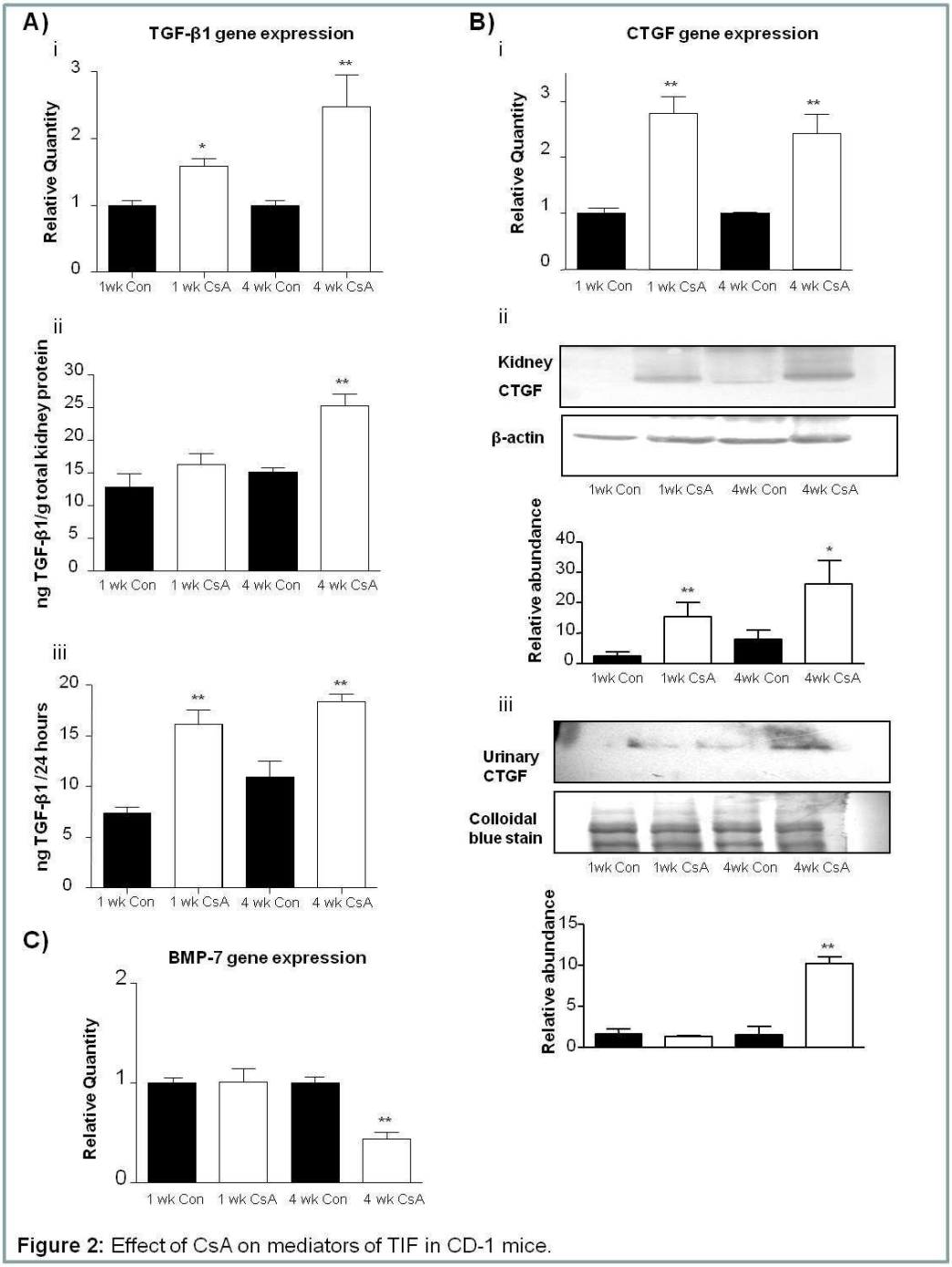

Figure 2: Effect of CsA on mediators of TIF in CD-1 mice.

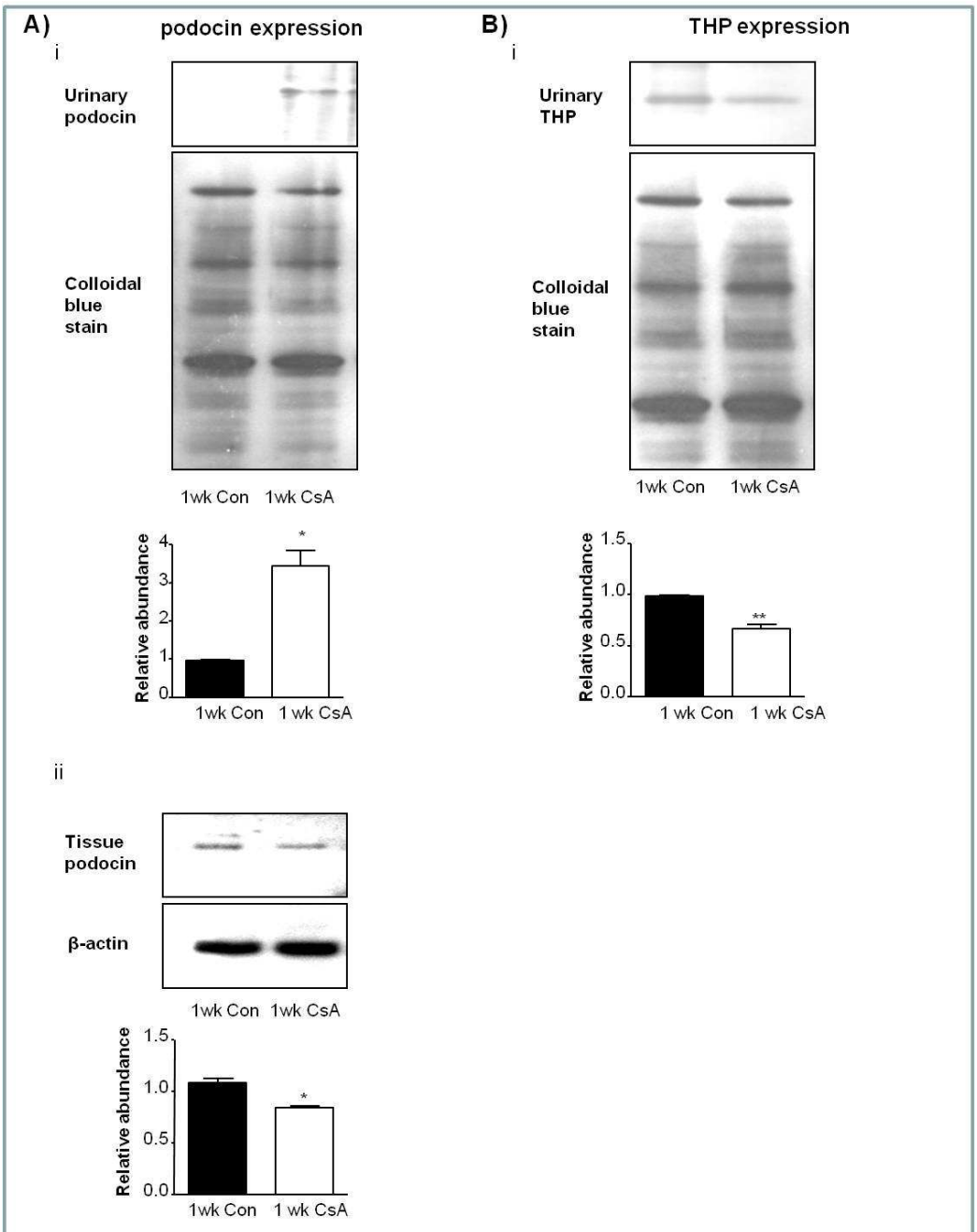

Figure 3: Evaluation of podocin and uromodulin as markers of CsA nephrotoxicity.

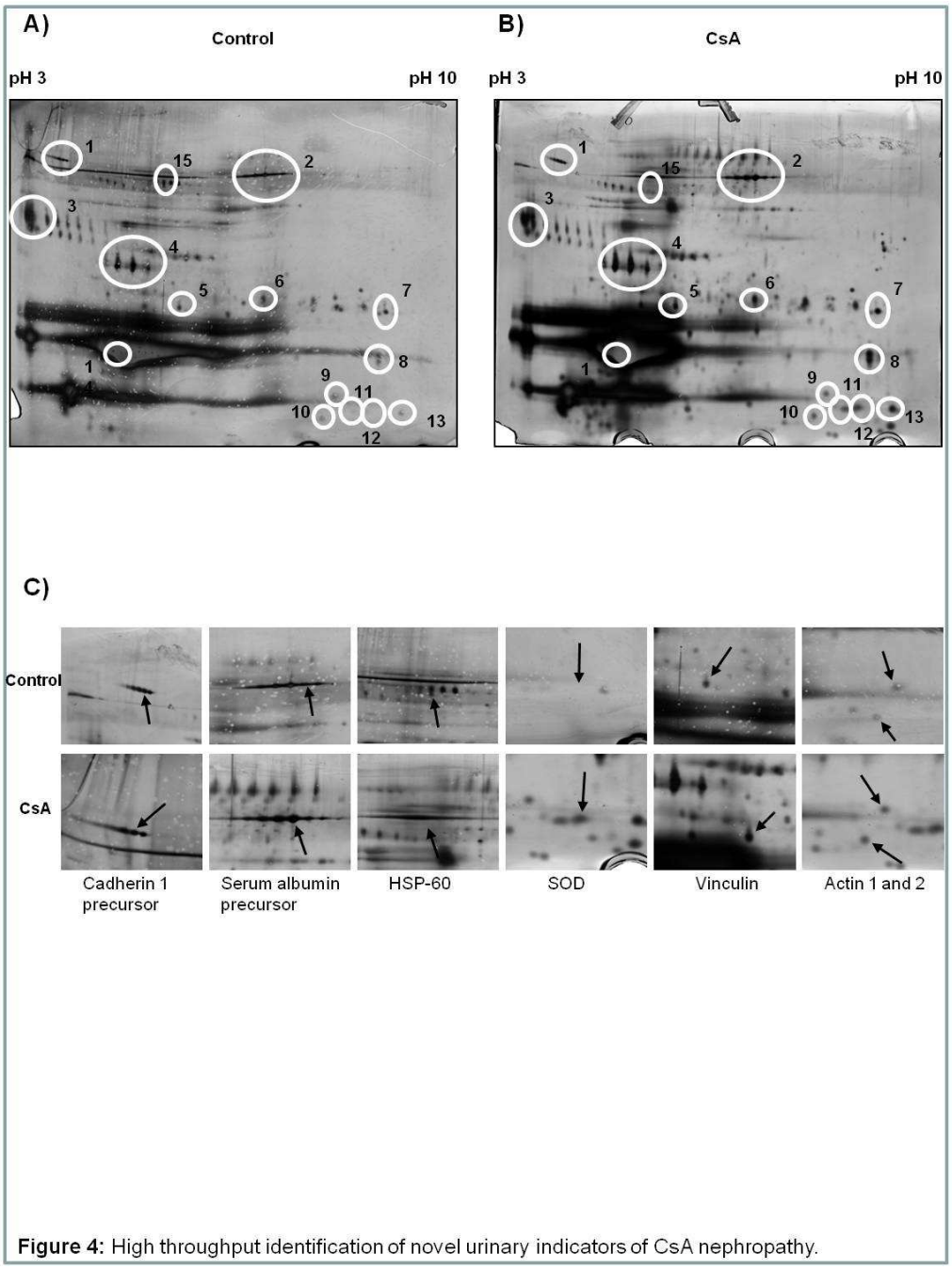

Figure 4: High throughput identification of novel urinary indicators of CsA nephropathy.

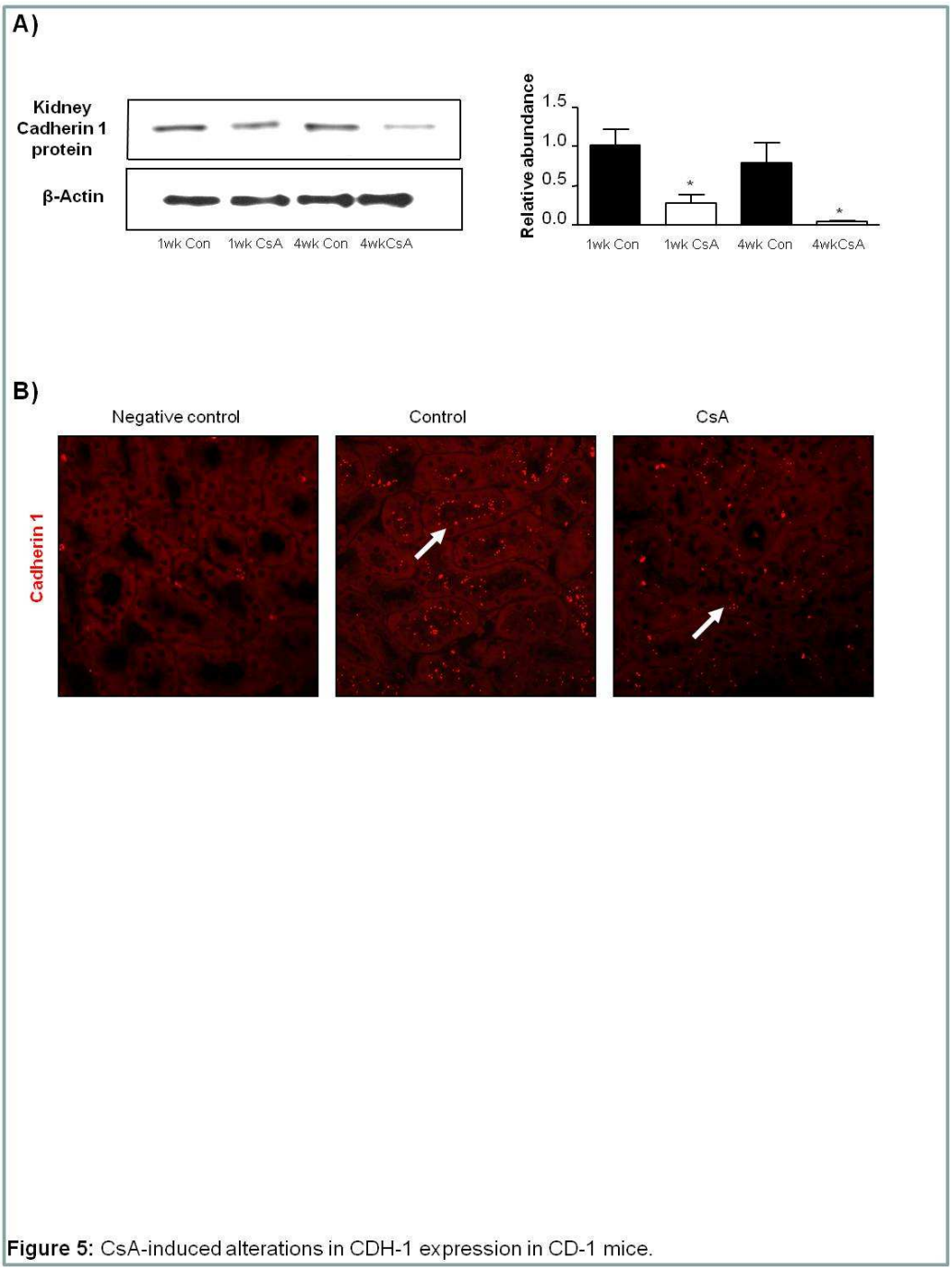

Figure 5: CsA-induced alterations in CDH-1 expression in CD-1 mice.