Cystinosis Research Foundation Final Progress Report

Title: ***Cysteamine effects on extracellular matrix accumulation in chronic kidney disease.***
Grant number: 412560090101
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Progress Report: December 31, 2010 to June 30, 2011

OVERVIEW

The project has progressed according to the original research plan with the most significant findings detailed in Aim 2 demonstrating the anti-fibrotic potential of cysteamine. Current efforts are focused on the investigating the effect of cysteamine on myofibroblasts, the primary cell producing extracellular matrix during kidney fibrosis. Furthermore, in our investigation of fibrogenic mechanisms in nephropathic cystinosis, we have discovered that *CTNS-/-* macrophages are skewed to a pro-fibrotic phenotype and is the subject of our recent CRF grant submission.

1) Aim #1: *Searching for a better mouse model of cystinosis-associated nephropathy:*

We hypothesized that the rate of progression of the renal phenotype in *Ctns-/-* mice may be attenuated by the endogenous expression of cysteamine synthesized via an enzymatic pathway that is encoded by the vanin-1 *(VNN1)* gene. We generated a colony of *Ctns/Vnn1* double knock-out mice. Similar to the study published by Dr. Antignac and colleagues on the *Ctns-/-* mice (*Nephrol Dial Transplant. 25; 2010*), we found significantly increased rates of polyuria in our double knock-out mice at 6 months, however, the difference diminished at 9 months and there was no difference in weight. There was a significant increase in BUN at 10 months in the *Ctns/Vnn1* double knock-out mice compared to heterozygous controls (*Heterozygous controls vs. Double knock-out,* =8/group: 17.6 ± 1.0 vs. 22.7 ± 1.4 mg/dL, *P* =0.01). Masson trichrome staining of the *Ctns/Vnn1* double knock-out did demonstrate glomerulosclerosis and mild interstitial fibrosis at 12 months. However, more in-depth fibrosis analysis (Total collagen, picrosirius red staining, Masson trichrome, and BUN) of the *Ctns/Vnn1* double knock-out mice did not suggest any increase in renal fibrosis with the addition of the *Vnn1* gene deletion compared to *Ctns-/-* mice. As discussed previously, this may be attributable to issues with the *Vnn1-/-* mice not being on a pure C57BL/6 background (inadvertent error by the sending institution, Dr. Terkaltaub-UCSD). We further investigated the role *Vnn1* in renal fibrosis through our unilateral ureteral obstruction (UUO) model in 8-week old *Vnn1-/-* male mice and demonstrated that *Vnn1*-deficiency alone does not alter the degree of kidney fibrosis after UUO. However, there was a non-significant trend toward more fibrosis in *Vnn1-/-* at advanced stages after UUO (day 21) and therefore future studies using a *Vnn1*-/- mice on a pure C57BL/6 background may be more promising.

Based on recent publications by Dr. Stephanie Cherqui's group suggesting the importance of interstitial macrophages (Mϕ) in nephropathic cystinosis and the inherent nature of these cells as professional phagocytes, we hypothesized that *Ctns*-/- Mϕs are genetically programmed to execute a more aggressive fibrotic response to renal injury (such as tubular apoptosis) than normal *Ctns*+/+ Mϕs that serves an essential role in the pathogenesis of cystinosis-associated chronic kidney disease. We have followed a cohort of *Ctns*-/- mice on a C57BL/6 background in our laboratory for 12+ months and documented that between 3 and 12 months of age both total kidney collagen significantly increased 2.3-fold and corresponded to an increase in F4/80+ interstitial Mϕs (Figure 1). When 3-month old *Ctns*-/- mice were subjected to UUO, they

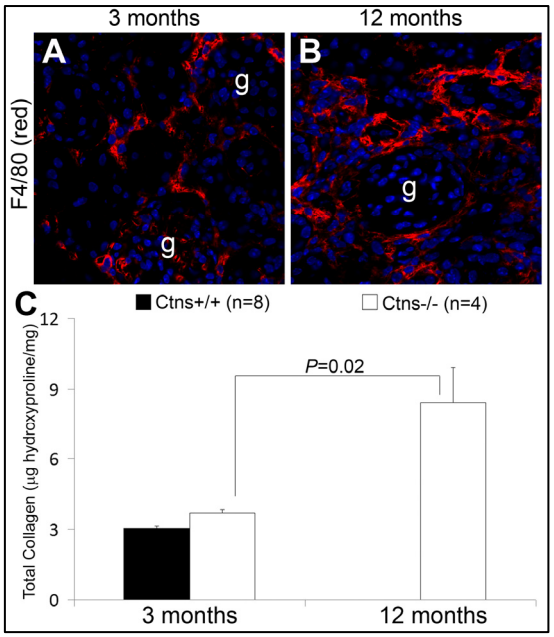

Figure 1: Macrophage infiltration in nephropathic cystinosis correlates with CKD progression. (A, B) Representative confocal images illustrate the increase in F4/80+ (red) interstitial macrophage infiltration in cystinotic kidneys with age. (C) Total collagen increases with age and correlates with deterioration of kidney function. (g = glomeruli)

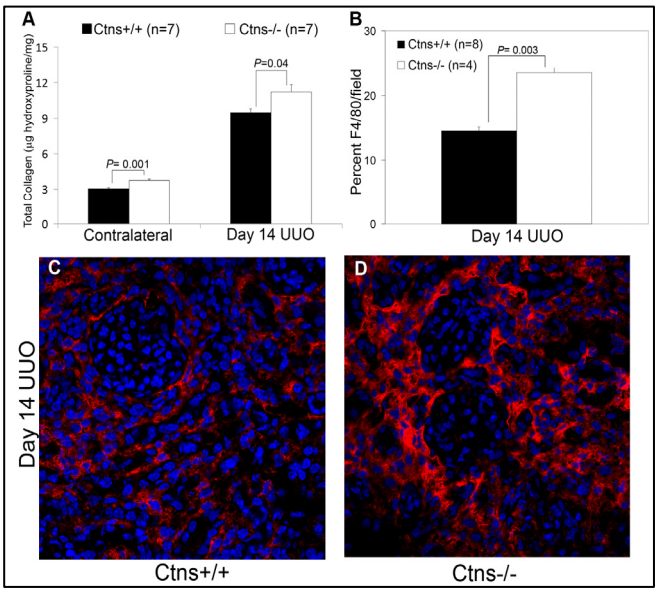

Figure 2: *Ctns*-deficiency leads to more severe fibrosis with increased macrophage infiltration in experimental CKD. Unilateral ureteral obstruction (UUO) was performed in 3 month old mice. (A) Total collagen levels were significantly increased in *Ctns*-/- UUO kidneys. (B-D) Graph summarizes quantification of the significant increase in F4/80+ area in *Ctns*-/- UUO kidneys and representative confocal micrographs below.

developed significantly worse fibrosis (19% higher) with more F4/80+ interstitial Mϕs (63%) (Figure 2). The expression pattern of cystinosin in chronically damaged kidneys has not been previously investigated. In the UUO model induced in *Ctns*+/+ C57BL/6 mice, we found by semi-quantitative real-time RT-PCR that *Ctns* gene expression initially declined (days 3 and 7), likely reflecting tubular injury, then increased at day 14, consistent with an influx of cystinosin+ Mϕ (Figure 3A); *in situ* hybridization and/or immunostaining studies will be necessary to confirm these assumptions. Using wild-type peritoneal Mϕ, *Ctns* mRNA expression was confirmed and shown to increase 4-fold after phagocytosis of apoptotic proximal tubular cells (Figure 3B).

In order to perform cytokine profiling studies to elucidate differences in macrophage function between *Ctns*+/+ and *Ctns*-/- Mϕ, with the assistance of Dr. Jeremy Duffield (University of Washington) we have developed antibody-based methods to isolate kidney CD11b+ Mϕ by both the AutoMACS® magnetic bead system and by flow cytometry (FACS). We have also developed an *in vitro* model to investigate the effects of apoptotic tubular cells on macrophage activation.

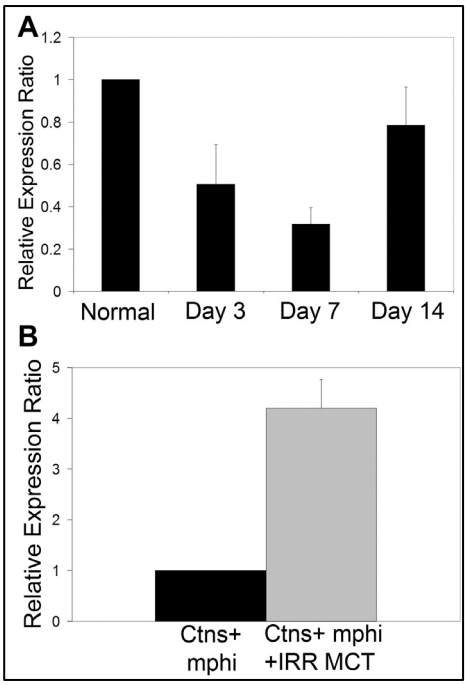

Figure 3: *Ctns* expression after UUO and after macrophage efferocytosis. Semi-quantitative real time RT-PCR was performed in kidneys and macrophages from *Ctns*+/+ mice and normalized to GAPDH. (A) Abundant *Ctns* expression was demonstrated in the contralateral kidney that decreased with UUO until advanced time points. (n=4/group) (B) Thioglycollate peritoneal macrophages were co-cultured with apoptotic renal tubular cells (+IRR MCT) and RNA isolated after 24 hours, demonstrating a large increase in macrophage *Ctns* expression.

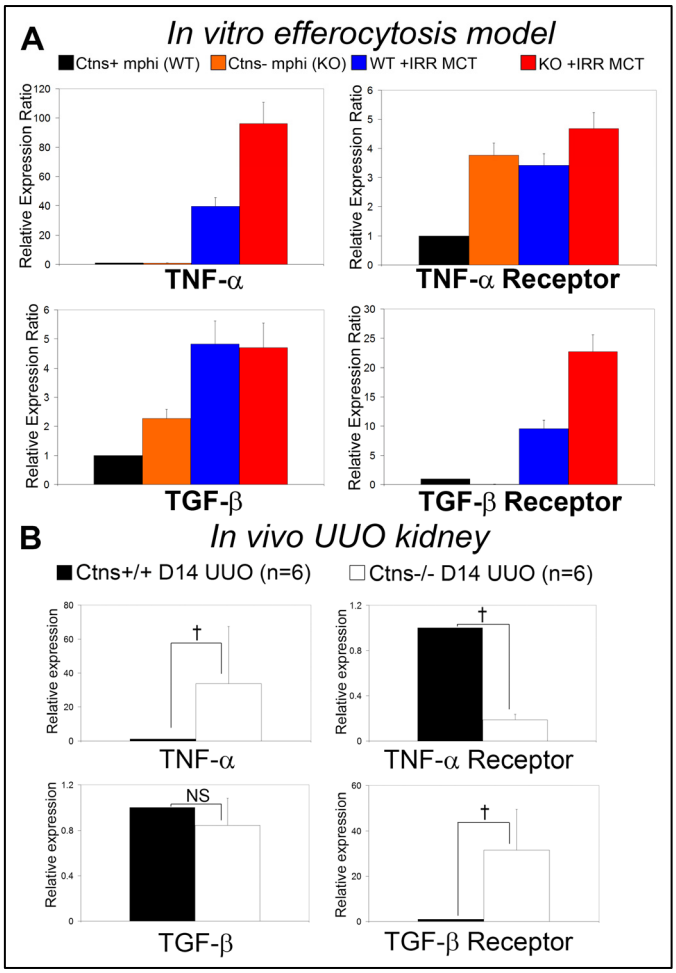

Figure 4: Dysregulated M1/M2 signaling in *Ctns* macrophage and UUO kidneys. Semi-quantitative real time RT-PCR was performed on RNA extracted from UUO kidneys and normalized to GAPDH and 18S. (A) Thioglycollate peritoneal macrophages were co-cultured with apoptotic mouse cortical tubular cells (MCT) cells for 24 hours in serum free media. Cells were harvested and RNA extracted for semi-quantitative real time RT-PCR normalized to GAPDH. Both TNF-α (M1) and TGF-β receptor (M2) were strongly up-regulated in *Ctns*-/- macrophages after efferocytosis compared to *Ctns*+/+ macrophages alone. (B) Graphs summarize the relative cytokine expression levels to *Ctns*+/+ UUO kidneys. Similar to our in vitro data, there was a signficant increase in both TNF-α (M1) and TGF-β receptor (M2) expression. († P<0.01, NS=not significant, n= 6/group).

Although tubular cell apoptosis is the most detrimental consequence of kidney fibrosis and correlates closely with functional decline, most nephropathic cystinosis studies have focused on the pathogenesis of tubular apoptosis, while less attention has been paid to the downstream effects that are triggered following their phagocytic clearance. In preliminary *in vitro* studies, we found that tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β receptor mRNA levels were significantly higher in *Ctns*-/- peritoneal Mϕ compared to *Ctns*+/+ Mϕ after incubation with apoptotic tubular cells for 24 hours (Figure 4A). Of note, similar cytokine differences were observed in *Ctns*-/- kidneys 14 days after UUO compared to *Ctns*+/+ kidneys (Figure 4B). Based on these data, we have recently submitted a CRF grant to investigate the role of *Ctns-/-* Mϕs in nephropathic cystinosis and chronic kidney disease.

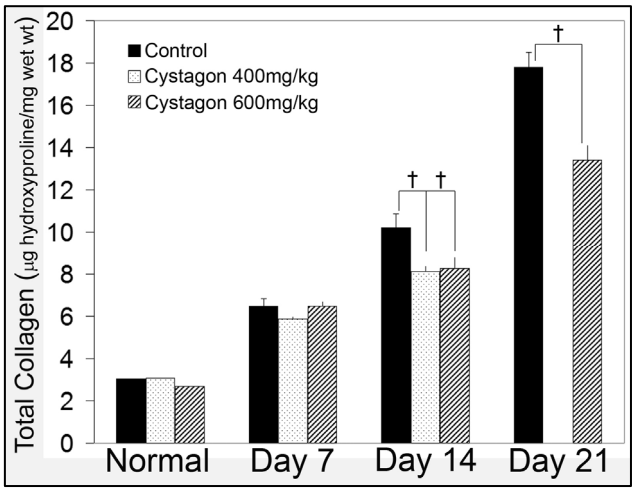

Figure 5: Cystagon attenuates renal fibrosis severity. Total kidney collagen as measured by hydroxyproline concentration was significantly decreased by 20-25% at day 14 and confirmed by Sirius red staining. † *P*<0.01, *n*= 8-10/group.

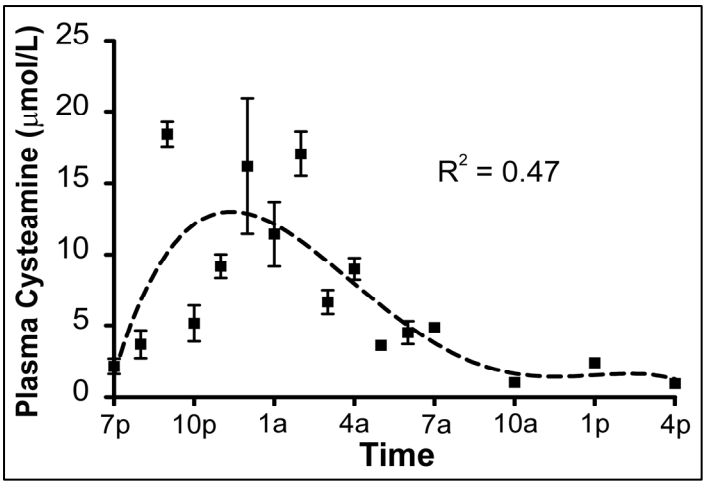

Figure 6: Plasma cysteamine levels in high dose Cystagon treated mice. C57BL/6 mice were placed on 600mg/kg of Cystagon in their drinking water changed daily for 3 days prior to sacrifice. Plasma cysteamine levels were measured by mass spectrometry. *n*= 4/group.

2) Aim #2: *Investigating the efficacy of cysteamine therapy for interstitial renal matrix protein reduction in chronic kidney disease and to determine its mechanism of anti-fibrotic action:*

Based on the findings from a pilot study, the effect of cysteamine (administered as Cystagon® added to the drinking water that was freshly made every 24h) on the degree of renal fibrosis was investigated using two doses: 400 and 600 mg/kg/day. Groups of mice (n = 8 at each time-point) were studied 3, 7, 14, and 21 days after the onset of chronic injury induced by UUO. Both doses were shown to significantly reduce kidney collagen levels by day 14 by 21 percent and by 25 percent at day 21 after UUO in the high dose Cystagon® group compared to the untreated group (Figure 5). Due to the high doses of Cystagon used in this study compared to typical doses in humans, we measured serum cysteamine levels of Cystagon® in mice by mass spectrometry in collaboration with Dr. Jon Gangoiti and Dr. Bruce Barshop at UCSD. Our initial studies on plasma levels of cysteamine taken at the time of sacrifice were low in the Cystagon® treated mice at day 14 after UUO (400mg/kg – 0.81 ± 0.09μmole/L; and 600mg/kg – 1.04 ± 0.15μmole/L; levels were undetectable in the vehicle alone group). This was thought to be attributable to the short half-life of cysteamine and the nocturnal feeding habits of the mice. Therefore, we performed a more detailed analysis on the high dose Cystagon group (600mg/kg) with plasma levels taken every hour during the evening (for 12 hours) and every four hours during the day (n=4/group). Our pharmacokinetic data confirms that the higher serum levels of cysteamine are achieved at night and lower levels during the day (Figure 6). In addition, C_{max} levels in mice were found to be between 15-20μmol/L, which is similar to the levels reported in humans (Dohil R et al, J Pediatr, 2006, 148:718-9). More formal area under curve analyses will be performed.

Kidney tissue studies are currently focusing on elucidating the mechanism by which Cystagon® reduces kidney fibrosis. One potential pathway may be its ability to reduce collagen synthesis rates. Extracellular matrix (ECM) gene mRNA levels were measured by semi-quantitative real-time qPCR in both doses at 3, 7 and 14 days after UUO. ECM gene transcription levels were significantly down-regulated in UUO kidneys of cysteamine-treated

mice: procollagen I mRNA levels were 56 percent lower in the mice treated with 600 mg/kg at day 14; and at day 7, despite no difference in total collagen, there was a nearly 40 percent reduction in kidney fibronectin and procollagen III mRNA levels in mice treated with 400mg/kg and a nearly 60 percent reduction in fibronectin, procollagen I and procollagen III at higher doses of cysteamine (600mg/kg). No difference was seen at day 3 in either dose of Cystagon®. The peak effect of Cystagon® on ECM synthesis was observed at 7 days in both treatment doses.

The interstial myofibroblast is the primary matrix-producing cell in response to kidney injury. We examined myofibroblast accumulation after UUO by measuring the expression of α-smooth muscle actin (α-SMA) a marker of myofibroblast activation. We found that there was a significant reduction in α-SMA + myofibroblasts in both doses at day 7 and day 14 after UUO (P<0.01); the largest reductions of 38% and 47% were seen at day 7 in the 400mg/kg and 600mg/kg group respectively (day 14: 400mg/kg – 24%; and 600mg/kg – 33%; Figure 7). In addition, there was a significant reduction in interstitial macrophage infiltration by 34% in mice treated with 600mg/kg/day (no difference was seen at day 7). Taken together, these data suggest that Cystagon® attenuates fibrogenic pathways primarily through modulating myofibroblast activation/proliferation but may have a secondary effect on macrophage accumulation. Further studies are currently underway to determine the mechanisms by which Cystagon® modulates myofibroblast behavior.

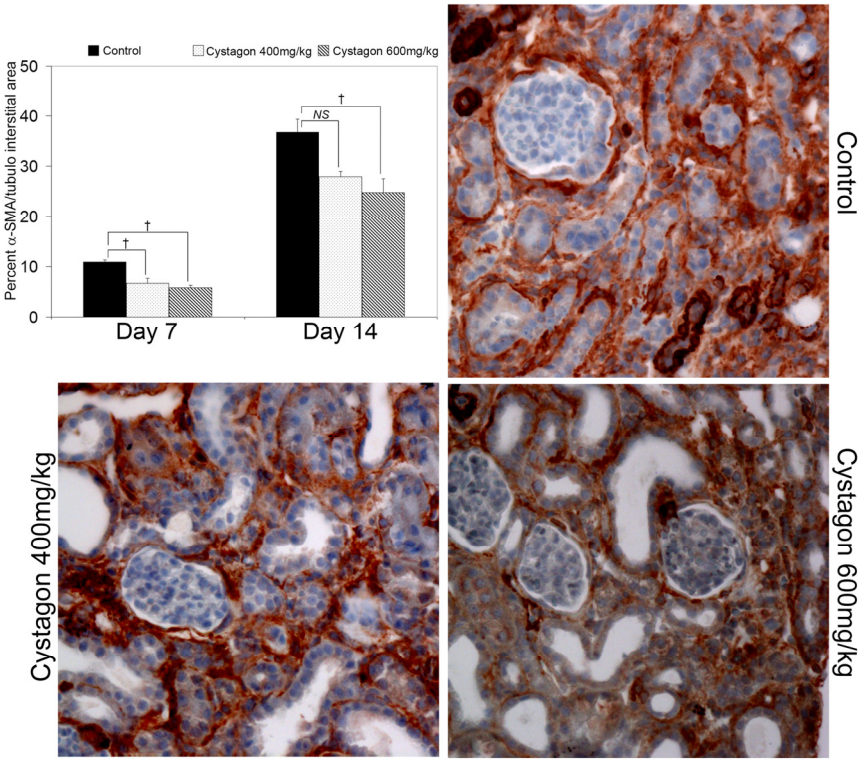

Figure 7: Myofibroblast accumulation attenuated in Cystagon treated mice after UUO. Representative a-SMA stained immunohistochemical photomicrographs (400x) are shown and graph in upper right panel summarizes the results of α-SMA-positive interstitial staining. Results are expressed as mean± SEM. NS – nonsignificant; †P<0.01.

Based on the above findings, we investigated pro-inflammatory and pro-fibrotic cytokine expression patterns at day 7 and day 14 in total kidney homogenate by semi-quantitive real time qPCR. The mRNA levels of the profibrotic cytokine TGF-β and the TGF-β receptor was significantly down-regulated by 47 percent and 64 percent, respectively, at day 14 at high doses of Cystagon® compared to control mice (*P*<0.05). Interestingly, at day 7 after UUO both TGF-β and the TGF-β receptor were significantly up-regulated by approximately 60 percent at high doses of Cystagon® compared to control mice (*P*<0.01), and suggests that the down-regulation of ECM gene transcription is TGF-β independent. There was no difference in expression levels of IL-1β, TNF-α, IL-6, Gal-3, and Endo180 at day 14. Interestingly, we did find a 1.9- and 2.3-fold increase IL-1β at day 7 in the 400mg/kg and 600mg/kg doses, respectively, however, there

was no difference in the remainder of the cytokines examined. Taken together, these data suggest that Cystagon® modulates interstitial cells independent of major cytokine modulation.

3) Aim #3: *To investigate the effect of cysteamine on apoptosis of renal tubular epithelial cells, oxidant stress, and other novel target pathways of chronic kidney disease.*

As originally proposed, the effects on apoptosis, glutathione activity and novel cysteamine targets will be the primary focus of these studies. Initial attempts to measure glutathione levels were fraught falsely elevated levels likely due to *ex vivo* oxidation. Therefore, we elected to measure total redox status during chronic kidney injury with total kidney thiol content, as a measure of antioxidant status. Total kidney thiol content in UUO tissue was significantly decreased 40 percent compared to the contralateral kidney in control dose mice (contralateral vs. UUO, n=5-6/group: 1397 vs. 838 mM thiol, $P<0.01$). At day 7 after UUO, total kidney thiol content remained at levels close to that of the contralateral kidney in the high dose Cystagon® treated mice compared to control (Figure 8). Currently, we are measuring oxidative stress through a carbonyl protein ELISA. In addition, there was no difference in the modulation of the oxidant and anti-oxidant genes *Nox2*, *Nox4*, and *SOD1* by semi-quantitative real time qPCR at day 7 and day 14. Future studies will investigate the specific thiol-dependent pathways modulated by cysteamine in myofibroblasts and macrophages during chronic kidney injury.

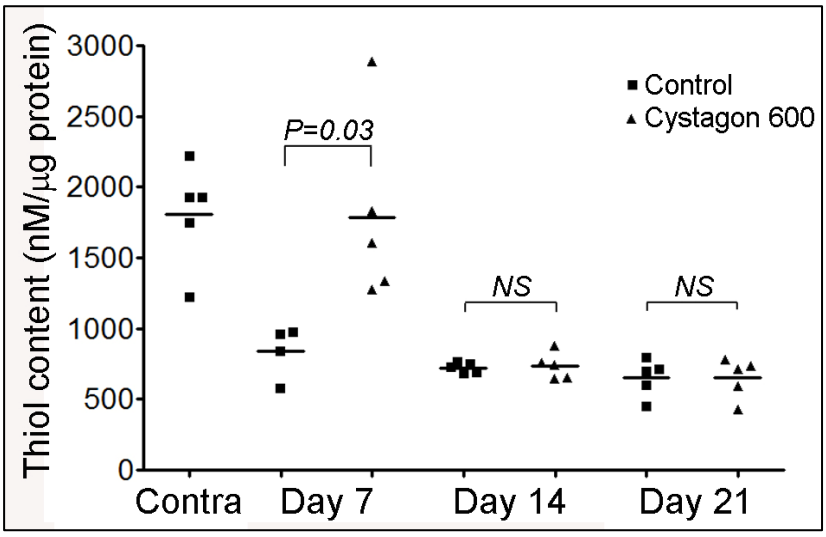

Figure 8. **Cysteamine modulates antioxidant status at early timepoints.** Contralateral and UUO kidneys from control and high dose Cystagon (600mg/kg) treated mice were processed in antioxidant buffer and analyzed for total thiol content (Measure iT – thiol assay kit, Invitrogen). Thiol content normalized to total protein. *n*= 5/group.

CONCLUSION

In summary, the data generated from this CRF grant suggest that Cystagon® may have significant anti-fibrotic effects that are applicable to all patients with chronic kidney disease in addition to patients with nephropathic cystinosis. Ongoing studies are planned to identify the anti-fibrotic mechanisms modulated by cysteamine during chronic kidney injury. In addition, new insights into the pathogenic effect of Ctns-/- macrophages based on our preliminary findings require further investigation.

BUDGET EXPENDITURES

January 1, 2010 - June 30, 2011

Category	Award *	Prior expenditures **	Expenditures this period ***	Anticipated expenditures ****	Total expenditures this period	Total expenditures to date	Remaining balance
Personnel	$ 181,099	$ 124,981	$ 28,044	$ 1,829	$ 29,873	$ 154,854	$ 26,245
Animals /Maintenance	$ 17,000	$ 21,320	$ 2,943	$ 550	$ 3,493	$ 24,813	$ (7,813)
Supplies	$ 40,181	$ 32,448	$ 7,045	$ 368	$ 7,413	$ 39,861	$ 320
Travel	$ 4,636	$ 34	$ -	$ -	$ -	$ 34	$ 4,602
Subtotal	$ 242,916	$ 178,783	$ 38,032	$ 2,748	$ 40,780	$ 219,563	$ 23,353
IDC	$ 24,292	$ 17,879	$ 3,803	$ 275	$ 4,078	$ 21,957	$ 2,335
Total	$ 267,208	$ 196,662	$ 41,835	$ 3,023	$ 44,858	$ 241,520	$ 25,688

* Award amount for 07/01/08-06/30/11
** Prior expenditures: 07/01/08-12/31/10
*** Expenditures this period: 01/01/11-06/29/11
*** Anticipated expenditures: 06/29/11-06/30/11

10% budget remaining