

## Cystinosis Research Foundation Progress Report

Title: ***Cysteamine effects on extracellular matrix accumulation in chronic kidney disease.***

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Funding Period: July 1, 2008 to June 30, 2011

Progress Report: July 1, 2010 to December 31, 2010

### OVERVIEW

The project is progressing according to the original research plan. The three mouse lines are all actively breeding (*Ctns*-/-, *vanin*-/- and *Ctns*-/-*vanin*-/- double knockouts). The animal work for the first cysteamine *in vivo* study has been completed and tissue analyses continue. We have sacrificed the cohort of 8-10 *Ctns*-/-*vanin*-/- double knockout and heterozygous control mice at 15 months of age. We are in the process of analyzing extracellular matrix deposition in the double knockout mice, heterozygous controls, and comparing them to the *Ctns*-/- mice. We are making excellent progress in our investigation of the anti-fibrotic mechanisms of Cystagon in progressive chronic kidney disease.

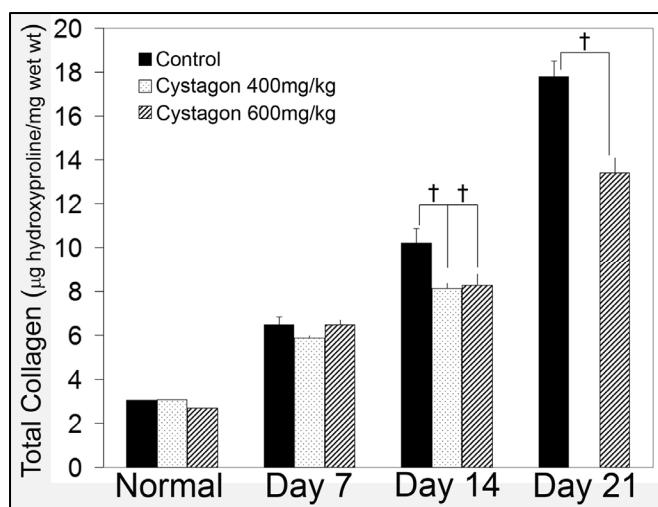
#### 1) Aim #1: Searching for a better mouse model of cystinosis-associated nephropathy:

We hypothesize 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* gene. We have generated a colony of *Ctns*-/- *Vanin*-1-/- double knock-out mice. Similar to the study published by Dr. Antignac and colleagues (*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 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 did suggest an increase in glomerulosclerosis and mild interstitial fibrosis at 12 months in the double knock-out mice. We are in the process of measuring serum creatinine levels by HPLC at 12 and 15 month old double knockout and heterozygous controls. We will also examine the histology of the double knock-out, heterozygous littermate controls, and *CTNS*-/- mice at 15 months for proximal tubules (lotus lectin), picrosirius red, in addition to PAS and trichrome stains.

Findings from a study of the unilateral ureteral obstruction (UUO) model in 8-week old male mice, comparing the degree of fibrosis between littermate controls (*Vanin*+/-) and *Vanin*-/- mice, suggest that vanin-deficiency alone does not alter the degree of kidney fibrosis. However, as reported in our last progress report there was a non-significant trend toward more renal fibrosis in *Vanin*-/- at day 21 after UUO (*Vanin*+/- vs. *Vanin*-/-). We did note that values for total collagen were much lower than typically seen in C57BL/6 mice and discovered that we did not receive the *Vanin*-/- mice on a pure C57BL/6 background as originally thought. Apparently this was an inadvertent error by the sending institution (Dr. Terkeltaub-UCSD). The backcross of the *Vanin*-/- onto a pure C57BL/6 background by Dr. Terkeltaub will be completed in January 2011 and we will arrange shipment of these mice. We plan to repeat these studies and cross these mice to the *Ctns*-/- mice since renal involvement in these mice are strain dependent.

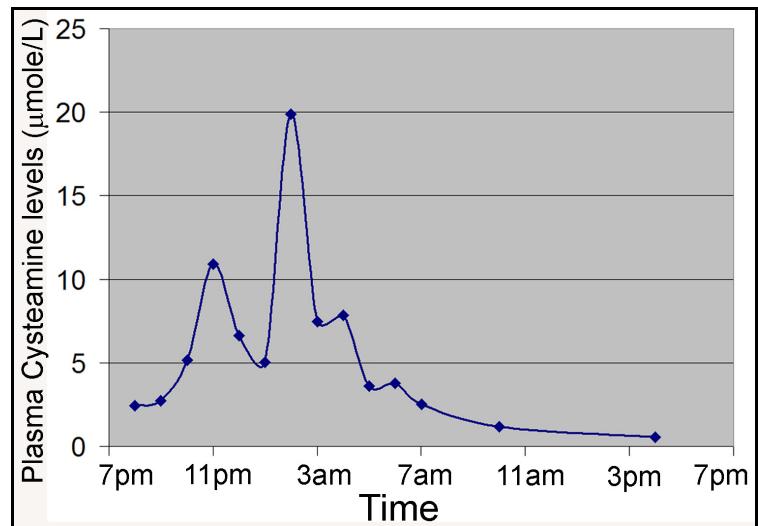
#### 2) Aim #2: Investigating the efficacy of cysteamine therapy for interstitial renal matrix protein reduction in chronic kidney disease and to determine its mechanism of anti-fibrotic action:

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 1). We designed studies to extend these observations to a glomerular model of chronic kidney disease and we are currently analyzing the data. 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 –  $0.81 \pm 0.09 \mu\text{mole/L}$ ; and 600mg/kg –  $1.04 \pm 0.15 \mu\text{mole/L}$ ; 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 ( $n=4/\text{group}$ ). Preliminary data on half the group confirms the higher levels at night and lower levels during the day (Figure 2). In addition,  $C_{\max}$  levels in mice were found to be  $20 \mu\text{M}$ , which is similar to the levels reported in humans (Dohil R et al, J Pediatr, 2006, 148:718-9). We will perform more formal area under curve analyses once the results are complete.



**Figure 1. 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.  $\dagger P<0.01$ ,  $n= 8-10/\text{group}$ .

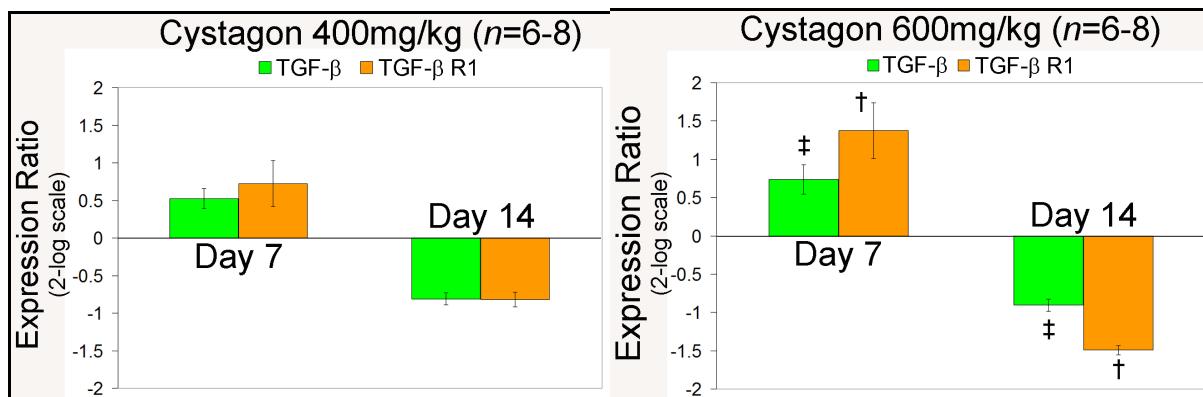


**Figure 2. Plasma cysteamine levels in high dose Cystagon treated mice.**

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.  $n= 4/\text{group}$ .

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 have now been measured by real-time qPCR in all mice 3, 7 and 14 days after UUO (See last progress report). 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

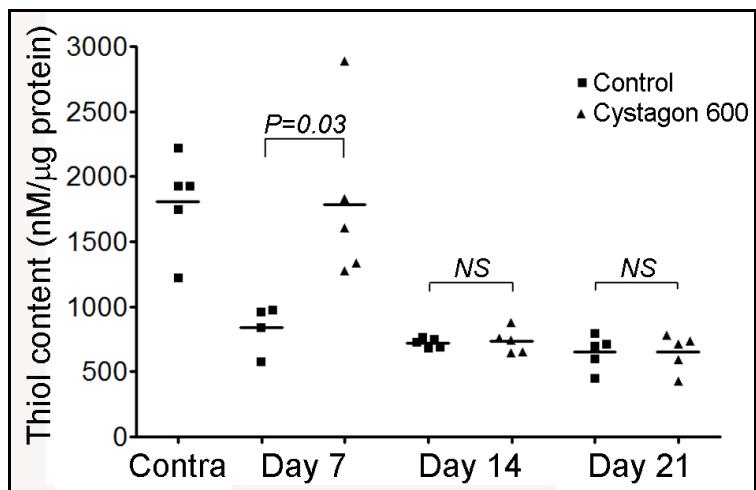
day 14; and at day 7, despite no difference in total collagen, there was a nearly 40 percent reduction in kidney fibronectin and procollagen III mRNA levels in mice treated with 400mg/kg and a nearly 60 percent reduction in fibronectin, procollagen I and procollagen III at higher doses of cysteamine (600mg/kg). The peak effect of Cystagon® was observed at 7 days at both treatment doses. Additional mechanisms must also contribute to the anti-fibrotic actions of Cystagon®. The mRNA levels of the profibrotic cytokine TGF- $\beta$  and the TGF- $\beta$  receptor was significantly down-regulated by 47 percent and 64 percent, respectively, at day 14 at high doses of Cystagon® compared to control mice (Figure 3,  $P<0.05$ ). Interestingly, at day 7 after UUO both TGF- $\beta$  and the TGF- $\beta$  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- $\beta$  independent.



**Figure 3. High dose Cystagon modulates profibrotic signaling at advanced timepoints.** Relative mRNA transcription of TGF- $\beta$  and TGF- $\beta$  receptor 1 genes in Cystagon treated mice (600mg/kg) relative to control mice. Semi-quantitative real-time qPCR data was analyzed with REST software using both GAPDH and 18S as reference genes. Each sample was performed in triplicate. ‡  $P<0.05$ , †  $P<0.01$ .

There was a significant reduction in  $\alpha$ -SMA positive myofibroblasts by nearly 30 percent in both doses at day 14 in cysteamine-treated mice. We have completed the  $\alpha$ -SMA immunostaining for all the groups for day 7 and we are currently analyzing the data. There was a significant reduction in interstitial macrophage infiltration by 34 percent in mice treated with 600mg/kg/day (no difference was seen at day 7). Therefore, it appears that Cystagon modulates both myofibroblast activation/proliferation and macrophage accumulation. Further studies are underway to determine the mechanisms by which Cystagon modulates these two key cells during chronic kidney injury.

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. In order to investigate the importance of total redox status during chronic kidney injury, we measured 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 4). Future studies will investigate the specific pathways modulated by cysteamine during chronic kidney injury.



**Figure 4. 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.

In summary, early data from the first Cystagon® treatment study establishes its significant anti-fibrotic effects. Ongoing studies are planned to identify the anti-fibrotic mechanisms modulated by cysteamine during chronic kidney injury. Over the next 6 months we will also be able to confirm the advanced renal phenotype of the *Ctns*-/- *vanin*-/- double knock-out mice compared to heterozygous control mice and *Ctns*-/- mice.

## BUDGET EXPENDITURES

July 1, 2010 to December 31, 2010

Category	Award *	Prior expenditures **	Expenditures this period ***	Anticipated expenditures ****	Total		Total expenditures to date	Remaining balance
					expenditures this period	expenditures this period		
Personnel	\$ 150,044	\$ 97,729	\$ 24,391	\$ 2,861	\$ 27,252	\$ 124,981	\$ 25,063	
Animals /Maintenance	\$ 14,083	\$ 14,422	\$ 6,093	\$ 465	\$ 6,558	\$ 20,980	\$ (6,898)	
Supplies	\$ 33,286	\$ 28,834	\$ 3,596	\$ 1,629	\$ 5,224	\$ 34,059	\$ (773)	
Travel	\$ 3,841	\$ 34	\$ -	\$ -	\$ -	\$ 34	\$ 3,806	
Subtotal	\$ 201,253	\$ 141,019	\$ 34,080	\$ 4,954	\$ 39,034	\$ 180,054	\$ 21,199	
IDC	\$ 20,126	\$ 14,102	\$ 3,408	\$ 495	\$ 3,903	\$ 18,006	\$ 2,120	
Total	\$ 221,378	\$ 155,121	\$ 37,488	\$ 5,450	\$ 42,938	\$ 198,059	\$ 23,319	

\* Award amount for 07/01/08-12/31/10

\*\* Prior expenditures: 07/01/08-06/30/10

11% budget remaining

\*\*\* Expenditures this period: 07/01/10-12/29/10

\*\*\*\* Anticipated expenditures: 12/29/10-12/31/10