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## Investigating Potential Therapies to Decrease the Rate of Cystine Stone Growth in *Slc3a1*<sup>-/-</sup> Mice

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# **Investigating Potential Therapies to Decrease the Rate of Cystine Stone Growth in *Slc3a1*<sup>-/-</sup> Mice**

A thesis submitted to the faculty of  
Dominican University of California and the Buck Institute for Research on Aging  
in partial fulfillment of the requirements  
for the degree

Masters of Science

in

Biology

By

Sruthi Damodar

San Rafael, California

May, 2015

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## Certification of Approval

I certify that I have read *Investigating Potential Therapies to Decrease the Rate of Cystine Stone Growth in Slc3A1<sup>-/-</sup> Mice* by Sruthi Damodar, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California and the Buck Institute for Research on Aging.

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## Abstract

Cystinuria is an autosomal recessive disorder characterized by a defective renal transporter involved in the reabsorption of cystine and other dibasic amino acids. This leads to an accumulation of cystine in the urine, resulting in cystine stones. The SLC3A1/SLC7A9 cystine transporter accounts for 90% of cystine reabsorption and mutations in this transporter result in the formation of cystine stones. For this study, micro-computed tomography ( $\mu$ CT) scanning was evaluated for its feasibility to track accurate volumetric measurements of *in vivo* cystine stone growth in the *Slc3a1*<sup>-/-</sup> cystinuric mouse model. Six pharmacological interventions – sulforaphane, methyl selenocysteine, homocysteine, tiopronin, TPEN and a zinc-supplemented diet– were also examined for their efficacy in reducing the rate of cystine stone growth.  $\mu$ CT analysis revealed stone growth proceeds linearly. Sulforaphane and TPEN supplementation resulted in a reduced rate of stone growth when compared to the respective vehicle controls; however, methyl selenocysteine and the zinc-supplemented diet displayed no effect on the rate or nature of stone formation. Homocysteine and tiopronin were shown to worsen stone growth rate. Sulforaphane and TPEN were effective interventions and our findings support both as a potential therapy for a cystinuric mouse model. A combination of treatments targeting the rate of cystine stone formation through similar agents appears to be a novel approach in further understanding cystine stone growth. Therapies that can manage the balance between these agents and adverse side effects provide an avenue to effectively treating cystinuria.

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# Table of Contents

Certification of Approval	iii
Abstract	iv
Acknowledgements	v
List of Figures	viii
List of Tables	x
Abbreviations	xi
Introduction	1
I.    Epidemiology of Cystinuria	3
II.   Pathophysiology	3
a.  rBAT-b <sup>0,+</sup> AT Transporter	3
b.  x <sub>c</sub> <sup>-</sup> Cystine-Glutamate Transporter	6
III. <i>Slc7a9</i> <sup>-/-</sup> and <i>Slc3a1</i> <sup>-/-</sup> Mouse Models	8
IV.   Diagnosis	8
V.    Treatments	9
a.  Dietary Modifications and Urine Alkalinization	9
b.  Pharmacological Intervention – Sulfhydryl Agents	10
c.  Prospects for New Interventions	11
d.  Surgical Intervention	11
VI.   Proposed Pharmacological Interventions	12
a.  Sulforaphane	12
b.  Methyl Selenocysteine	13
Methods	
I.    Overview	15
II.   Genotyping – DNA Extraction and Amplification	15
III.  Experimental Design	18
I.  Drug Preparation	18
II. Cystine Growth Detection Using the Micro-Computed Tomography <i>in vivo</i> (μCT) Scanner	20
III. Cystine Stone Growth Analysis	20
IV.   Statistical Analysis	22
Results	
I.    Confirmation of Cystine Stone Formation	23
II.   Effect of the Pharmacological Treatments in Reducing the Rate of Cystine Stone Growth	27

Discussion and Conclusions	35
I. Micro-Computed Tomography ( $\mu$ CT) Scanning as a Novel Approach to Tracking Stone Growth	35
II. Sulforaphane as a Potential Therapeutic in Reducing the Rate of Cystine Stone Growth	36
III. Methyl Selenocysteine Did Not Show Any Effect In Reducing Cystine Stone Growth	37
IV. Future Directions	38
Addendum	40
Introduction	40
Effect of Homocysteine and Tiopronin in Reducing the Rate of Cystine Stone Growth	40
Effect of TPEN and Zinc-Supplemented Diet in Reducing the Rate of Cystine Stone Growth	46
Discussions and Conclusions	53
I. Homocysteine Worsened Cystine Stone Growth	53
II. Currently Prescribed, Tiopronin, Did Not Reduce the Rate of Cystine Stone Growth	53
III. Zinc Bioavailability May Affect Cystine Stone Growth	54
IV. Future Directions	54
References	55



## List of Figures

#	Figure Name	Page Number
1	Kidney Nephron Structure	2
2	Cystine Reabsorption in an Unaffected Individual and in a Cystinuric Patient	5
3	Cystine Reabsorption in the $x_c^-$ Cystine-Glutamate System	7
4	Metallome Analysis of Human and Mouse Cystine Stones	14
5a	Representative $\mu$ CT scan	21
5b	3D Model	21
6	Volumetric Analysis of Cystine Stones in the Bladder of <i>Slc3a1</i> <sup>-/-</sup> Mice	24
7	Single Stone Tracker Contributes to Stone Accumulation	26
8	Body Weights per Treatment Group	29
9	The Rate of Stone Growth for the Treatment Groups	30
10	Sulforaphane Reduced the Rate of Cystine Stone Growth by 15%	31
11	Sulforaphane Displayed a Similar Reduction Pattern in the Rate of Stone Growth	32
12	Interventions Do Not Show Preventative Properties	32 33
13	Sulforaphane Produces More Medium Sized Stones	
14	Homocysteine Worsens Cystine Stone Growth	44
15	Tiopronin Worsens Cystine Stone Growth Rate	45
16	Zinc Detection in Cystine-Based Stones	47
17	TPEN Reduced the Rate of Cystine Stone Growth by 18%	51

18

Zinc-Supplemented Diet Did Not Display Any Effect  
on Cystine Stone Growth

52

## List of Tables

#	Table Name	Page Number
1	PCR Master Mix	16
2	PCR Primers	17
3	Preparation and Doses for Sulforaphane and Methyl Selenocysteine Hydrochloride	19
4	Rate of Stone Growth per Treatment Group	25
5	Preparation and Doses for Homocysteine and Tiopronin	42
6	Preparation and Doses for TPEN and Zinc-Supplemented Diet	49

## **Abbreviations**

**Nrf2** – Nuclear Factor-Like 2

**ARE** – Antioxidant Response Element

**μCT** – Micro-computed Tomography

**Se-methyl selenocysteine** – Methyl Selenocysteine

**L-CDME** – L-cystine dimethylester

**L-CME** – L-cystine methylester

**3D** – Three-Dimension

**Nrecon** – Reconstruction Package

**CTAn** – CT Analysis Package

**ROI** – Regions of Interest

**CTvol** – CT Volume Package

**N, N, N', N'-tetrakis (2-pyridylmethyl)-ethyenediamin** - TPEN

## **Introduction**

Cystinuria is an autosomal recessive disorder characterized by mutation(s) in the genes that code for the cystine transporter. This disorder causes a defect in the proper reabsorption of cystine (or cystine dimmers) in the proximal tubule of the nephron (Figure 1). Cystine is produced endogenously from methionine or from a disulfide bond between two molecules of cysteine. Due to the defect in cystine transport, cystine accumulates in the urine and eventually forming small stones and larger stones. Without proper intervention, this leads to renal colic (flank pain) while passing, urinary obstructions and lastly, renal failure if not treated (Evan, 2010).

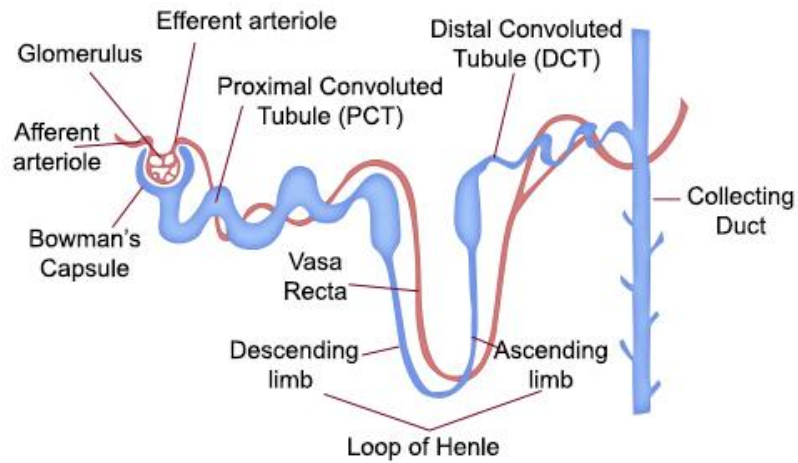


Figure 1: Kidney Nephron Structure (Tikekar, 2014). The diagram depicts the parts of the nephron. This study focuses mainly on the proximal convoluted tubule.

## **I. Epidemiology of Cystinuria**

Cystinuria accounts for one to two percent of all kidney stone cases in adults and six to eight percent in children (Botzenhart, 2002; Eggermann, 2012). It is one of the more common inherited genetic disorders (Matoo, 2008) that eventually leads to renal failure due to a 60% recurrence rate in at least 50% cystinuric patients (Claes, 2012). Five percent of women and twelve percent of men develop a cystine-based stone at least once during their lifetime with 40% of familial history matching in children cases (Eggermann, 2012; Pearle, 2005). These figures may be an underestimation of the true prevalence since cystinurics may not actually develop a stone in their lifetime and therefore, evade statistical prevalence.

## **II. Pathophysiology**

Stone formation is thought to arise from one of three pathways: overgrowth on interstitial plaques, formation of crystal deposits in the renal tubules or crystallization in free solution (Claes, 2012; Coe, 2010; Evan, 2006). Cystine stones mainly arise from the third pathway. The defective transporter leads to accumulation of cystine in the urine. Due to its poor solubility in urine, a supersaturated solution is created, where homogenous or heterogeneous nucleation can occur. This eventually leads to the formation of crystals that plug the ducts of Bellini; however, this mechanism of the anchorage is poorly understood. The crystals are thought to aggregate to form free-floating cystine-based stones that eventually grow in size.

### **A. rBAT-b<sup>0,+</sup> AT Transporter**

The rBAT-b<sup>0,+</sup> AT transporter, composed of the SLC3A1 and SLC7A9 protein subunits, is characterized as a cystine and dibasic amino acid reabsorption transporter. The SLC3A1 subunit encodes for rBAT protein while the SLC7A9 subunit encodes for the b<sup>0,+</sup>, AT (Calonge,

1994; Feliubadalo, 1999). This transporter accounts for an estimated 90% of cystine reabsorption (Silbernaglet, 1988). In an unaffected individual, cystine and other dibasic amino acids are filtered by the glomerulus and are reabsorbed across the apical membrane of the proximal tubule through the rBAT-b<sup>0,+</sup> AT transporter. Once cystine and the dibasic amino acids are transported in the proximal tubule cell, intracellular cystine is readily reduced to two molecules of cysteine (Figure 2). In a cystinuric patient, cystine is not reabsorbed through the rBAT-b<sup>0,+</sup> AT transporter and therefore, accumulates in the urine, leading to the formation of its stones.



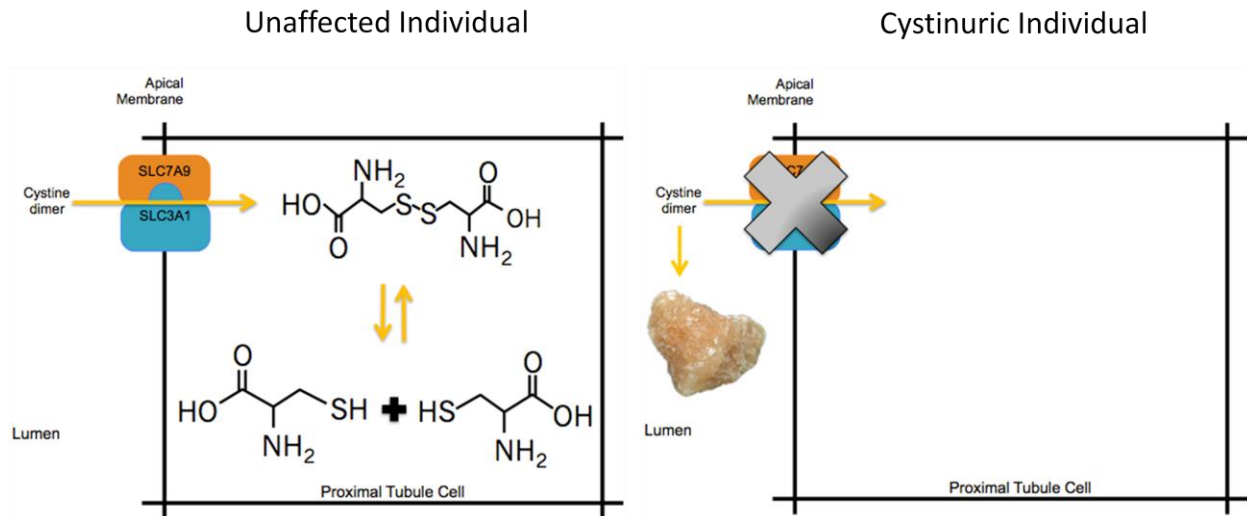


Figure 2: Cystine Reabsorption in an Unaffected Individual and in a Cystinuric Patient. Adapted from Mattoo, 2008. Cystine and the dibasic amino acids are transported across the apical membrane through the rBAT-  $b^{0,+}$  AT transporter, the SLC3A1 and SLC7A9 heterodimer. Once inside, cystine is reduced to two molecules of cysteine. Mutations in either the SLC3A1 or SLC7A9 subunit cause a defective transporter. Due to the defective transporter, cystine is not reabsorbed, leading to its accumulation in the urine and eventually, the formation of cystine-based stones.

## **B. $x_c^-$ Cystine-Glutamate Transporter**

In addition to the rBAT-  $b^{0,+}$  AT transporter, an alternate transport system, known as the  $x_c^-$  Cystine-Glutamate Transporter, is classified as a high affinity low capacity system (Palacin, 2001; Fernandez, 2002). It is composed of the SLC3A2 and SLC7A11 protein subunits and is responsible for approximately 10% of cystine reabsorption in the apical membrane of the S3 segment of the proximal tubule (Mattoo, 2008). This transporter works by exchanging cystine with glutamate. In addition, studies have shown that SLC7A11 is regulated by the transcription factor, Nuclear Factor-like 2 - (Nrf2) (Ishii, 2000) (Figure 3). Nrf2 is commonly in the cytoplasm bound to Keap1, its inhibitor. However, through the dissociation of the Nrf2-Keap1 complex, Nrf2 is translocated into the nucleus, where its binding to the Antioxidant Response Element (ARE) motif, leads to its activation (Sasaki, 2002). This activation then leads to increased transcription of many genes and pathways including the  $x_c^-$  Cystine-Glutamate Transporter (Johnson, 2008; Singh, 2006).

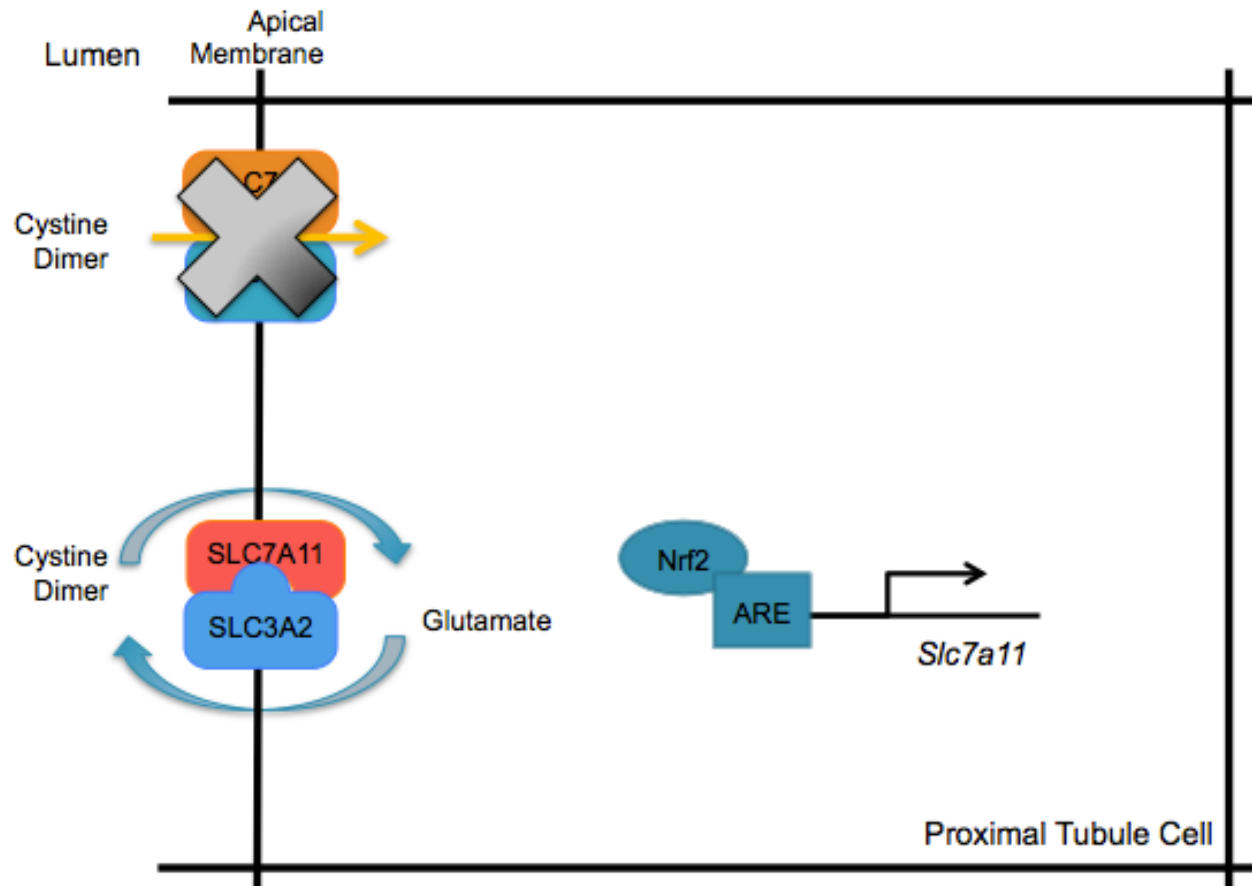


Figure 3: Cystine transport in the  $x_C^-$  Cystine-Glutamate System (Porcheray, 2006; Ishii, 2000; Sasakim 2002). This process accounts for 10% of cystine reabsorption. Cystine is exchanged with glutamate. Once in the tubular cell, cystine is reduced to two molecules of cysteine and exits into the bloodstream.

### III. *Slc7a9*<sup>-/-</sup> and *Slc3a1*<sup>-/-</sup> Mouse Models

To study the rBAT-b<sup>0,+</sup> AT transporter *in vivo*, *Slc7a9*<sup>-/-</sup> mice were generated and have become a model for cystinuria and cystine urolithiasis (Feliubadalo, 2003). In this study, the expression of the rBAT protein and b<sup>0,+</sup> AT protein was evaluated. The *Slc7a9*<sup>-/-</sup> mice were shown to express large amounts of rBAT protein and decreased levels of b<sup>0,+</sup> AT protein. In addition, at least 42% of the *Slc7a9*<sup>-/-</sup> mice formed cystine stones that continued to grow throughout the lifespan of the mouse.

Recently, the *Slc3a1*<sup>-/-</sup> mouse model was created and tested for its efficacy in studying cystinuria as well (Ercolani, 2010). All the male *Slc3a1*<sup>-/-</sup> mice had shown the formation of cystine stones that were visualized under a  $\mu$ CT scanner and upon dissection of the bladders, the number of stones ranged from a few to over 15 in number. Bladder function was also evaluated and partial outlet obstruction was found due to poor compliance and contractile responses. With both models, cystinuria can be further studied and advances can be made to target either gene deficiency.

### IV. Diagnosis

Diagnostic approaches are mainly focused on metabolic screens and imaging evaluations. Metabolic screens include spot urine samples, fasting blood samples and 24-hour urine collections to measure cystine excretion, volume, etc. 24-hour urine collections typically exhibit high cystine concentrations during the night. Due to this, cystine analysis of separate day and night samples may be needed to detect proper cystine excretion levels (Fjellstedt, 2001). Normal cystine excretion is 30 mg/L per day. However, cystinuric patients excrete cystine anywhere from 300-400 mg/L per day.

Imaging screens include microscopic evaluation, ultrasound and computed tomography. Microscopic evaluation reveals the characteristic hexagonal crystal formations of cystine stones (Knoll, 2005). The ultrasound is one of the preferred routes and is used for newly developed stones, while computed tomography is preferred for characterization of stone appearance as rough or smooth. This rough or smooth distinction is then used to determine the efficacy of extracorporeal shock wave lithotripsy, a process used for stone dissolution.

## **V. Treatments**

While the pathophysiology and genetics are well characterized, there's been little progress in finding an effective drug treatment. Medical management primarily consists of reducing the free cystine concentration and/or increasing cystine solubility in the urine through dietary modifications, urine alkalinization, sulfhydryl agents and surgical interventions. Current medications rely heavily on sulfhydryl agents that have the ability to bind to cysteine and increase the solubility of the asymmetric disulfides formed. Agents such as D-penicillamine (DP),  $\alpha$ -mercaptopyrionyl-glycine (MPG), captopril, and tiopronin are frequently used yet the efficacy is limited. However, even with these techniques, poor compliance, adverse side effects and the limitations with current medications still do not prevent cystine stone formation (Barbey, 2000).

### **A. Dietary Modification and Urine Alkalinization**

Cystinuric patients are recommended a diet consisting of: hyperhydration (4-5 L per day), a sodium-restricted diet (2g per day) and a protein-restricted diet (1g per day) (Knoll, 2005). The goal of hyperhydration is to supersaturate urinary cystine at physiological pH. Previous studies have correlated hyperhydration, before bed and upon awakening, with a reduction in cystine saturation in the urine (Claes, 2012; Monnens, 2000; Fjellstedt, 2001). This ensures dilute urine

overnight. A low sodium intake has been previously shown to lower cystine excretion and is recommended for cystinurics; the mechanistic action of this effect is unknown. However, the known precursor to cysteine is methionine (Knoll, 2005) that is found low amounts in protein-rich foods (Fattah, 2014; Rodman 1984).

In addition, urine alkalization has been shown to be a beneficial treatment for cystinurics. Cystine solubility is known to increase by threefold with an increase in pH (Dent, 1955). High pH of 7.5 for cystinurics is recommended. However, a pH higher than 8 usually runs the risk of forming calcium phosphate stones (Knoll, 2005). Potassium citrate and sodium bicarbonate are compounds given to increase pH in urine (Fjellstedt, 2001). Sodium bicarbonate is mainly administered to renal insufficient patients, while potassium citrate is recommended for most other cases.

### **B. Pharmacological Intervention: Sulfhydryl Agents**

Chelating and sulfhydryl agents have been used in the past when other treatment measures failed. However, they now are becoming the primary choice. These agents are often employed when cystine concentration reaches 2000  $\mu\text{mol/L}$  or greater. Agents such as D-penicillamine (DP),  $\alpha$ -mercaptoproprionyl-glycine (tiopronin), captopril etc. are recommended for their ability to bind to cysteine, forming asymmetric disulfides that are hypothesized to be more soluble in urine (Harbar, 1986; Pak, 1986; Crawhall, 1963; Xu, 2013). This increased solubility reduces the formation of cystine stones.

Tiopronin has been shown to present fewer side effects such as edema, hypouresis and nephrotic syndrome than DP and is frequently recommended by physicians (Pak, 1986; Fattah, 2014). A study conducted by Chow *et al.* (1966) revealed that patients who did not experience

the effects of hyperhydration and alkalization treatments displayed a reduced rate of cystine stone growth when treated with DP and tiopronin. Lastly, captopril is an angiotensin-converting enzyme inhibitor that contains a thiol group. A study conducted by Asplin *et al.* (2013) revealed that administration of captopril increases cystine solubility while increasing urinary pH in *in vitro* studies; however its effectiveness is debated (Chow, 1996; Cohen, 1995; Goldfarb, 2006; Asplin, 2013). Combinations of sulfhydryl agents have been suggested though haven't been researched yet (Tiselius, 2001).

### **C. Prospects for New Interventions**

There are four steps in the formation of cystine stones – nucleation, crystallization, aggregation and adhesion to nearby cells (Mandal, 2013). Current medications are known to mediate the effects of nucleation by reducing the cystine accumulation in the urine or by increasing cystine solubility. A study by Rimer *et al* (2010) revealed a new molecular approach in preventing cystine stones by focusing on the crystallization and aggregation steps. The design was based on an *in vitro* crystal growth inhibition model that was attained through the binding of L-cystine dimethylester (L-CDME) and L-cystine methylester (L-CME) to cystine crystals. Different binding modes were revealed in cystine crystals when treated with L-CDME and L-CME, indicating that similar compounds can be administered to affect the binding and packing modes of the cystine crystals. In a recent study conducted by Lee *et al.* (2015), administration of CDME and CME to the *Slc3a1*<sup>-/-</sup> mice model displayed both candidates to be effective inhibitors of the crystallization stage in cystine crystal growth. This novel approach to understanding cystinuria through binding and packing modes of cystine crystals provides newer avenues to targeting the steps of cystine stone formation.

### **D. Surgical Intervention**

Surgical interventions, such extracorporeal shock wave lithotripsy (SWL), ureteroscopy (URS) and percutaneous nephrolithotripsy (PNL), are commonly suggested due to their high success rate and their safety. Computed tomography reveals the appearance of cystine stones as either rough or smooth. From this, SWL, the most preferred route by physicians, is employed. This non-invasive procedure is used for patients with a stone in the upper urinary tract (Kim, 2007; Kachel, 1991; Brinkmann, 2001; Muslumanoglu, 2003; Delakas, 2001). Previously, cystine stones were thought to be resistant to shock wave lithotripsy (SWL) (Harada, 1992). However, a study conducted by Bhatta *et al.* (1989) and later confirmed by Elkoushy *et al.* (2011) reported that cystine stones with rough morphology were more susceptible to SWL than those with smooth morphology. URS is also used for patients with stones in the upper urinary tract. However, optimal results occur in patients with distal ureter stones (Schuster, 2002; Choong, 2000). Lastly, PNL is used in cases where there are larger sized stones.

## **VI. Proposed Pharmacological Interventions**

### **A. Sulforaphane**

Sulforaphane is an isothiocyanate molecule and is part of the organosulfur group. It is known to exhibit anticancer, anti-diabetic and antioxidant properties due to its Nrf2 activator role (Zheng, 2011; Hwang, 2015). Studies have shown that sulforaphane, when administered to wild-type and Nrf2<sup>-/-</sup> mice, can be detected in the small intestine, lungs, and kidney (Clarke, 2011; Elbarbry, 2011; Cui, 2012). A recent study conducted by Zheng *et al.* (2011) has shown that sulforaphane rescues diabetic nephropathy through an Nrf2-dependent pathway. This suggests that sulforaphane is likely to be deliverable to the kidney and can activate Nrf2 in the renal epithelial cells of the proximal tubule. By administering sulforaphane, Nrf2 activation is hypothesized to promote the expression of *Slc7a11* and thus, increase cystine reabsorption (Fig.



4). From this, we predict that the defective cystine reabsorption will be rescued by promoting the use of the  $x_c^-$  system

### **B. Methyl Selenocysteine**

Metallomics or kidney stone metal profiling has revealed amounts of selenium (Se) in human and mouse cystine-based stones (Killilea, unpublished, Figure 4). Se is known to exist in trace amounts in biological systems in organic (predominantly as selenocysteine and selenomethionine) or inorganic (selenite,  $\text{SeO}_3^{2-}$  and selenate,  $\text{SeO}_4^{2-}$ ) forms (Sunde, 2006; Zhang 2008). However, its appearance in both human and mouse cystine stones provides an avenue for testing a new *in vivo* approach to cystinuria.

An *in vitro* study by Rimer *et al* (2010) revealed a new molecular approach in preventing cystine stones. The design was based on *in vitro* crystal growth inhibition attained through the binding of L-cystine dimethylester (L-CDME) and L-cystine methylester (L-CME) to cystine crystals (Rimer, 2010). Different binding modes were revealed in cystine crystals treated with these interventions. For the purpose of our study, Se-methyl selenocysteine (methyl selenocysteine) was similarly hypothesized to readily convert to selenocysteine and interact with endogenous renal cysteine to generate asymmetric cysteine-selenocysteine disulfides. The difference in atomic radii between selenium and sulfur is expected to impact the packing of these heterogenous cystine stones, resulting in a reduced rate of cystine stone growth.

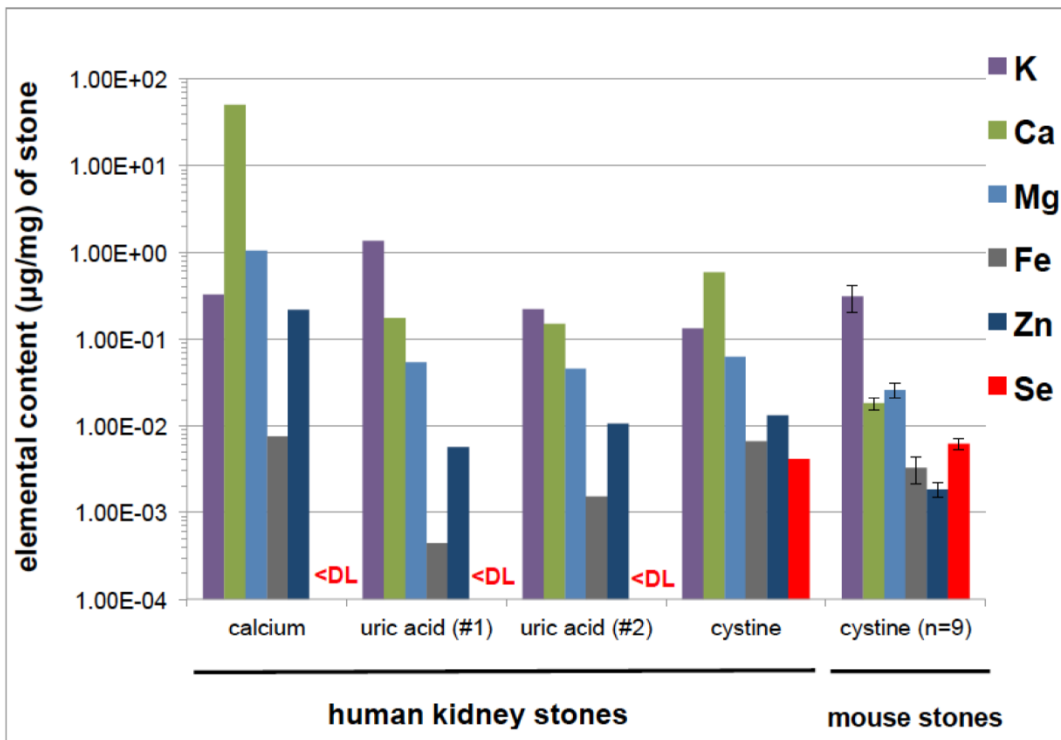


Figure 4: Metallome Analysis of Human and Mouse Cystine Stones. (Killilea, unpublished data). Kidney stone metal profiling revealed amounts of selenium in cystine-based stones when compared to the calcium and uric acid stones. <DL : Below Detection Limit.

## Methods

### I. Overview

The *Slc3a1*<sup>-/-</sup> mouse, in a mixed genetic background of A129 and C57Bl/6J, develops cystine stones in the bladder (Ercolani, 2010). These stones develop in the first 2-3 months of life and grow throughout the lifespan of the mouse. All procedures and protocols were conducted according to the Institutional Animal Care and Use Committee (IACUC), approved by the Buck Institute for Research on Aging.

### II. Genotyping – DNA Extraction and Amplification

The *Slc3a1*<sup>-/-</sup> mice were genotyped using a modified version of the REDEExtract-N-Amp Tissue PCR protocol (Sigma-Aldrich, St. Louis, MO) (Amrik Sahota, Rutgers). 2mm tail clips were obtained and added to 100µL extraction buffer solution and 25 µL tissue preparation solution, following incubation at room temperature for 10 minutes and then inactivation at 95°C for 3 minutes. Samples were then neutralized by adding 100µL neutralization buffer solution.

For each PCR reaction, 2µL DNA sample were mixed with 8µL of the PCR Master Mix (Table 1) to a final volume of 10µL. All primers used in this study were synthesized by IDT technologies, San Diego and are listed in Table 2. The PCR cycling parameters were set to 3 minutes at 94°C (94°C, 40 seconds, 58°C, 40 seconds, 72°C, 60 seconds X 35 cycles) and 7 minutes at 72°C. This standard PCR protocol was used to amplify the wild-type and *Slc3a1*<sup>-/-</sup> alleles. 1.5% Agarose gel electrophoresis was conducted to identify the products.

Table 1: PCR Master Mix.

<b>PCR Mix</b>	<b>Amount per Reaction</b>
Water	2.7 $\mu$ L
REDExtract-N-Amp PCR Reaction Mix	5 $\mu$ L
10 uM Primer 1	0.1 $\mu$ L
10 uM Primer 2	0.1 $\mu$ L
10 uM Primer 3	0.1 $\mu$ L
DNA sample	2 $\mu$ L

Table 2: PCR Primers.

<b>Primer</b>	<b>Primer Sequence(5'-3')</b>	<b>Molecular Weight (Base Pairs)</b>
Primer 1	AGAATGTCTTCACTTCTGCCA	6356.2
Primer 2	CGAGACTAGTGAGACGTGCTA	7088.6
Primer 3	CTGCCTCCCGCATGCTGAGAT	6975.6

### **III. Experimental Design**

#### **A. Drug Preparation**

All drugs were freshly prepared daily prior to administering. Each cohort received the respective dose administered daily or three times per week through intraperitoneal (IP) injections for the duration of the study. R,S-sulforaphane (IDT Technologies, San Diego, CA) was kept in a stock solution of 40mg/mL DMSO and was administered in 6.25% DMSO in PBS at 25mg/kg body weight. Methyl selenocysteine hydrochloride (IDT Technologies, San Diego, CA) was dissolved in 6.25% DMSO in PBS and administered at 100 $\mu$ g/kg body weight. Control mice received vehicle only (DMSO+PBS). Mice were randomized to avoid bias before being placed in the treatment groups. Preparation and dosage are summarized in Table 3.

Table 3: Preparation and Doses for Sulforaphane and Methyl Selenocysteine Hydrochloride.

<b>Treatment</b>	<b>Preparation</b>	<b>Dosage</b>
R,S-Sulforaphane	Stock Solution in 40mg/mL DMSO Administered in 6.25% DMSO in PBS	25mg/kg body weight
Methyl Selenocysteine Hydrochloride	Dissolved in 6.25% DMSO in PBS	100µg/kg body weight

## **B. Cystine Stone Growth Detection Using the Micro-Computed Tomography *in vivo*( $\mu$ CT)Scanner**

A noninvasive method to detect stone growth was employed using the  $\mu$ CT scanner (SkyScan 1176, Bruker, Belgium) for this study. All mice received general anesthesia through inhalation of 1.5-4% isoflurane before and during the scanning period and were placed in a supine position in the scanner. Low resolution (35  $\mu$ m) scans were performed at the level of the bladder using the following settings: X-ray voltage = 50 kV, anode current = 500  $\mu$ A, exposure time = 4 minutes, rotation step = 0.3°, averaging = 2, image pixel size = 35  $\mu$ m, and filter = Al 0.5 mm.  $\mu$ CT scans were obtained weekly for the duration of the studies to track and measure the rate of cystine stone growth.

## **C. Cystine Stone Growth Analysis**

The stones are radiolucent and appear on the  $\mu$ CT scan (Figure 5a). Images were reconstructed as cross-sectional three-dimensional (3D) image stacks using the software, Nrecon (Nrecon v1.6.9.8, Bruker-MicroCT, Belgium). The dynamic range parameter (-0.002 to 0.08) was set to minimize background noise and ensure the consistency between each individual mouse scans. The 3D image stacks were exported to the image analysis software, CT Analysis (CTAn v1.14, Bruker-MicroCT, Belgium). A threshold between 70 and 120 was chosen to obtain better contrast between the bladder wall and stones. Following, circular regions of interest (ROI) were drawn individually for each scan to enclose the bladder region. The rate of stone growth was detected and quantified into 3D volumetric measurements, provided by the CTAn package. 3D models of the stones were visualized using CT Volume (CTvol v2.0, Bruker-MicroCT, Belgium) (Figure 5b).



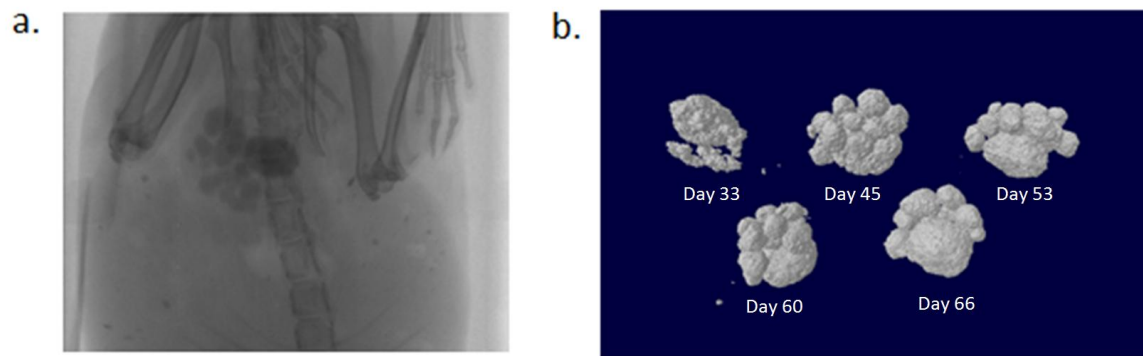


Figure 5: A) Representative  $\mu$ CT scan of a bladder from a control male cystinuric with stones. B) 3D model of a control male mouse tracked over a month.

#### **IV. Statistical Analysis**

Linear mixed model with random intercept was used for analyzing and modeling longitudinal data to track the rate of stone growth by accounting for each individual mouse and the different baselines the stone events occurred at. The Kaplan Meier estimate was employed to track the rate of stone occurrence after a given time point and to determine whether interventions had preventative properties. The statistical package, R, was used to analyze the collected data.  $p < 0.05$  was considered statistically significant.

## Results

### I. Confirmation of Cystine-Based Stone Formation

A total of 17 male cystinuric mice were observed under the  $\mu$ CT scanner to ensure the formation of cystine stones prior to the study initiation. Stone formation in the bladder was detected in all the *Slc3a1*<sup>-/-</sup> mice prior to treatment. The number of stones and sizes varied among the mutant mice. At the age of three months, the *Slc3a1*<sup>-/-</sup> mice were placed in three cohorts – vehicle control (n=6), sulforaphane (n=5) and methyl selenocysteine (n=6). The interventions were administered daily with IP injections and observed weekly for one month using the  $\mu$ CT scanner.

Linear mixed model with random intercept was used to compare the rates of stone growth between the treatment groups. Stone growth for the treatment groups proceeded at a linear rate (Figure 6). Sulforaphane was significantly different from methyl selenocysteine (p=0.02). However, neither sulforaphane nor methyl selenocysteine were statistically different from the vehicle control group (Table 4). However, an interesting finding revealed that single stones may increase in size as the total stone volume increases, contributing to large stone accumulation. By tracking a single stone in a control mouse for one month, the rate of a single stone was shown to increase linearly (Figure 7) with a correlation coefficient ( $R^2$ ) of 0.96.

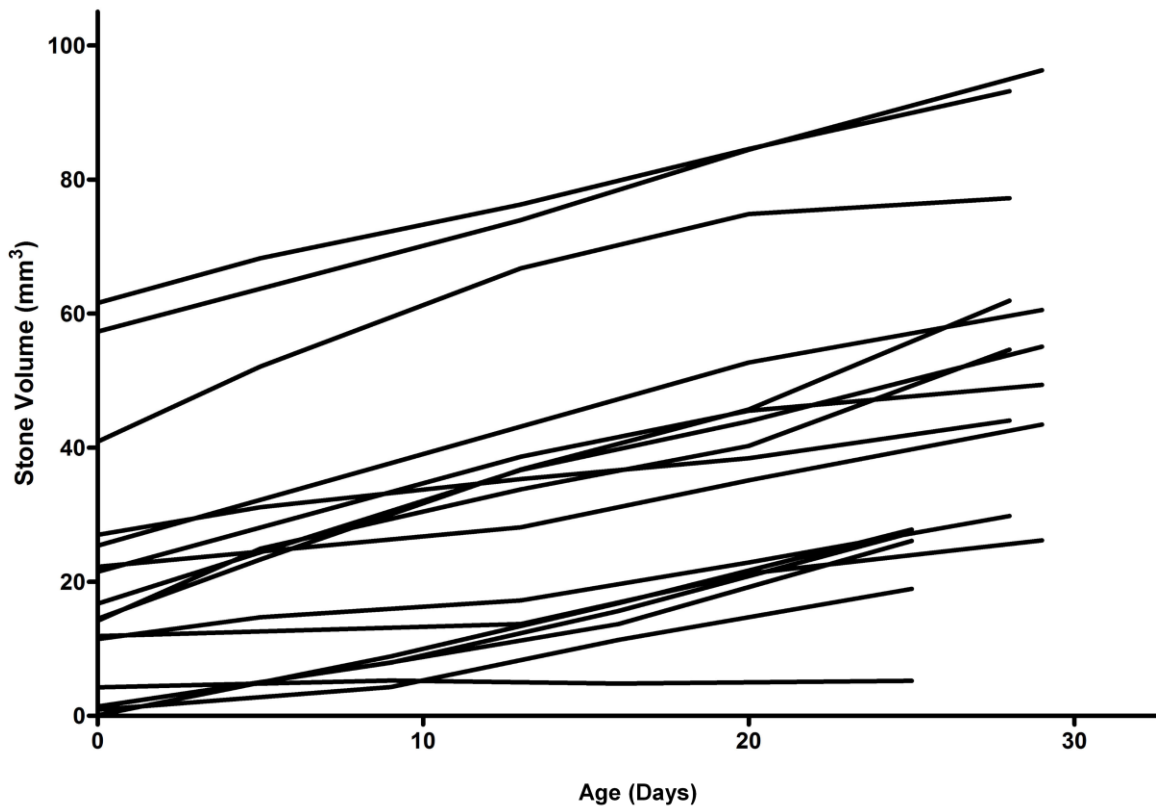


Figure 6: Volumetric Analysis of Cystine Stones in the Bladder of *Slc3a1*<sup>-/-</sup> Mice. Regions of Interest (ROIs) were drawn encompassing the bladder region in the CTAn package. The rate of stone growth was tracked over a one month. At the end of the study, the linear mixed model with random intercept was employed. Our data suggests that the rates of all three treatment groups were similar and linear.

Table 4: Rate of Stone Growth per Treatment Group. N.S. – Not Significant

Treatment Group	Number of Mice	Rate of Stone Growth (mm <sup>3</sup> /day)	p-value
Vehicle Control	6	0.981	
Sulforaphane	5	0.843	N.S.
Methyl Selenocysteine	6	1.170	N.S.

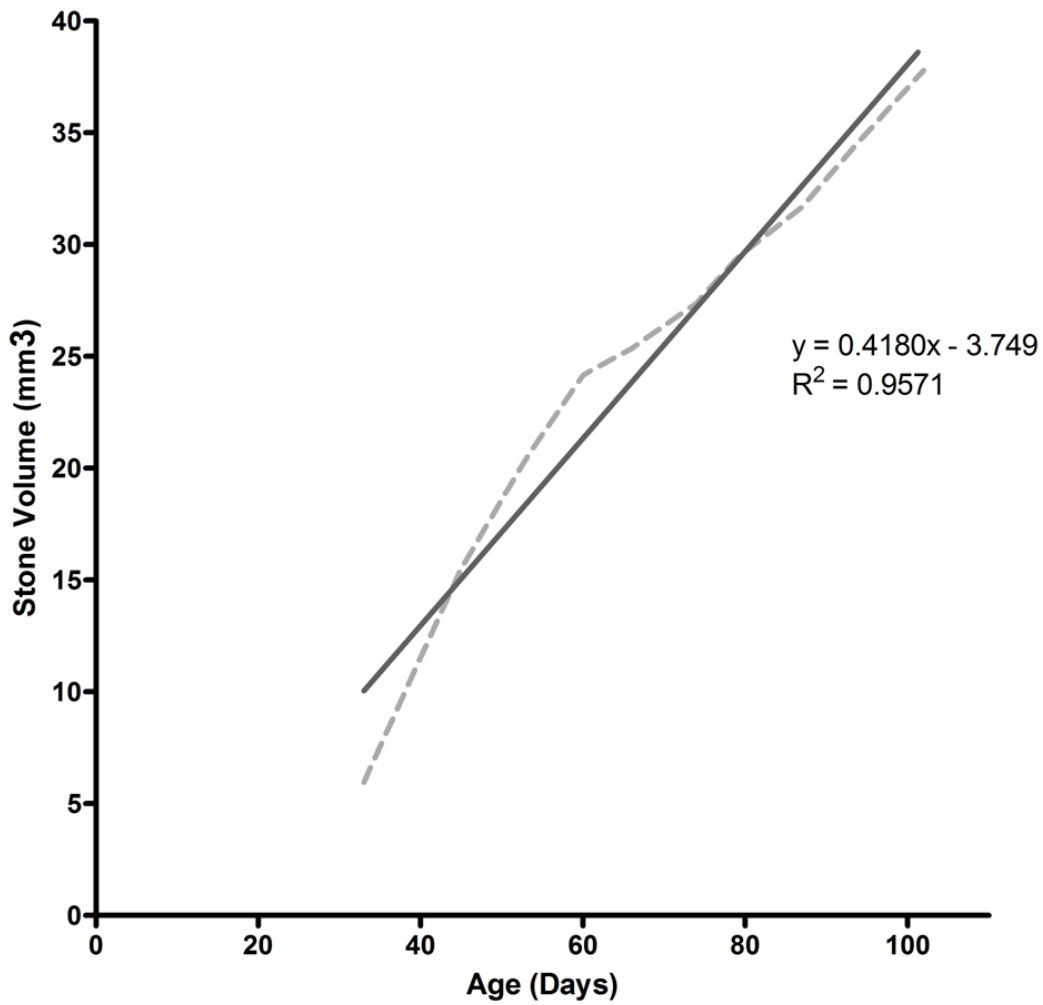


Figure 7: Single Stone Tracker Contributes to Stone Accumulation. A single stone was tracked weekly using the  $\mu$ CT scanner in a control *Slc3a1*<sup>-/-</sup> mouse. Linear regression revealed that the rate of stone growth increased linearly with total stone volume. The correlation coefficient ( $R^2$ ) was 0.96.

## II. Effect of the Pharmacological Treatments in Reducing the Rate of Cystine Stone Growth

To understand the effects of the interventions further, *Slc3a1*<sup>-/-</sup> mice without stones were studied. In this study, the mice were treated intraperitoneally with vehicle control (n=6), sulforaphane (n=6) and methyl selenocysteine(n=6) four weeks after birth. The doses were based on previous studies. The dose of sulforaphane was based on a study conducted by Zheng *et al.* (2011) that confirmed 12.5 mg/kg of the compound affects Nrf2 expression in the kidney. The dose of methyl selenocysteine was based on a study conducted by Cao *et al.* (2014) that confirmed the selective protective properties of Se- seleno methylcysteine against antitumor activity and anticancer drugs.

The mice were treated three times a week and imaged weekly for four months using the  $\mu$ CT scanner. Eventually, all the *Slc3a1*<sup>-/-</sup> mice formed stones. Body weights were recorded weekly and remained consistent between the treatment groups (Figure 8). To compare the rates of stone growth between the treatment groups, linear mixed model with random intercept was employed and a linear correlation was found, indicating that the stone volume increased at a linear rate (Figure 9). There was a statistical difference between the vehicle control and sulforaphane groups (chisq=28.62, df=1, p<0.001) (Figure 10) and between sulforaphane and methyl selenocysteine group (chisq=20.42, df=1, p<0.001). However, the difference between the vehicle control and methyl selenocysteine group (chisq=2.74, df=1, p=0.098) was not statistically significant. Overall, the slopes between the treatment groups were significantly different (chisq=34.48, df=2, p<0.001) and sulforaphane showed a 15% reduction in the rate of stone growth.

This study was repeated using 20 *Slc3a1*<sup>-/-</sup> mice – 10 in vehicle control and 10 in a sulforaphane treated group – for the duration of five months. Sulforaphane exhibited a similar reduction of 18% in the rate of stone growth when compared to the vehicle control (chisq=4, df=1, p=0.046) (Figure \_\_\_\_), further confirming sulforaphane as a potential pharmacological intervention for cystinurics.

In order to understand the probability of having another stone event after a given time point, a Kaplan-Meier survival plot was constructed. There were no significant differences across the treatment groups (chisq=4.4, df=2. p=0.109) (Figure 12), indicating these interventions do not possess preventative properties in cystine stone growth. Interestingly, by selecting for each individual stone as a ROI in the CTAn package, individual stone volumes and the numbers of stones in each size group were obtained and plotted in Figure 13. Stone size was then separated into six size fractions (<1, 1-5, 5-10, 10-15, 15-20, 20-25, >25 mm<sup>3</sup>). Our data suggests that sulforaphane produces medium sized stones.



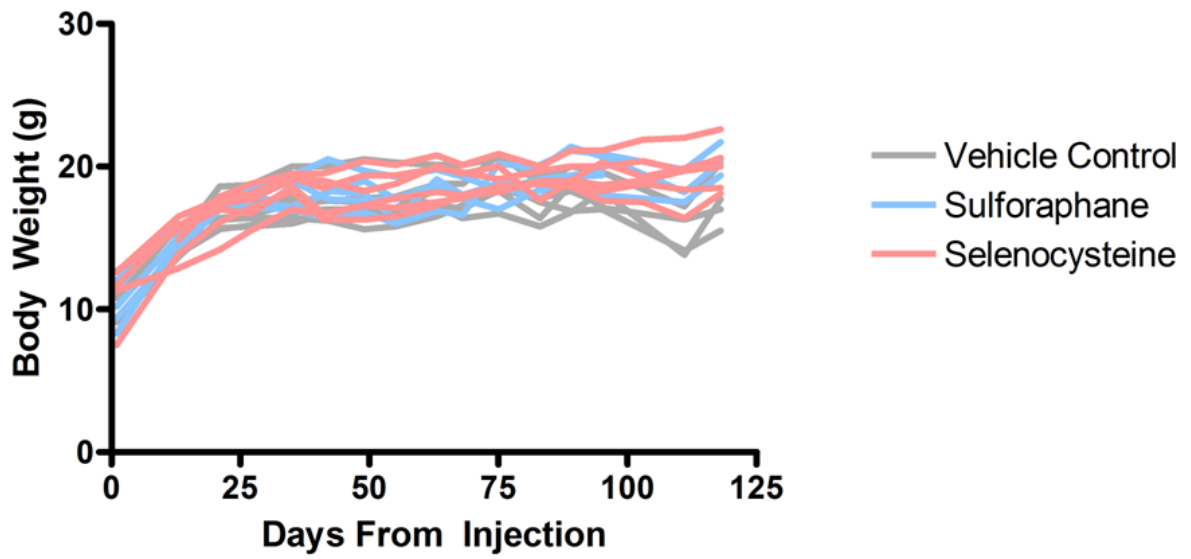


Figure 8: Body Weights per Treatment Group. Body weights were tracked weekly. There is no significant difference between the body weights for the three treatment groups, indicating that the treatment had no effect on body weight.

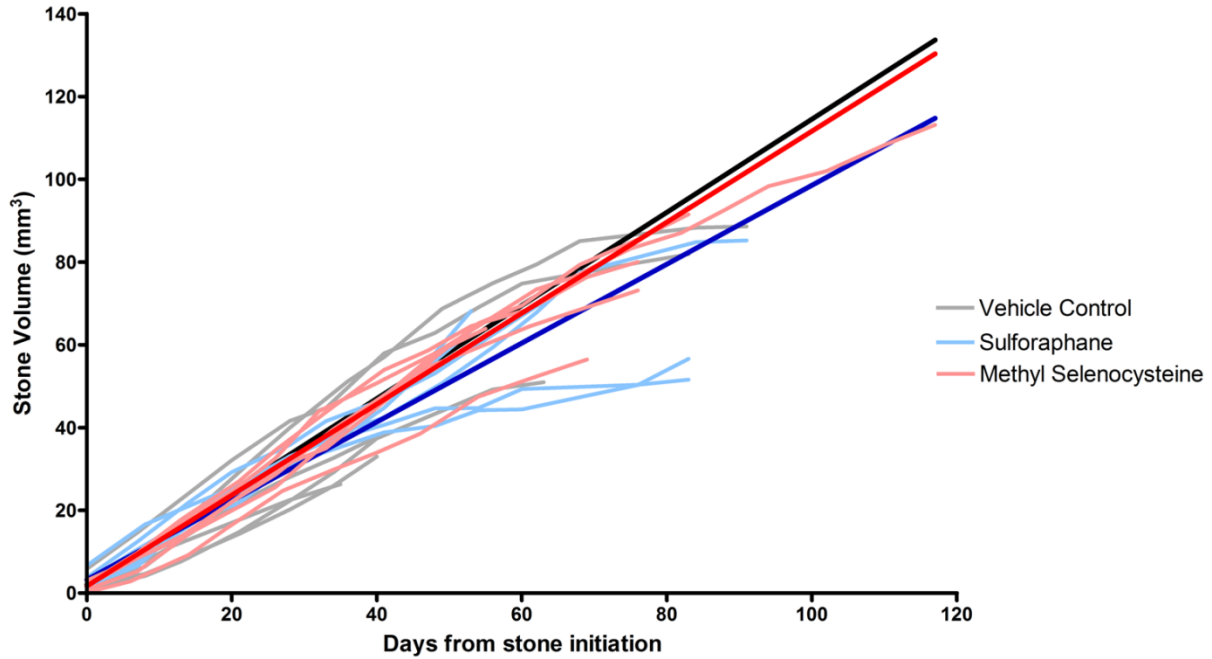


Figure 9: The Rate of Stone Growth for the Treatment Groups. Each line represents an individual *Slc3a1*<sup>-/-</sup> mouse in its respective treatment group. The thick solid lines are the estimated trajectories for the rate of stone growth. Statistical differences were noted between the vehicle control and sulforaphane groups and between the sulforaphane and methyl selenocysteine groups. However, no significant difference was found between the methyl selenocysteine and vehicle control groups.

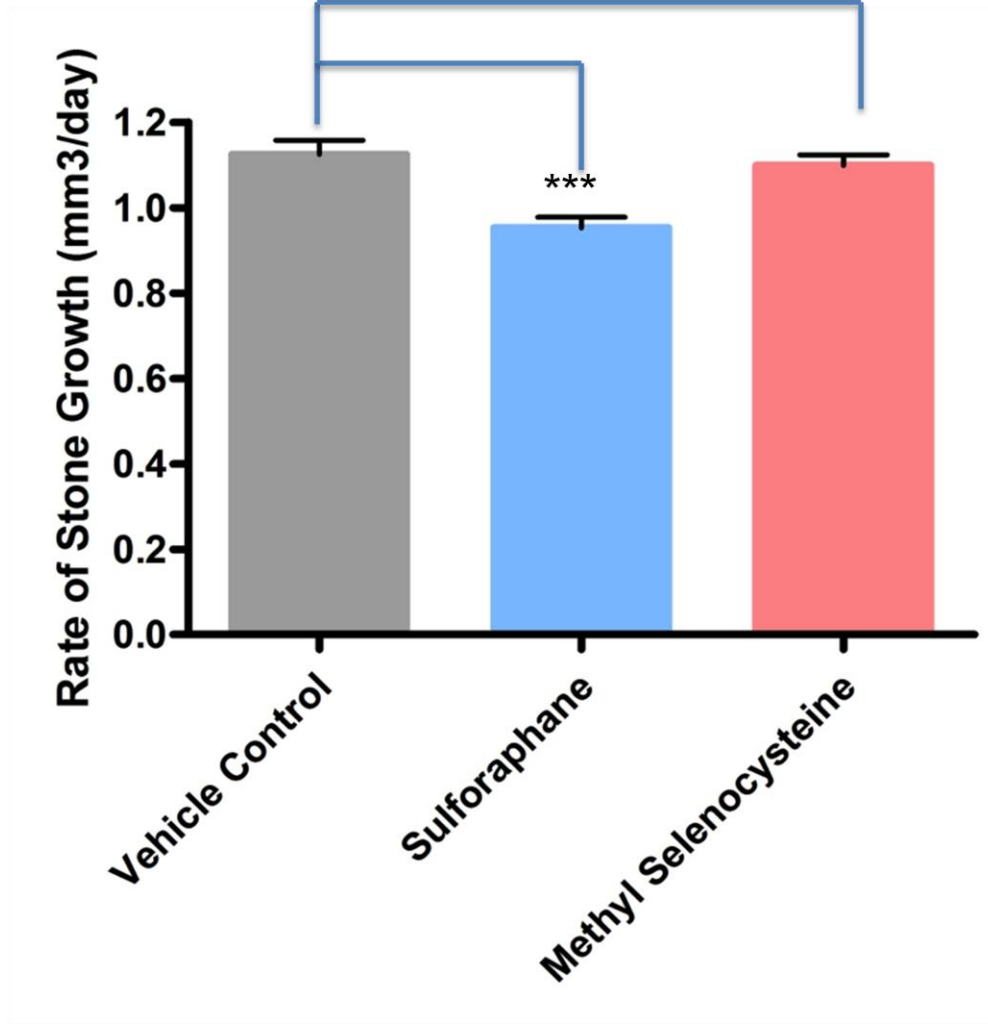


Figure 10: Sulforaphane Reduced the Rate of Stone Growth by 15%. Vehicle control and methyl selenocysteine did not significantly reduce the rate of stone growth. However, sulforaphane showed a 15% reduction in the rate of stone growth. \*\*\* Significantly different from the vehicle control and methyl selenocysteine treatment groups ( $p < 0.001$ ).

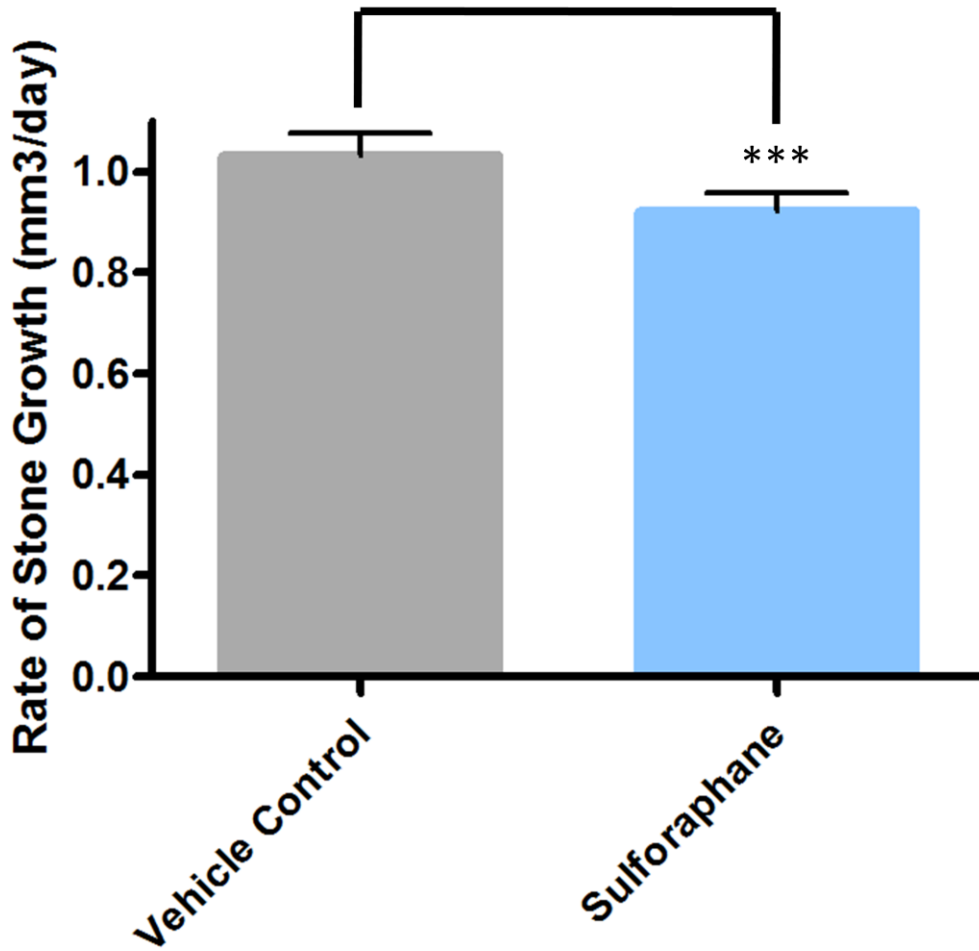


Figure 11: Sulforaphane Displayed a Similar Reduction Pattern in the Rate of Stone Growth. The study was repeated with vehicle control and sulforaphane treated groups. Statistical differences were found between the vehicle control and sulforaphane. \*\*\* Significantly different from the vehicle control group ( $p < 0.001$ ).

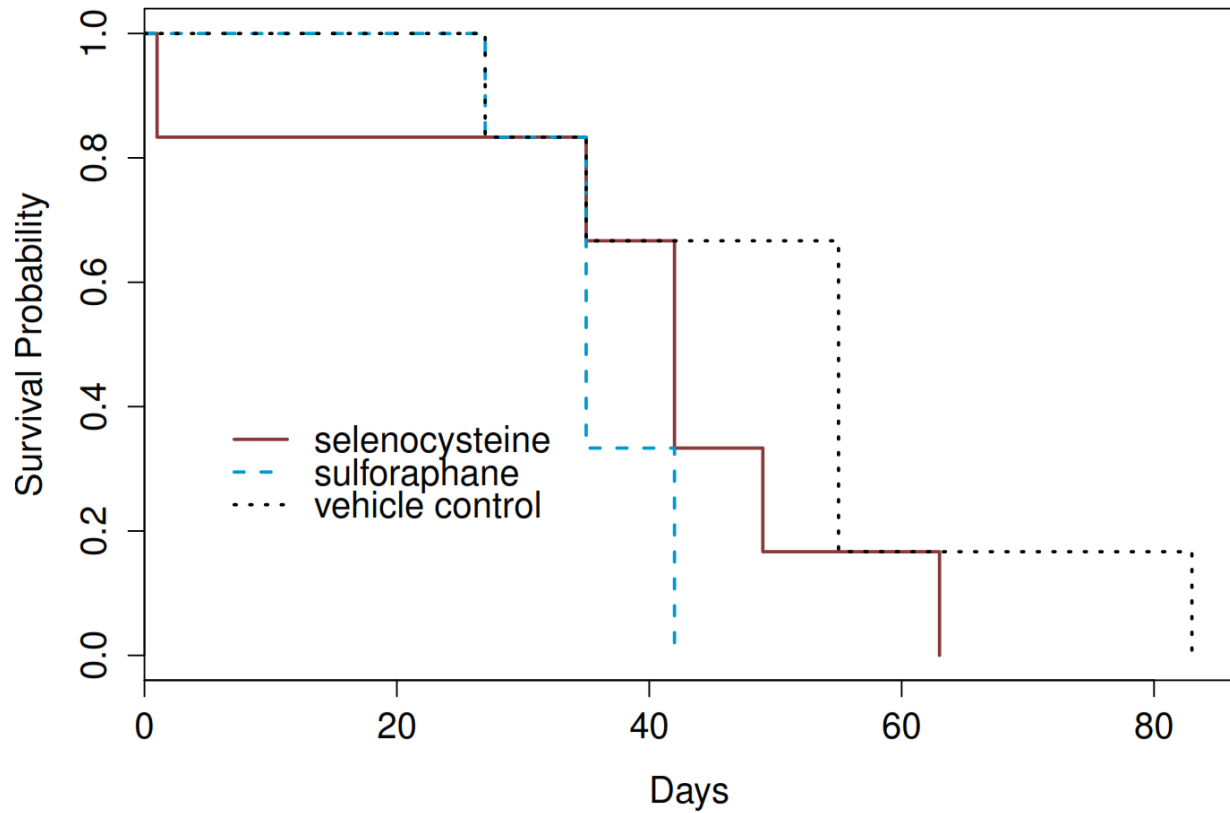


Figure 12: Proposed Interventions Do Not Show Preventative Properties. Kaplan Meier plot was used to determine the probability of having a stone event after a given period of time. Differences between the treatment groups were not found, indicating that these interventions do not possess preventative measures in reducing the rate of cystine stone growth.

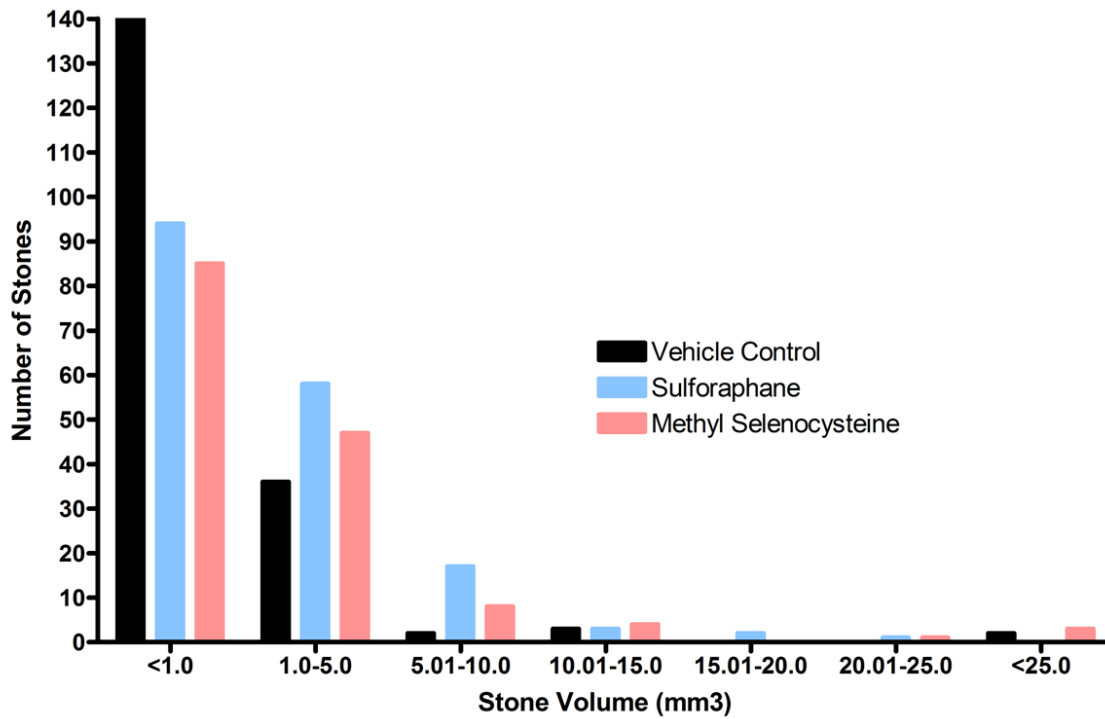


Figure 13: Sulforaphane Produces More Medium Sized Stones. Size distribution for each individual mouse in all three treatments was performed by selecting for the region of interests (ROIs) and using the CTAn package to obtain the volumetric measurements. Our results suggest that sulforaphane has a preference for medium sized stones.

## Discussion and Conclusions

Cystinuria has been researched and redefined over the years. Medical management currently consists of dietary modifications and urinary alkalization. Cystinurics are now frequently given cystine-binding thiol drugs (CBTD) to reduce cystine accumulation and/or increase cystine solubility in the urine. With the creation of the *Slc3a1*<sup>-/-</sup> mouse model for cystinuria, questions regarding treatment options - what pharmacological interventions should be investigated and what treatments should be given to reduce the rate of cystine growth - can be better answered. In this study, we determined a) the feasibility of using the  $\mu$ CT scanner in measuring 3D volumetric growth of the cystine stones and b) the efficacy of two pharmacological interventions on the *Slc3a1*<sup>-/-</sup> mouse model in reducing the rate of cystine stone growth.

### I. **Micro-Computed Tomography ( $\mu$ CT) Scanning as a Novel Approach to Tracking Stone Growth**

The  $\mu$ CT scanner has become a popular model for mapping the internal structures and surfaces in smaller organisms (Cavanaugh, 2004). The scanner has also been used in many longitudinal studies requiring quantitative information of 3D volumetric measurement changes during the lifespan of a small animal. While previous studies have employed the scanner to measure trabecular and cortical bone volume and morphology in small animals at high resolution (Yamashita, 2000; Bouxsein, 2010), measuring volumetric changes in stone volume at low resolution in live animals has not been attempted. However, a study conducted by Ercolani *et al.* (2010) revealed that the  $\mu$ CT scanner can be employed to visualize bladder stones in the *Slc3a1*<sup>-/-</sup> mouse model. Based on this, we developed a protocol using the CTAn package to measure 3D cystine stone volume over time and to also accurately measure the rate of cystine stone growth.

From the developed protocol, three treatment groups – vehicle control, sulforaphane and methyl selenocysteine – that had stones present in the bladder region were evaluated for at least a month. Comparison of stone growth rate was evaluated using linear mixed model with random intercept.

Stone growth modeled by this system was shown to proceed linearly in all the treatment groups (Figure 6 and Figure 9). To further confirm the volumetric advances in stone volume, the size of a single stone was used as a marker for stone growth in the bladder. The single stone also displayed linear growth with increases in total observed stone volume, indicating that this growth contributes to the existing supersaturated solution in the bladder (Figure 7). This suggests that a supersaturated environment may rise as a contributor to increased stone volume over time. A previous study conducted by Ercolani *et al.* (2010) indicated that the environment provided by the bladder may yield to crystal precipitation and eventually, intensify stone formation. With the  $\mu$ CT scanner and the analysis packages, the scanner has provided valuable insight by delivering accurate measurements of stone volume over a period of time in live small animals. Our results support that the  $\mu$ CT scanner is a feasible instrument in determining the rate of stone growth longitudinally.

## **II. Sulforaphane as a Potential Therapeutic in Reducing the Rate of Cystine Stone Growth**

With the use of this novel approach, the comparison of the rate of stone growth were calculated and evaluated using the linear mixed model with random intercept model. Our results suggest that sulforaphane was shown to reduce the rate of stone growth by 15% when compared to the vehicle control and methyl selenocysteine groups (Figure 10). This suggests that sulforaphane is deliverable to the kidneys (Zheng, 2011) and may upregulate SLC7A11



expression in the  $x_c^-$  system. A previous study reported that the Nrf2 had shown significant upregulation in SLC7A11 expression in the human bladder cancer cell line, T24 (Ye, 2014). Further studies investigating the link between Nrf2 and SLC7A11 may provide insights to the mechanism through which this intervention functions. Also, understanding the mechanism of action and the role of other Nrf2 activators in this mechanism are of interest.

An interesting observation from the study revealed that sulforaphane is associated with medium sized stones when compared to the vehicle control and methyl selenocysteine groups (Figure 12). This suggests that sulforaphane may be acting to promote medium size stone formation and prevent the growth of large stones. This was seen in parallel with a study conducted by Rimer *et al.* (2010) that showed CDME (l-cystine dimethylester) to promote small stone formation *in vitro*. Because sulforaphane showed a reduced effect on the rate of stone growth and the growth of medium sized stones, we conclude that sulforaphane may be a potential therapeutic for cystinurics.

### **III. Methyl Selenocysteine Did Not Show Any Effect in Reducing Cystine Stone Growth**

Methyl selenocysteine did not show a significant reduction in the rate of stone formation. This suggests that methyl selenocysteine may not be delivered to the kidneys and may not have converted to selenocysteine to dimerize with cysteine. Studies have suggested that synthesis of selenocysteine is analogous that of cysteine; however, there is no evidence to support this claim (Esaki 1981). Selenocysteine and cysteine also have been shown to affect glutathione activity in mice and rats; however, no evidence was found indicating a conversion of selenocysteine to

cysteine (Watts, 2014). Further understanding is needed in determining the conversion of methyl selenocysteine to cysteine in mammalian and mice tissue.

In order to obtain a chemical structure of the packing in cystine-based stones, understanding the arrangement of atoms in cystine stones and its chemical composition is of utmost importance. We hypothesized that selenocysteine would dimerize with cysteine and that the packing of the cystine stones was thought to be disrupted. However, our results suggest that selenocysteine may not have affected the packing of heterogeneous cystine stones and therefore, may not have displayed a significant change to the overall structure of the stones. When the crystals are packed closely together, the atomic radii may have assumed a larger distance between the molecules, giving rise to non affected cystine stones. Therefore, no significant changes were recorded in the *Slc3a1*<sup>-/-</sup> mouse model. Further studies will need to be done to confirm this possibility. Finally, a Kaplan-Meier plot was constructed to determine the probability of having another stone event (Figure 11). No significant differences were found between the treatment groups, indicating that these interventions do not possess preventative measures. Further analysis is still needed in understanding the preventative properties of cystinuria.

#### **IV. Future Directions**

While the pathophysiology of the cystine transport systems are widely known, studies can be conducted in evaluating deficiencies in the rBAT-b<sup>0,+</sup> AT transporter and the x<sub>c</sub><sup>-</sup> system. In addition, understanding how these interventions affect the cystine transporters is also of interest. The interventions used in this study were chosen based on previous studies. Understanding the mechanism of how the interventions are received and processed in the kidney might provide insight in determining and evaluating newer interventions, such as other Nrf2

activators. Further studies should also evaluate the Nrf2 properties should be determined in whether Nrf2 can increase the expression of the  $x_c^-$  transporter and cystine. A combination of these therapies may also yield to progression in understanding treatment options for cystinurics.

With the developed protocol, this study was able to use the *in vivo*  $\mu$ CT scanner in confirming the feasibility of tracking stone growth longitudinally. By using the analysis packages, the study was also able to evaluate the efficacy of the interventions in reducing the rate of cystine stone growth in the *Slc3a1*<sup>-/-</sup> mice model. These approaches displayed a linear growth in total stone volume that can be visualized through 3D models and quantified through the CTAn package. Changes in stone size distribution provided insight into the phenotypic effects of the interventions used. The intervention, sulforaphane, showed promise as a preventative therapeutic for cystinurics by displaying a reduction in the rate of stone growth. With this approach, evaluating potential therapeutics in the *Slc3a1*<sup>-/-</sup> cystinuric mouse model will further our understanding and will provide newer interventions for cystinurics.

## **Addendum**

### **Introduction**

By using the established protocol, three additional interventions – homocysteine, tiopronin and TPEN – and a zinc-supplemented diet were evaluated for their ability to prevent cystine stone formation and/or reduce the rate of cystine stone growth.

### **Effect of Homocysteine and Tiopronin in Reducing the Rate of Cystine Stone Growth**

Homocysteine is a homologue of the amino acid, cysteine. It is formed from methionine and is an intermediate in the methionine pathway. Homocysteine can be removed through the remethylation or trans-sulfuration pathway (Wu, 2012). The trans-sulfuration pathway is most related to cystinuria, in which homocysteine is readily converted to cystathionine and subsequently to cysteine, a process that mainly takes place in the kidneys. Based on the Rimer *et al.* (2010) study and the proposal for methyl selenocysteine, homocysteine is hypothesized to generate an asymmetric cysteine-homocysteine dimer. The packing of the stones will be disrupted, yielding to a reduced rate of cystine stones.

Tiopronin is an amino thiol, antioxidant compound that is currently administered to cystinuric patients (Barbey, 2000; Chow, 1966). Tiopronin functions in many capacities including acting as a chelating agent, and has cardio- and radio-protecting properties (Penugonda, 2004). Due to the free sulfhydryl group, it is highly sensitive to oxidation and can readily form disulfide dimers with other sulfhydryls (Leroy, 1991). This suggests that administering tiopronin can break the disulfide bond in cystine and form a bond with cysteine (Barbey, 2000). For the basis of our study, tiopronin is thought to also form asymmetric cysteine –tiopronin dimer, leading to a reduction in the rate of stone growth.

Homocysteine and Tiopronin were freshly prepared daily prior to administering. Each cohort received the respective dose, administered three times per week through intraperitoneal (IP) injections for the duration of the study. Homocysteine and tiopronin were dissolved in PBS. Control mice for the homocysteine and tiopronin groups received PBS only. Mice were randomized to avoid bias before being placed in the treatment groups. Preparation and dosage are summarized in Table 5.

Table 5: Preparation and Doses for Homocysteine and Tiopronin.

<b>Treatment</b>	<b>Preparation</b>	<b>Dosage</b>
Homocysteine	Dissolved in PBS	8.11mg/kg
Tiopronin	Dissolved in PBS	18.4mg/kg

A total of 11 male cystinuric mice were observed under the  $\mu$ CT scanner to ensure that the *Slc3a1*<sup>-/-</sup> mice had not formed cystine stones prior to the initiation of the study. At two months of age, the *Slc3a1*<sup>-/-</sup> mice were randomly placed in three cohorts – vehicle control (n=5), Homocysteine (n=2) and Tiopronin (n=4). The doses were chosen based on previous studies. The dose of homocysteine was based on dosage administered to humans and adjusted for mice. The dose of Tiopronin was based on the calculation used to determine L-CDME administration in the Rimer *et al.* (2010) study. The mice were imaged weekly for three months using the  $\mu$ CT scanner. Eventually, all the *Slc3a1*<sup>-/-</sup> mice formed stones. To compare the rates of stone growth between the treatment groups, linear mixed model with random intercept was employed.

Linear mixed model with random intercept was used to compare the rate of stone growth between the treatment groups. Stone growth for the treatment groups proceeded at a linear rate. Both homocysteine (chisq=28.05, df=1, p<0.001) (Figure 14) and Tiopronin (chisq=2.81, df=1, p=0.094) (Figure 15) were statistically different from the vehicle control. However, both compounds increased the rate of stone growth as opposed to reducing the rate.

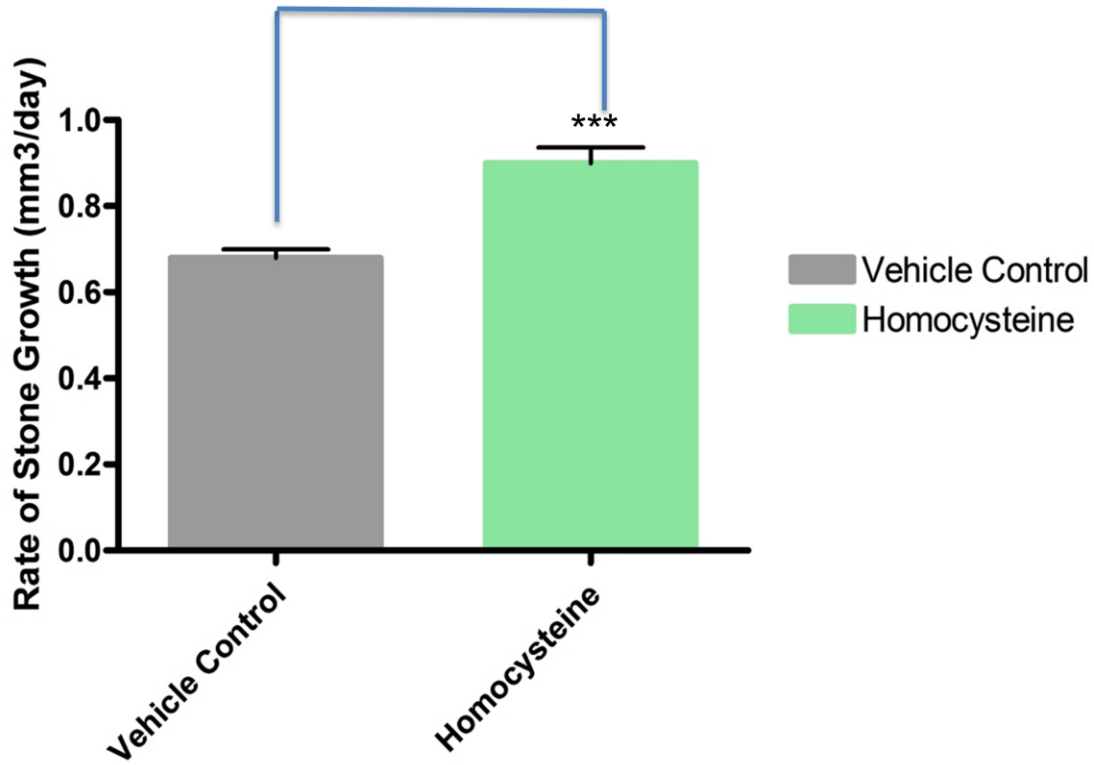


Figure 14: Homocysteine Worsens Cystine Stone Growth. Statistical differences were found when the homocysteine treatment group was compared to the vehicle control group. However, the differences displayed an increased rate of stone growth as opposed to a reduction.



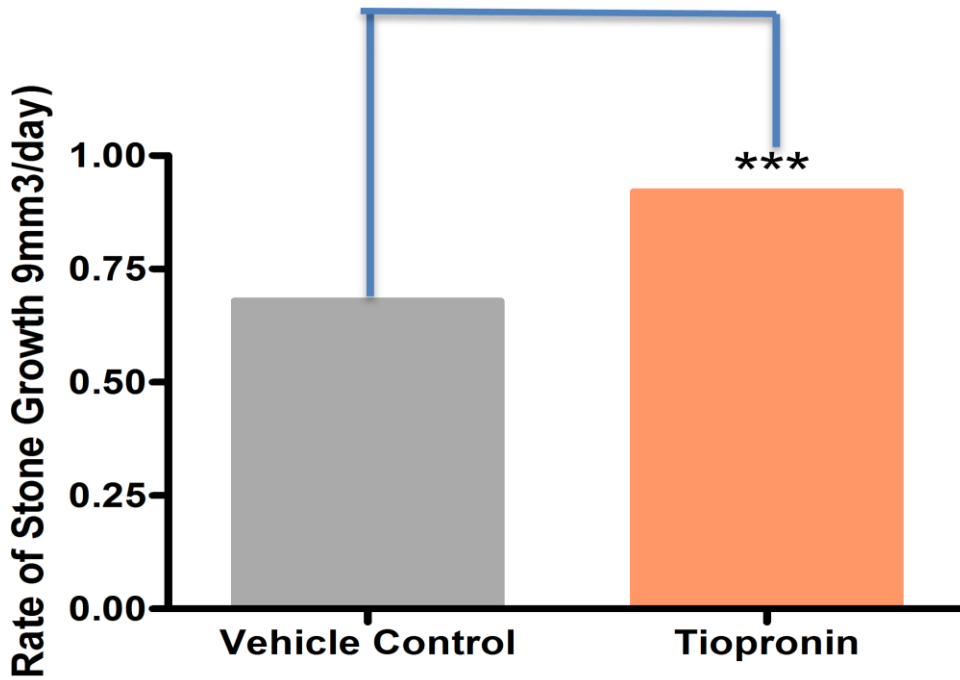
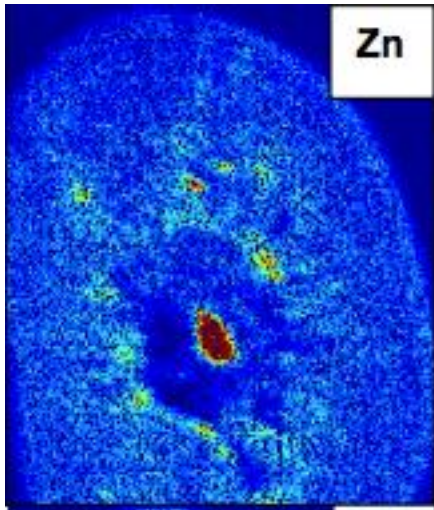


Figure 15: Tiopronin Worsens Cystine Stone Growth Rate. Statistical differences were found between the Tiopronin treatment groups and the vehicle control group. However, the differences displayed an increased rate of stone growth as opposed to a reduction.

## **Effect of TPEN and Zinc-Supplemented Diet in Reducing the Rate of Cystine Stone Growth**

N, N, N', N'-tetrakis (2-pyridylmethyl)-ethylenediamine (TPEN) is a  $Zn^{2+}/Fe^{2+}$  chelator (Shumaker, 1998). Studies have shown that the administration of TPEN decreases zinc levels in the kidney (Li, 2013; Li, 2014; Hamon, 2014). Zinc is an essential trace element that has antioxidant properties and is associated with diabetes. In the Kapahi lab (Chi, unpublished data), zinc was found to promote urinary concretions in a *Drosophila Melanogaster* (fruit fly) model of chronic stone disease. In addition, zinc was detected in the center of the stones (Figure 16). TPEN and a zinc-supplemented diet were hypothesized to affect the bioavailability of zinc in the *Slc3a1*<sup>-/-</sup> mice, thereby reducing the rate of stone growth.



20% cystine  
80% CaP

Figure 16: Zinc Detection in Cystine Stones. Zinc was shown to be present at the center of in a cystine stone.

TPEN was freshly prepared daily prior to administering. The TPEN and vehicle control cohorts received the respective dose, administered four to five times per week through intraperitoneal (IP) injections for the duration of the study. TPEN was kept in stock solution of 100% EtOH and diluted to 5% with the addition of PBS. Control mice in this group received 5% EtOH in PBS only. The zinc-supplemented diet was prepared by Harlan Laboratories (Haslett, MI). Control mice for this diet received Harlan Irradiated Pellet Rodent Diet (chow diet). All animals from zinc-supplemented cohort were fed weekly. Animals were randomized to avoid bias before being placed in the treatment groups. Preparation and dosage are summarized in Table 6.

Table 6: Preparation and Doses for TPEN and Zinc-Supplemented Diet.

<b>Treatment</b>	<b>Preparation</b>	<b>Dosage</b>
TPEN	Stock Solution in 100% EtOH.  Administered in 5%EtOH in  PBS	25mg/kg
Zinc-Supplemented Diet	Prepared by Harlan  Laboratories	250 ppm

A total of 33 male *SLC3A1*<sup>-/-</sup> mice were observed under the  $\mu$ CT scanner to ensure that cystine stones had not formed prior to the initiation of the study. At the age of two months, the *Slc3a1*<sup>-/-</sup> mice were placed in two cohorts – vehicle control (n=8) and TPEN (n=7). At one month of age, another cohort of *Slc3a1*<sup>-/-</sup> mice were placed in two groups – vehicle control (n=8) and zinc-supplemented diet (n=10). The mice were imaged weekly for three months using the  $\mu$ CT scanner. Eventually, all of the *Slc3a1*<sup>-/-</sup> mice formed stones.

Linear mixed model with random intercept was employed, yielding the rate of growth. Stone growth for both treatments proceeded at a linear rate. There was a statistical difference between the vehicle control and TPEN group (chisq=10.71, df=1, p=0.001), indicating that TPEN may be an effective treatment for cystinurics. TPEN was shown to reduce the rate of cystine stone growth by 18% (Figure 17). However, no statistical difference was found in the zinc-supplemented diet (chisq=0.005, p=0.943) (Figure 18).

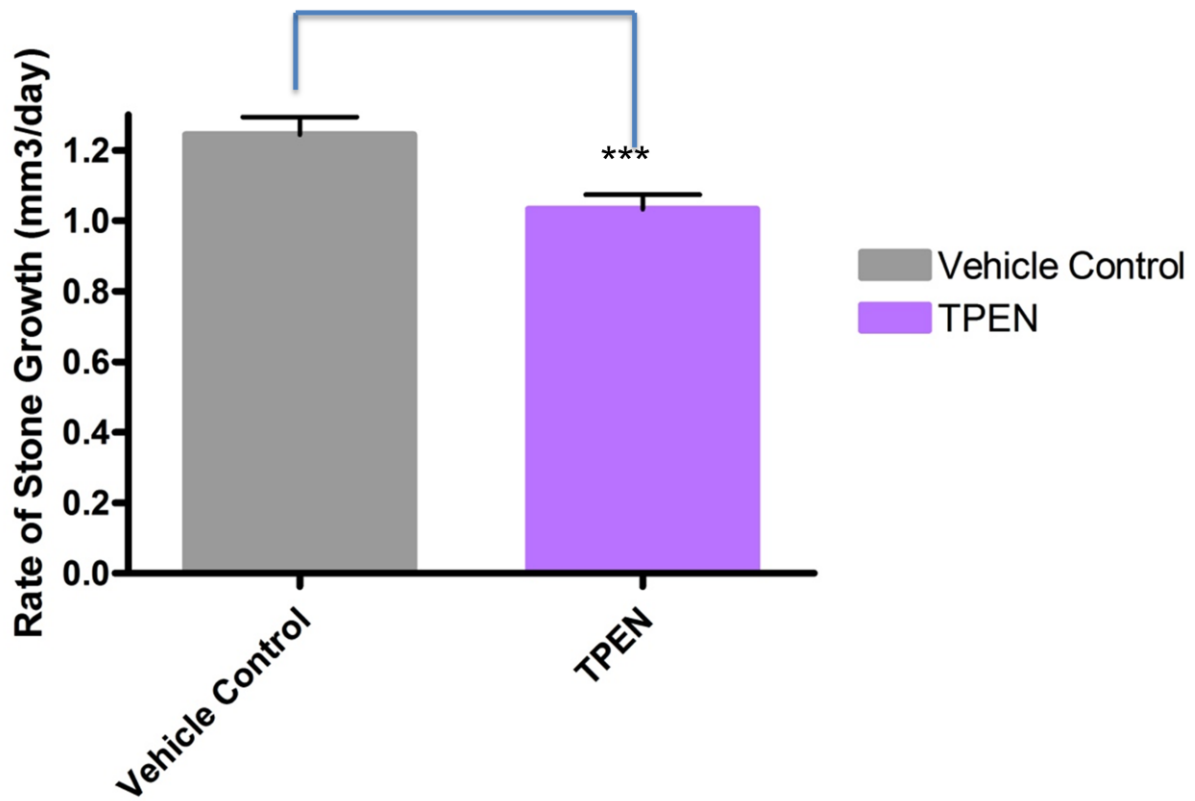


Figure 17: TPEN Reduced the Rate of Cystine Stone Growth by 18%. Statistical differences were found between the TPEN and vehicle control treatment groups.

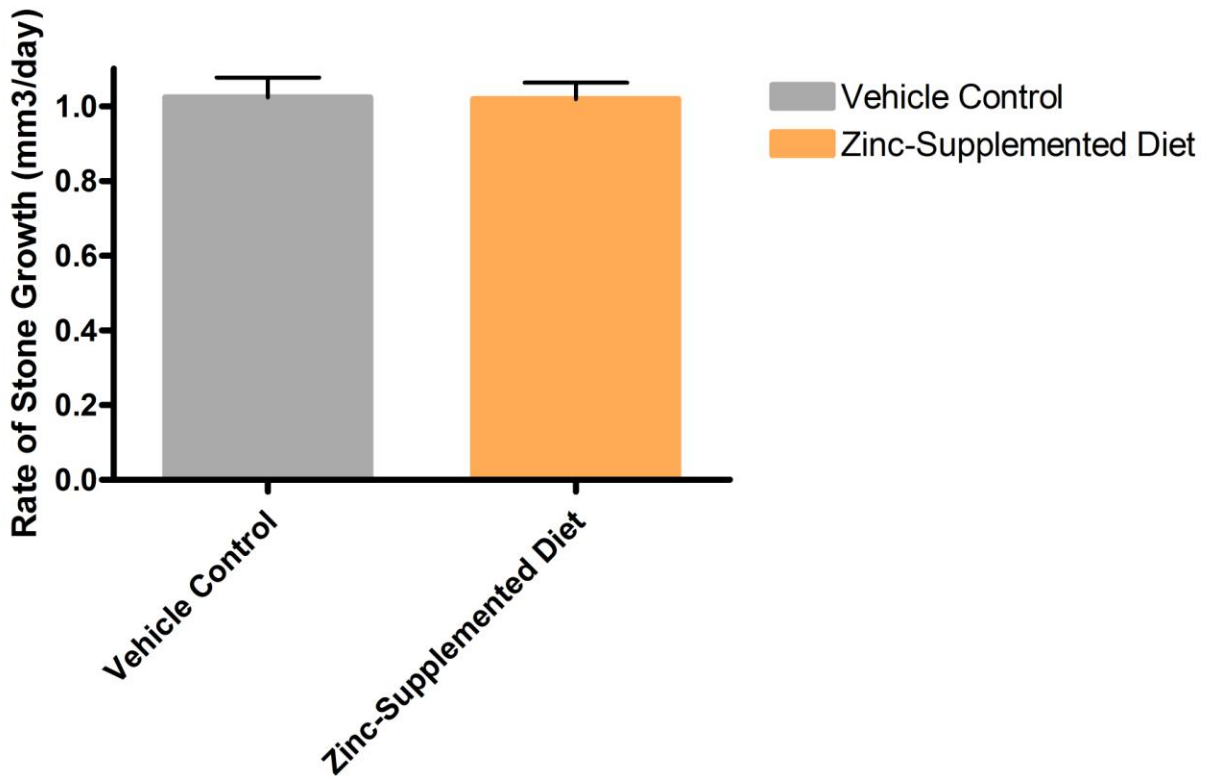


Figure 18: Zinc-Supplemented Diet Did Not Display Any Effect on Cystine Stone Growth Rate. There were no statistical differences between the zinc-supplemented diet group and the vehicle control group.



## **Discussion and Conclusions**

### **I. Homocysteine Worsened Cystine Stone Growth**

In this study, we were able to evaluate three additional interventions— homocysteine, tiopronin and TPEN - and a zinc-supplemented diet – in reducing the rate of cystine stone growth. All interventions and the supplemented diet displayed a linear growth pattern. However, homocysteine and tiopronin were shown to increase the rate of stone formation in our *Slc3a1*<sup>-/-</sup> cystinuric model. This suggests that homocysteine may not have converted to cysteine to form a dimer or may have prompted the formation of cystine. Homocysteine has also been shown to bind to proteins, allowing its exportation out of the cell (Svardal, 1986). This suggests that protein-protein interactions may have taken place within the cell. Further understanding is needed in the proteins interactions within and between cystine, cysteine and homocysteine in the urine.

### **II. Currently Prescribed, Tiopronin, Did Not Reduce the Rate of Cystine Stone Growth**

In addition to homocysteine, tiopronin, a prescribed medication for cystinuric patients, also displayed an increased rate of stone growth. This is contradicted by multiple studies that have shown that tiopronin treatment is critical to treatment of cystinuria. These studies have shown that with tiopronin treatment, cystine excretion was remarkably higher in cystinuric patients (Joly, 1999). However, its effectiveness is debated. Toxicity was a possibility; however a study conducted by Zhang *et al.* (1999) revealed that with tiopronin treatment, nephrotoxic effects in *in vivo* rat kidneys were protected. This indicates that tiopronin may have acted to protect kidney function as opposed to forming cysteine dimmers.

### **III. Zinc Bioavailability May Affect Cystine Stone Growth**

Our results confirmed the efficacy of TPEN in reducing the rate of stone growth and that zinc-supplemented displayed no effect. This suggests that TPEN is deliverable to the kidneys (Tainer, 1982) and the chelating properties of zinc may have affected zinc bioavailability in the cystine stones. Interestingly, a study conducted by Ho *et al.* (2003) revealed that p53 expression, a tumor suppressor, is increased by zinc depletion. Another study conducted by Jiang *et al.* (2015) revealed that p53 plays a role in inhibiting cystine uptake through the reduced of SLC7A11. In addition, zinc homeostasis is heavily regulated, indicating that zinc may have not been absorbed and therefore, may have been excreted; however, further studies will need to be conducted to confirm the possibility.

### **IV. Future Directions**

Future studies in understanding the roles of homocysteine, TPEN and zinc bioavailability may provide further knowledge in the role of zinc and p53 in cystinuria as well as the mechanistic action that is taking place. Finally, evaluating early stones and late stones may provide new knowledge in the how the treatments are configured in the mouse cystinuric model. With the evaluation of the presented and future therapeutics, the incident of stone formation will decrease for cystinurics and will be a presentable treatment option.

## References

- Asplin DM & Asplin JR (2013). The interaction of thiol drugs and urine pH in the treatment of cystinuria. *The Journal of Urology*, 189(6), 2147-2151.
- Barbey F, Joly D, Rieu P, Mejean A, Daudon M, & Jungers P (2000). Medical treatment of cystinuria: Critical reappraisal of long-term results. *The Journal of Urology*, 163(5), 1419-1423.
- Bhatta KM, Prien EL & Dretler SP (1989). Cystine calculi-rough and smooth: A new clinical distinction. *The Journal of Urology*, 142(4), 937-940.
- Botzenhart E, Vester U, Schmidt C, Hesse A, Halber M, Wagner C, Lang F, Hoyer P, Zerres K, & Eggermann T (2002). Cystinuria in children: Distribution and frequencies of mutations in the SLC3A1 and SLC7A9 genes. *Kidney International*, 62(4), 1136-1142.
- Brinkmann OA, Griehl A, Kuwertz-Broking E, Bulla M & Hertle L (2001). Extracorporeal shock wave lithotripsy in children efficacy, complications and long-term follow-up. *European Urology*, 39(5), 591-597.
- Calonge MJ, Gasparini P, Chillaron J, Chillon M, Gallucci M, Rousaud F, Zelante L, Testar X, Dallapiccola B, & Di Silverio F (1994). Cystinuria caused by mutations in rBAT, a gene involved in the transport of cystine. *Nature Genetics*, 6(4), 420-425.
- Choong S, Whitfield H, Duffy P, Kellett M, Cuckow P, Van't Hoff W & Corry D (2000). The management of paediatric urolithiasis. *BJU International*, 86(7), 857-860.

Chow GK & Strem SB. (1996). Medical treatment of cystinuria: Results of contemporary clinical practice. *The Journal of Urology*, 156(5), 1576-1578.

Chow GK & Strem SB. (1996). Medical treatment of cystinuria: Results of contemporary clinical practice. *The Journal of Urology*, 156(5), 1576-1578.

Claes D & Jackson E. (2012).Cystinuria: Mechanisms and management. *Pediatric Nephrology*, 27(11), 2031-2038.

Clarke JD, Hsu A, Williams DE, Dashwood RH, Stevens JF, Yamamoto M & Ho E (2011). Metabolism and tissue distribution of sulforaphane in Nrf2 knockout and wild-type mice. *Pharmaceutical Research*, 28(12), 3171-3179.

Coe FL & Asplin JR. (2010).Medicine.stopping the stones. *Science (New York, N.Y.)*, 330(6002), 325-326.

Coe FL, Evan AP, Worcester EM, Lingeman JE (2010). Three pathways for human kidney stone formation. *Urological Research*, 38(3), 147-160.

Cohen TD, Strem SB, & Hall, P. (1995).Clinical effect of captopril on the formation and growth of cystine calculi. *The Journal of Urology*, 154(1), 164-166.

Crawhall JC, Scowen EF, & Qatts, RW.(1963). Effect of penicillamine on cystinuria. *British Medical Journal*, 1(5330), 588-590.

- Cui W, Bai Y, Miao X, Luo P, Chen Q, Tan Y, Rane MJ, Miao L & Cai L (2012). Prevention of diabetic nephropathy by sulforaphane: Possible role of Nrf2 upregulation and activation. *Oxidative Medicine and Cellular Longevity*, 2012, 821936.
- Delakas D, Karyotis I, Daskalopoulos G, Lianos E, Mavromanolakis E (2003). Independent predictors of failure of shockwave lithotripsy for ureteral stones employing a second-generation lithotripter. *Journal of Endourology / Endourological Society*, 17(4), 201-205.
- Dello SL, Pras E, Pontesilli C, Beccia E, Ricci-Barbini V, De Sanctis L, Ponzzone A, Gallucci M, Bisceglia L, Zelante L, Jimenez-Vidal M, Font M, Zorzano A, Rousaud F, Nunes V, Gasparini P, Palacin M & Rizzoni G (2002). Comparison between SLC3A1 and SLC7A9 cystinuria patients and carriers: A need for a new classification. *Journal of the American Society of Nephrology : JASN*, 13(10), 2547-2553.
- Dent CE & Senior B. (1955). Studies on the treatment of cystinuria. *British Journal of Urology*, 27(4), 317-332.
- Eggermann T, Venghaus A & Zerres K. (2012). Cystinuria: An inborn cause of urolithiasis. *Orphanet Journal of Rare Diseases*, 7, 19-1172-7-19.
- Elbarbry F & Elrody N. (2011). Potential health benefits of sulforaphane: A review of the experimental, clinical and epidemiological evidences and underlying mechanisms. *Journal of Medicinal Plants Research*, 5(4), 473-484.

- Elkoushy MA, Nady M, Abdel Hafez ES (2011). Predicting the fragility of renal calculi in response to shock wave lithotripsy through their radiographic appearance. *African Journal of Urology*, 17(2), 37-42.
- Ercolani M, Sahota A, Schuler C, Yang M, Evan AP, Reimer D, Barone JG, Tischfield JA, & Levin RM (2010). Bladder outlet obstruction in male cystinuria mice. *International Urology and Nephrology*, 42(1), 57-63.
- Esaki N, Nakamura T, Tanaka H, Suzuki T, Morino Y & Soda K (1981). Enzymic synthesis of selenocysteine in rat liver. *Biochemistry*, 20(15), 4492-4496.
- Evan AP. (2010). Physiopathology and etiology of stone formation in the kidney and the urinary tract. *Pediatric Nephrology (Berlin, Germany)*, 25(5), 831-841.
- Evan AP, Coe FL, Lingeman JE, Shao Y, Matlaga BR, Kim SC, Bledsoe SB, Sommer AJ, Grynepas M, Phillips CL, & Worcester EM (2006). Renal crystal deposits and histopathology in patients with cystine stones. *Kidney International*, 69(12), 2227-2235.
- Fattah H, Hambaroush Y & Goldfarb DS.(2014). Cystine nephrolithiasis. *Translational Andrology and Urology*, 3(3), 228-233.
- Feliubadalo L, Arbones ML, Manas S, Chillaron J, Visa J, Rodes M, Rousaud F, Zorzano A, Palacin M & Nunes V (2003). Slc7a9-deficient mice develop cystinuria non-I and cystineurolithiasis. *Human Molecular Genetics*, 12(17), 2097-2108.
- Feliubadalo L, Font M, Purroy J, Rousaud F, Estivill X, Nunes V, Golomb E, Centola M, Aksentijevich I, Kreiss Y, Goldman B, Pras M, Kastner DL, Pras E, Gasparini P, Bisceglia

L, Beccia E, Gallucci M, De Santis L, Ponzzone A, Rizzoni GF, Zelante L, Bassi MT, George AL, Manzoni M, De Grandi A, Riboni M, Endsley JK, Ballabio A, Borsani G, Reig N, Fernandez E, Estevez R, Pineda M, Torrents D, Camps M, Lloberas J, Zorzano A, & Palacin M (1999). Non-type I cystinuria caused by mutations in SLC7A9, encoding a subunit (bo,+AT) of rBAT. *Nature Genetics*, 23(1), 52-57.

Fernandez E, Carrascal M, Rousaud F, Abian J, Zorzano A, Palacin M & Chillaron J (2002). rBAT-b(0,+AT) heterodimer is the main apical reabsorption system for cystine in the kidney. *American Journal of Physiology.Renal Physiology*, 283(3), F540-8.

Fjellstedt E, Denneberg T, Jeppsson JO, Christensson A & Tiselius HG (2001). Cystine analyses of separate day and night urine as a basis for the management of patients with homozygous cystinuria. *Urological Research*, 29(5), 303-310.

Font MA, Feliubadalo L, Estivill X, Nunes V, Golomb E, Kreiss Y, Pras E, Bisceglia L, d-Adamo AP, Zelante L, Gasparini P, Bassi MT, George AL, Manzoni M, Riboni M, Ballabio A, Borsani G, Reig N, Fernandez E, Zorzano A, Bertran J, & Palacin M (2001). Functional analysis of mutations in SLC7A9, and genotype-phenotype correlation in non-type I cystinuria. *Human Molecular Genetics*, 10(4), 305-316.

Font-Llitjos M, Feliubadalo L, Espino M, Cleries R, Manas S, Frey IM, Puertas S, Collet G, Palomo S, Aranda J, Visa J, Palacin M & Nunes V (2007). Slc7a9 knockout mouse is a good cystinuria model for antilithiasic pharmacological studies. *American Journal of Physiology.Renal Physiology*, 293(3), F732-40.

- Friedman AN, Bostom AG, Selhub J, Levey AS & Rosenberg IH (2001). The kidney and homocysteine metabolism. *J Am Soc Nephrol*, 12, 2181-2189.
- Goldfarb DS. (2011). Potential pharmacologic treatments for cystinuria and for calcium stones associated with hyperuricosuria. *Clinical Journal of the American Society of Nephrology : CJASN*, 6(8), 2093-2097.
- Goldfarb DS, Coe FL & Asplin JR. (2006). Urinary cystine excretion and capacity in patients with cystinuria. *Kidney International*, 69(6), 1041-1047.
- Hamon R, Homan CC, Tran HB, Mukaro VR, Lester SE, Roscioli E, Bosco MD, Murgia CM, Ackland ML, Jersmann HP, Lang C, Zalewski PD & Hodge SJ (2014). Zinc and zinc transporters in macrophages and their roles in efferocytosis in COPD. *PloS One*, 9(10), e110056.
- Harada M, Zhao-Ren KO & Kamidono S. (1992). Experience with extracorporeal shock wave lithotripsy for cystine calculi in 20 renal units. *Journal of Endourology*, 6(3), 213-215.
- Harbar JA, Cusworth DC, Lawes LC, Wrong OM (1986). Comparison of 2-mercaptopropionylglycine and D-penicillamine in the treatment of cystinuria. *The Journal of Urology*, 136(1), 146-149.
- Ho E, Courtemanche C & Ames BN (2003). Zinc deficiency induces oxidative DNA damage and increases p53 expression in human lung fibroblasts. *J. Nutri.*, 133(8): 2543-2548.



- Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S & Yamamoto M (2000). Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *The Journal of Biological Chemistry*, 275(21), 16023-16029.
- Jiang L, Kon N, Li T, Wang SJ, Su T, Hibshoosh H, Baer R & Gu W (2015). Ferroptosis as a p53-mediated activity during tumor suppression. *Nature*, 520(7545), 57-62.
- Johri N, Cooper B, Robertson W, Choong S, Rickards D & Unwin R (2010). An update and practical guide to renal stone management. *Nephron. Clinical Practice*, 116(3), c159-71.
- Joly D, Rieu P, Mejean A, Gagnadoux MF, Daudon M & Jungers P (1999). Treatment of cystinuria. *Pediatr Nephrol*, 13(9), 945-950.
- Johnson JA, Johnson DA, Kraft AD, Calkins MJ, Jakel RJ, Vargas MR & Chen PC (2008). The Nrf2-ARE pathway: An indicator and modulator of oxidative stress in neurodegeneration. *Ann. N.Y. Acad. Sci.*, 1147/; 61-69.
- Kachel TA, Vijan SR & Dretler SP. (1991). Endourological experience with cystine calculi and a treatment algorithm. *The Journal of Urology*, 145(1), 25-28.
- Kim SC, Burns EK, Lingeman JE, Paterson RF, McAteer JA, & Williams JC (2007). Cystine calculi: Correlation of CT-visible structure, CT number, and stone morphology with fragmentation by shock wave lithotripsy. *Urological Research*, 35(6), 319-324.
- Knoll T, Zollner A, Wendt-Nordahl G, Michel MS, & Alken P (2005). Cystinuria in childhood and adolescence: Recommendations for diagnosis, treatment, and follow-up. *Pediatric Nephrology (Berlin, Germany)*, 20(1), 19-24.

- Leroy P, Nicolas A, Gavriloff C, Matt M, Netter P, Bannwarth B, Hercelin B & Mazza M (1991). Determination of 2-mercaptopropionylglycine and its metabolite, 2-mercaptopropionic acid, in plasma by ion-pair reversed-phase high-performance liquid chromatography with post-column derivatization. *Journal of Chromatography*, 564(1), 258-265.
- Li B, Tan Y, Sun W, Fu Y, Miao L & Cai L(2013). The role of zinc in the prevention of diabetic cardiomyopathy and nephropathy. *Toxicology Mechanisms and Methods*, 23(1), 27-33.
- Li B, Cui W, Tan Y, Luo P, Chen Q, Zhang C, Qu W, Miao L & Cai L (2014). Zinc is essential for the transcription function of Nrf2 in human renal tubule cells in vitro and mouse kidney in vivo under the diabetic condition. *Journal of Cellular and Molecular Medicine*, 18(5), 895-906.
- Lotz M & Bartter FC.(1965). Stone dissolution with D-penicillamine in cystinuria. *British Medical Journal*, 2(5475), 1408-1409.
- Mandal T & Ward MD (2013). Determination of specific binding interactions at L-cystine crystal surfaces with chemical force microscopy. *Journal of the American Chemical Society*, 135(15), 5525-5528.
- Mattoo A & Goldfarb DS. (2008). Cystinuria. *Seminars in Nephrology*, 28(2), 181-191.
- Meschi T, Maggiore U, Fiaccadori E, Schianchi T, Bosi S, Adorni G, Ridolo E, Guerra A, Allegri F, Novarini A & Borghi L (2004). The effect of fruits and vegetables on urinary stone risk factors. *Kidney International*, 66(6), 2402-2410.

- Muslumanoglu AY, Tefekli A, Sarilar O, Binbay M, Altunrende F & Ozkuvanci U (2003). Extracorporeal shock wave lithotripsy as first line treatment alternative for urinary tract stones in children: A large scale retrospective analysis. *JUrol*, 170, 2405-2408.
- Pak CY, Fuller C, Sakhaee K, Zerwekh JE & Adams BV (1986). Management of cystine nephrolithiasis with alpha-mercaptopyronylglycine. *JUrol*, 136, 1003-1008.
- Palacin M, Fernandez E, Chillaron J & Zorzano A (2001). The amino acid transport system b(o,+) and cystinuria. *Molecular Membrane Biology*, 18(1), 21-26.
- Pearle MS, Calhoun EA & Curhan GC (2005). Urologic diseases in America project: Urolithiasis. *The Journal of Urology*, 173(3), 848-857.
- Penugonda S, Wu W, Mare S & Ercal N (2004). Liquid chromatography analysis of N-(2-mercaptopyronyl)-glycine in biological samples by ThioGlo 3 derivatization. *Journal of Chromatography.B, Analytical Technologies in the Biomedical and Life Sciences*, 807(2), 251-256.
- Rimer JD, An Z, Zhu Z, Lee MH, Goldfarb DS, Wesson JA & Ward MD(2010). Crystal growth inhibitors for the prevention of L-cystine kidney stones through molecular design. *Science (New York, N.Y.)*, 330(6002), 337-341.
- Rodman JS, Blackburn P, Williams JJ, Brown A, Pospischil MA & Peterson CM (1984).The effect of dietary protein on cystine excretion in patients with cystinuria. *Clinical Nephrology*, 22(6), 273-278.

Sunde RA, Raines AM, Barnes KM & Evenson JK (2011). Selenium status highly-regulates selenoprotein mRNA levels for only a subset of the selenoproteins in the selenoproteome.

*Biosci Rep*, 29(5), 329-338.

Sasaki H, Sato H, Kuriyama-Matsumura K, Sato K, Maebara K, Wang H, Tamba M, Itoh K, Yamamoto M & Bannai S (2002). Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression. *The Journal of Biological Chemistry*,

277(47), 44765-44771.

Schuster TG, Russell KY, Bloom DA, Koo HP & Faerber GJ (2002). Ureteroscopy for the treatment of urolithiasis in children. *JUrol*, 167, 1813.

Shumaker DK, Vann LR, Goldberg MW, Allen TD & Wilson KL (1998). TPEN, a Zn<sup>2+</sup>/Fe<sup>2+</sup> chelator with low affinity for Ca<sup>2+</sup>, inhibits lamin assembly, destabilizes nuclear architecture and may independently protect nuclei from apoptosis in vitro. *Cell Calcium*, 23(2-3), 151-164.

Silbernagl S. (1988). The renal handling of amino acids and oligopeptides. *Physiological Reviews*, 68(3), 911-1007.

Singh A, Misra V, Thimmulappa RK, Lee H, Ames S, Hoque MO, Herman JG, Baylin SB, Sidransky D, Gabrielson E, Brock MV & Biswal S (2008). Dysfunctional Keap1-Nrf2 interaction in non-small-cell lung cancer. *PLoS Med*, 3(10): e420.

- Svardal A, Refsum H & Ueland PM. (1986). Determination of in vivo protein binding of homocysteine and its relation to free homocysteine in the liver and other tissues of the rat. *J Biol Chem*, 261, 3156-3162.
- Tainer LB, Sokoloff J, Halpern SE & Taylor A (1982). Limitations of renal function scanning in acute obstruction. *International Journal of Nuclear Medicine and Biology*, 9(3): 181-183, 185-187.
- Tiselius HG, Ackermann D, Alken P, Buck C, Conort P & Gallucci M (2001). Guidelines on urolithiasis. *EurUrol*, 40, 362-371.
- Watts SD, Torres-Salazar D, Divito CB & Amara SG (2014). Cysteine transport through excitatory amino acid transporter 3 (EAAT3). *PloS One*, 9(10), e109245.
- Wu CC, Zheng CM, Lin YF, Lo L, Liao MT & Lu KC(2012). Role of homocysteine in end-stage renal disease. *Clinical Biochemistry*, 45(16-17), 1286-1294.
- Xu H, Zisman AL, Coe FL & Worcester EM (2013). Kidney stones: An update on current pharmacological management and future directions. *Expert Opinion on Pharmacotherapy*, 14(4), 435-447.
- Ye P, Mimura J, Okada T, Sato H, Liu T, Maruyama A, Ohyama C & Itoh K (2014). Nrf2- and ATF4-dependent upregulation of xCT modulates the sensitivity of T24 bladder carcinoma cells to proteasome inhibition. *Molecular and Cellular Biology*, 34(18), 3421-3434.

Zhang J, Wang X & Xu T (2008). Elemental selenium at nano size (nano-se) as a potential chemopreventive agent with reduced risk of selenium toxicity: Comparison with selenomethylselenocysteine in mice. *Toxicological Sciences*, 101(1), 22-22.

Zhang JG (1999). Tiopronin protects against nephrotoxicity of cisplatin in the rat. *Hum Exp Toxicol*, 18(12), 713-717.

Zheng H, Whitman SA, Wu W, Wondrak GT, Wong PK, Fang D & Zhang DD(2011). Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy. *Diabetes*, 60(11), 3055-3066.