A Potential New Method to Estimate Tissue Cystine Content in Nephropathic Cystinosis

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Objectives To evaluate intestinal mucosal cystine crystal (CC) load as a way to estimate tissue cystine content in children with cystinosis.

Study design Intestinal mucosal biopsies were obtained endoscopically from children (ages 2-18 years) with cystinosis. Using a special processing technique, CC within histiocytes were easily visible and enumerable in the mucosal tissue. Mean CC counts, calculated from stomach and duodenum combined (CC-GD), were correlated with duration of cysteamine treatment, estimated glomerular filtration rate (eGFR), and mean white blood cells (WBC) cystine levels.

Results Seventeen subjects (6 male) were enrolled in 2 studies from 2001 and 2003. The CC-GD count (mean 12.5 ± 1.41 crystals/histiocyte) was lower than the colonic crystal count (mean 23.6 ± 3.38, $P = .0031$). Nine of 17 subjects underwent repeated endoscopy 2 years later and the trend for CC-GD was to decrease over time ($P = .065$). Biopsies, however, were never completely depleted of CC. In subjects who were diagnosed before age 18 months, the percent change from baseline of both eGFR and CC-GD were inversely correlated ($P = .026$). Mean WBC cystine levels were positively correlated with CC-GD ($P = .023$).

Conclusions CC are easily visible in the intestinal mucosa. CC-GD counts appear to correlate with eGFR and may help monitor response to treatment. Even when mean WBC cystine levels are low, the mucosal CC are not depleted suggesting that tissue cysteamine levels may not achieve therapeutic efficacy. *(J Pediatr 2012; ■: ■-■).*

Nephopathic cystinosis, a rare autosomal recessive disorder characterized by a membrane transport defect, causes intralysosomal accumulation of cystine and is associated with progressive renal failure.[1] Cystine accumulation occurs in all body tissues and can often be seen in crystal form. The aim of therapy is to reduce tissue cystine, which can be achieved by administering the aminothiol agent cysteamine (Cystagon; Mylan, Morgantown, West Virginia) every 6 hours. Cysteamine reacts with accumulated cystine to form the mixed disulfide of cysteamine and cysteine, which then leaves the lysosome through a different transport system.[1,2] Lifelong regular cysteamine therapy is challenging because of the nocturnal dosing as well as the drug's known side effects such as odor and gastrointestinal (GI) symptoms.[3,4] Compliance with therapy may therefore be a problem leading to earlier renal transplantation.[5,6]

In clinical practice, the response to cysteamine is determined by measuring pre-dose white blood cell (WBC) cystine levels. WBC cystine measurements, however, may not reliably reflect body tissue cystine levels. A single dose of cysteamine can transiently normalize WBC cystine levels,[7,8] and circulating peripheral WBCs may be exposed to higher concentrations of cysteamine than would be found in body tissues. Unfortunately, a method to evaluate tissue cystine levels and long-term response to therapy does not exist for cystinosis. The most accurate way to measure tissue cystine accumulation would be to biopsy organs such as kidney, skin, or muscle, but this would not be feasible on a regular basis. Cystine crystals (CC) within histiocytes have recently been identified in endoscopically obtained intestinal mucosal biopsies.[4,9] In the same way that corneal CC density is used to monitor response to topical ocular cysteamine therapy, intestinal mucosal CC load may provide comparable information.

This study examined the CC content of intestinal tissue as a potential method for monitoring tissue cystine content in nephropathic cystinosis. We describe a method for preparing endoscopically obtained mucosal tissue to visualize CC and also to correlate intestinal CC load with duration of cysteamine therapy, estimated glomerular filtration rate (eGFR), and mean WBC cystine levels.

CC	Cystine crystal
CC-GD	Mean CC counts, calculated from stomach and duodenum combined
CC-5H	Mean CC load from the 5 histiocytes with the highest number of crystal was also calculated
eGFR	Estimated glomerular filtration rate
GI	Gastrointestinal
LM	Light microscopy
WBC	White blood cell

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Methods

In 2001 and 2003, 2 studies were undertaken in children with cystinosis to assess the effect of cysteamine bitartrate on the intestinal tract.[4,9] As part of these studies, subjects underwent upper GI endoscopy with biopsies. Some subjects also underwent flexible proctosigmoidoscopy. The studies were approved by the University of California at San Diego Human Subjects Committee, and informed consent was obtained for each participant. Study patients were transported and admitted to the University of California at San Diego General Clinical Research Center.

Children with cystinosis, 2-18 years of age, who were reported to be taking regular 6 hourly cysteamine bitartrate participated in the study. Subjects had a mean WBC cystine level of <2.0 nmol half-cystine per mg protein for at least 1 year before entering the study. All available WBC cystine levels were obtained for each subject. Acid suppressants, antibiotics, nonsteroidal anti-inflammatory drugs, pro-kinetic agents, and antihistamines were discontinued 2 weeks prior to admission. None of the patients had undergone renal transplantation.

Upper GI Endoscopy and Proctosigmoidoscopy with Biopsies

These were performed under general anesthesia using an Olympus GIF 100 or P140 endoscope (Olympus, Melville, New York). Endoscopic findings were reported in a conventional manner. Three mucosal biopsies were taken from each of the esophagus, gastric body, duodenum, and, when applicable, from the rectum/sigmoid colon. Formalin-fixed biopsies were processed routinely, cut at 4 u, stained with hematoxylin and eosin and examined by light microscopy (LM) at ×400 magnification. CC are not visible in the hematoxylin and eosin stained sections. Biopsies were therefore also fixed in glutaraldehyde, embedded in epoxy resin, cut at 1 u, and stained with toluidine blue. Using this technique, CC within histiocytes were clearly visible using LM at ×1000 magnification (Figure 1, A). The toluidine blue-stained sections were scanned at ×400 and areas with cystine-laden histiocytes in the lamina propria were reviewed under oil at ×1000 magnification. CC were counted in each of first 15 consecutively visualized histiocytes. A mean value of CC per histiocyte was calculated. If less than 15 CC-laden histiocytes were visualized per 1 mm^2 biopsy specimen, the mean of the lesser number was taken. A combined mean CC load calculated from stomach and duodenum (CC-GD) was obtained for gastric and duodenal biopsies. Mucosal biopsies from the esophagus and rectum/sigmoid colon were also evaluated. CC were of different size; therefore, we estimated the mean cell cytosol volume occupied by the CC/histiocyte for the 15 histiocytes and correlated this with the mean CC load. The mean CC load from the 5 histiocytes with the highest number of crystal was also calculated (CC-5H). Gastric and duodenal biopsies from 3 patients were processed for full electron microscopic evaluation (Figure 1, B).

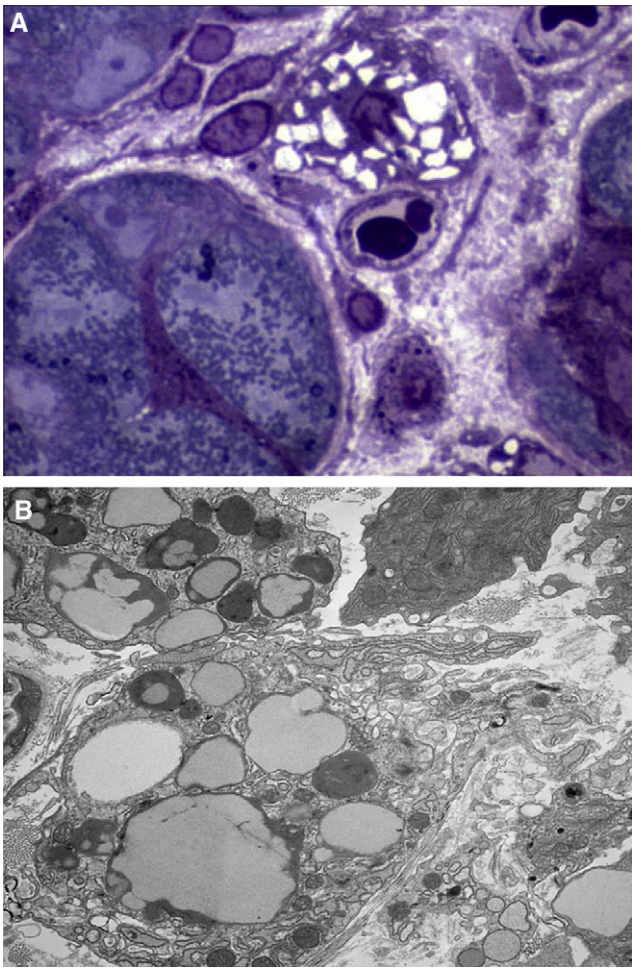

Figure 1. **A,** Gastric mucosal biopsies were fixed in glutaraldehyde, prepared as 1 u thick cuts, and stained with toluene blue. Numerous CC within a histiocyte are clearly visible with LM at ×1000 magnification. **B,** Electron micrograph (×7900) of colonic mucosa showing CC surrounded by a rim of dense material consistent with a lysosomal envelope. No extracellular CC were visualized.

Data and Laboratory Analyses

WBC cystine measurements were provided by the Cystine Determination Laboratory (San Diego, California). Serum creatinine was measured and used to estimate the glomerular filtration rate (eGFR calculated using 0.413 length in cm/plasma creatinine in mg/dL).[10]

Statistical analysis was performed using GraphPad Prism (GraphPad Software; San Diego, California). Paired Student *t* tests were used to compare CC-GD in 2001 and in 2003 as well as eGFR values in the same patients. Unpaired t-tests were also used to compare CC counts. All test statistics were 2-tailed. Linear regression analysis was performed and correlation coefficients (r) were

calculated. A P value of <.05 was considered statistically significant.

Results

Seventeen children (6 male) were enrolled, of whom 9 participated in both studies, in 2001 and 2003. Their ages ranged from 2-12 (mean 6.9) years. The mean age at diagnosis of all subjects was 1.8 (range 0-5.5) years. All patients started regular cysteamine therapy at the time of diagnosis and were reported to be compliant with therapy. At the time of the subjects' enrollment into their initial study, the mean duration of treatment with cysteamine was 5.2 years (range 2.1-10.9 years). For the 9 subjects who participated in both studies, the mean duration of treatment at the time of the first and second study was 3.6 years and 5.5 years, respectively. In 2003, 9 subjects underwent both upper GI endoscopy and proctosigmoidoscopy.

For all study subjects, the mean CC load, calculated at all time-points, was higher in the gastric (mean ± SEM 13.6 ± 1.06 crystals/histiocyte, n = 27) and duodenal (10.2 ± 1.24, n = 21) than in the esophageal (2.2, n = 2), but were highest in the rectal/colonic biopsies (23.6 ± 3.38, n = 9; Figure 2). Of the total 70 intestinal biopsies evaluated, 6 biopsies (3 gastric and 3 duodenal) had <15 identifiable histiocytes, but also had fewer CC per histiocyte; the mean gastric and duodenal CC counts were 4 and 3.9 crystals/histiocyte. In the 9 subjects who had colonic biopsies, there was a significant difference between colonic crystal load and their CC-GD (mean 12.5 ± 1.41) measured at the same time-point (P = .0031). Nine subjects had upper GI biopsies in 2001 and 2003 and their mean CC-GD were 13.1 ± 1.03 and 14.3 ± 2.3, respectively; they were not significantly different (P = .57). The CC-5H count (mean 22.1 ± 1.88, n = 27) was also calculated and was about 70% higher than the CC-GD (mean 12.43 ± 0.97, P < .0001).

Although the CC were different sizes, the mean CC-GD (r = 0.44, P < .0001) and the CC-5H (r = 0.42, P < .0001) correlated well with the estimated cell cytosol volume that was occupied by these crystals.

Our study patients were between 0.5-10.9 years post-diagnosis, and those treated for longer trended towards a lower CC (r = 0.13, P = .065; Figure 3; available at www.jpeds.com). This trend also failed to reach significance when subjects with a mean WBC cystine >1 nmol 1/2 cystine per mg protein (up to the specific time-point) were removed from the calculation (r = 0.26, P = .072).

Nine subjects participated in the 2001 and 2003 studies, and the mean eGFR was 76.9 ± 8.65 and 66.3 ± 12.6 mL/min/1.73 m^2, respectively; they were not significantly different (P = .122). The percent change from baseline for the CC-GD trended towards an inverse relationship with percent change from baseline of eGFR (r = 0.22, P = .23); however, this was only significant when 2 subjects with late diagnosis (2 and 5.5 years) were excluded from the analysis (r = 0.6, P = .048; Figure 4, A). Seven of the 9 subjects were diagnosed before age 18 months, and 3 of these 7 had a >30% reduction in actual eGFR and an increase in mean CC-GD from 12.7 to 16.6 crystals/histiocyte. Statistical analysis was impacted by the small group size, and there was no clear relationship between the percent changes of CC-GD and eGFR from baseline (r = 0.74, P = .34). The other 4 subjects had a mean CC-GD reduction from 11.7-8.7 crystals/histiocyte and had no change in percent change of eGFR from baseline (P = .98).

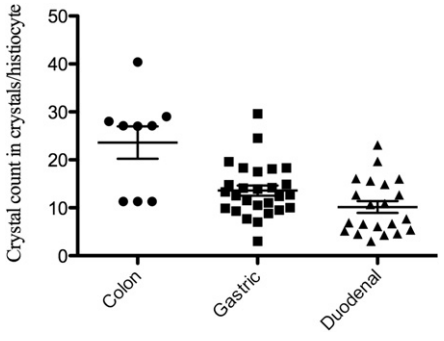

Figure 2. CC counts in all biopsies taken from 2001 and 2003. A significant difference existed between colon and both gastric (P = .0006) and duodenal crystal counts ($P \leq .0001$). Gastric crystal loads were also higher than duodenal loads (P = .041). Mean and SE bars are shown.

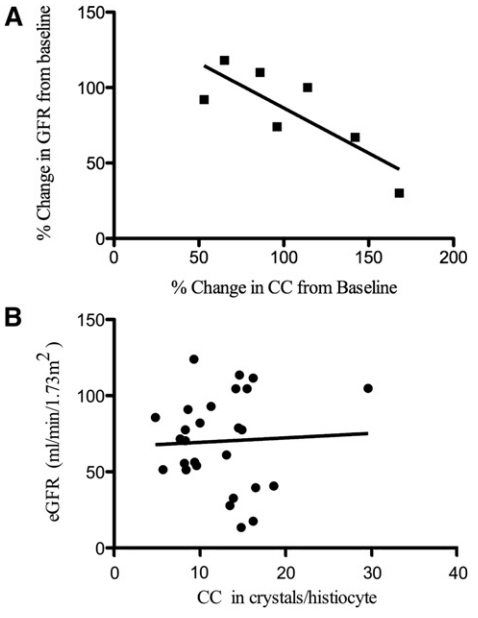

Figure 4. **A,** Percentage changes of eGFR from baseline against percent changes of CC-GD from baseline in 7 patients who were diagnosed and started cysteamine therapy before age 18 months. A positive correlation is shown (r = 0.66, P = .026). **B,** eGFR and absolute CC-GD estimated at the same time-points (n = 27). There was no significant association (P = .8), although all CC-GD of <12 crystals/histiocytes (n = 14) were associated with an eGFR of >50 eGFR mL/min/1.73 m^2.

Estimated GFR and absolute CC-GD were also calculated at the same time-points in all samples obtained in 2001 and 2003 (n = 27). There was no significant trend, although, all CC-GD of <12 crystals/histiocytes (n = 14) were associated with an estimated glomerular filtration rate of >50 GFR mL/min/1.73 m^2 (Figure 4, B).

Mean WBC cystine levels were calculated for each patient 4-30 (mean 16) using previous measurements. Each CC-GD measured in 2001 and 2003 was correlated with the mean of the WBC cystine levels up to that time-point. These mean WBC cystine levels were significantly correlated with the CC-GD only after 1 outlier with delayed diagnosis and very high CC-GD was excluded (P = .023; Figure 5). Those patients who had low mean WBC counts of <1 nmol 1/2 cystine per mg protein still had detectable CC in the intestinal mucosal biopsies.

One subject, diagnosed at age 18 months had a gastrostomy tube placement and fundoplication shortly afterwards. He underwent upper GI endoscopy in 2001, 2003, and again in 2011, and his mean WBC cystine levels at each of these time-points was 1.3, 1.0, and 0.84, respectively. Mean CC-GD at these times were 14.5, 7.7, and 13.5 crystals/histiocyte, respectively, and eGFR was 105, 95.3, and 37 mL/min/1.73 m^2, respectively. His dose of cysteamine was 42 mg/kg/d at the time of his last biopsy.

Discussion

Nephropathic cystinosis is a rare disorder characterized by intralysosomal accumulation of cystine and progression to renal failure. Regular cysteamine therapy can significantly deplete intracellular cystine by up to 90% (as measured in circulating WBCs) and this has been shown to reduce the rate of progression to kidney failure and also to obviate the need for thyroid replacement therapy.[1,6,11-13] WBC cystine levels are presently the best and most readily available surrogate marker for tissue cystine accumulation. However, WBC cystine measurements only reflect short-term compliance with therapy as levels may normalize after a single dose of cysteamine.[8,14] In addition, achievable tissue cysteamine concentrations may not be as high as plasma concentrations and WBC cystine levels may, therefore, be lower than actual tissue cystine levels. This may explain why patients who maintain WBC cystine levels of <2 nmol 1/2 cystine per mg protein still eventually progress to renal failure and transplantation, albeit more slowly than untreated patients.[6,12]

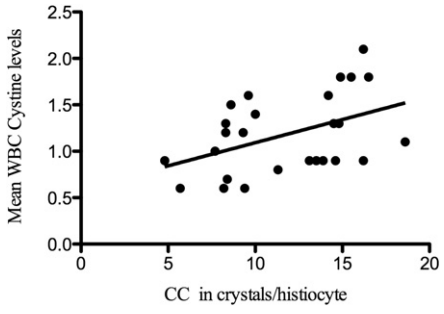

Figure 5. Individual patients' CC-GD were measured in 2001 and 2003 and correlated with their mean WBC cystine up to that point. One patient with delayed diagnosis and extremely high initial CC-GD was considered an outlier and was excluded from the analysis (r = 0.2, P = .023).

Our data show that despite adequately controlled WBC cystine levels, the intestinal mucosal CC had not completely dissolved in any of the subjects, even after 10 years of reported regular cysteamine therapy (Figure 4). It is unclear whether this is typical for other organs, such as the kidneys, although, cystine in circulating WBC and crystals in the cornea are shown to be completely depleted with cysteamine therapy.[15] Corneal cystine depletion, however, does require regular and frequent use of topical cysteamine eye drops, which dissolve the crystals within months. The concentration of cysteamine in the eye drops is probably much higher than that achievable or tolerable in the plasma or body tissues following oral cysteamine ingestion.[16] The currently available formulation of cysteamine may not be able to achieve adequate cysteamine levels required to prevent crystal formation or to dissolve existing crystals. In addition, the present formulation may be unable to deliver the drug to the body tissues for long enough. Ongoing animal studies have provided preliminary data showing that tissue (including kidney) cysteamine levels are elevated above baseline levels for only 60-90 minutes after direct intraduodenal administration at conventional drug doses (Dohil et al, unpublished data).

Using the histologic processing technique described above, CC were easily visible and enumerable at ×1000 magnification (Figure 1, A). CC counts were significantly higher in the colonic mucosal biopsies than in the gastric or duodenal biopsies. Possible explanations for this would include better absorption of cysteamine in the stomach and duodenum than in the colon, resulting in higher local mucosal concentrations of cysteamine in the upper intestine.[16] Histiocytes are relatively common in the rectum/colon.[17,18] This, however, is unlikely to account for the increased CC load in the colon compared with stomach/duodenum. In our study, >15 histiocytes were visualized in all but 6 biopsies (from the stomach and duodenum, which also had lower mean crystal/histiocyte counts. Visualizing <15 CC-laden histiocytes per biopsy was most likely due to lower CC count (and possibly lower tissue cystine levels) than to lower histiocyte counts in the upper GI tract.

Our study shows that the CC-GD counts (n = 27), from all time-points, was positively correlated with the subjects' mean WBC levels (Figure 5). There was no clear correlation between eGFR and the same CC-GD counts (Figure 4, B). Seven of 9 subjects who underwent repeat endoscopy in 2001 and 2003 were diagnosed before age 18 months. These 7 showed a significant correlation between % CC-GD and % eGFR change from baseline values (Figure 4, A). Three of the 7 had a >30% reduction in eGFR in 2 years and >30% rise in the mean CC-GD,

whereas the other 4 subjects whose eGFR did not deteriorate had a >25% reduction in mean CC-GD. Although our data are derived from a small study group, they suggest that estimating CC-GD at regular intervals may be of value in monitoring response to cysteamine therapy and may possibly provide a better estimate of organ cystine load than circulating WBC cystine levels. It should also be noted that at the time of each endoscopy, multiple mucosal biopsies can be obtained safely and painlessly from the same intestinal site. Multiple biopsy analysis can reduce sampling error that might be associated with the patchy distribution of tissue CC.

Histiocytes, derived from monocytes, have the ability to phagocytose and can survive for prolonged periods of time in the lamina propria of the intestinal mucosa. Histiocytes are typically difficult to see but, when laden with CC, they are easily recognized at ×1000 magnification following specific histologic preparation (**Figure 1**, A). The natural history of CC laden histiocytes is unclear. It is possible that the histiocytes might die and shed their crystal load, which could then be phagocytosed by other histiocytes. This, however, would seem unlikely as free extracellular CC were not seen in any of the specimens analyzed with electron microscopy or LM at ×1000 magnification. In addition, many of the crystals within the histiocytes were surrounded by a rim of dense material consistent with a lysosomal envelope (**Figure 1**, B). This would be in keeping with the mechanism of cystine accumulation previously described in cystinosis, rather than CC being phagocytosed.

Crystal counts may provide a relatively simple way to estimate cystine content in other less accessible organs and also to monitor response to long-term therapy. A correlation between CC-GD counts and eGFR may exist. It is also feasible that the CC will never completely disappear or will do so only when better treatments become available. CC measurements cannot be considered an alternative to leukocyte cystine measurements but may possibly be used as a supportive test in monitoring long-term response to therapy. Further studies to evaluate the use of intestinal CC levels as a marker of therapeutic efficacy in patients with nephropathic cystinosis are very much warranted. ■

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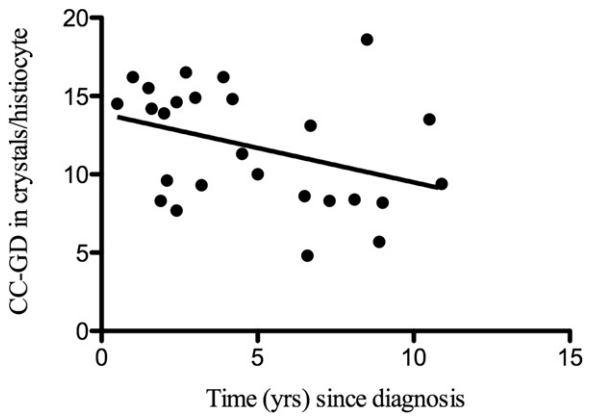

Figure 3. CC-GD is measured at different timepoints (in years) following diagnosis. There is a trend towards reduction in CC-GD but significance was not achieved (r = 0.13, *P* = .065).