Biochemical evaluations at two time points in 15 patients with nephropathic cystinosis under cysteamine treatment

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Background
Nephropathic cystinosis (NC) (MIM #219800) is a rare autosomal recessive disorder that is characterized by accumulation of cystine in many tissues and organs, especially kidney and eyes. It occurs in ~1–2 of 100 000 live births. NC is caused by mutations in the *CTNS* gene encoding the lysosomal membrane transporter cystinosin. Cystinosin deficiency results in lysosomal cystine accumulation in different organs and systems. Cysteamine is the mainstay treatment to deplete the cystine accumulation. The aims were to establish white blood cell cystine measurement using mass spectrometry and evaluate the effect of treatment through measurement of some biochemical markers.

Patients and methods
The study included 15 patients with NC referred from the cystinosis outpatient clinic of Department of Pediatrics, Cairo University, over the period from June 2018 to January 2019 and 25 healthy normal participants as controls. Venous blood samples were taken for studying the effect of treatment through measurement of cystine level, chitotriosidase activity, thyroid profile, free carnitine, and acylcarnitine concentrations at two time points of evaluation. The basic evaluation was started in the beginning of June 2018, and the follow-up was started in the beginning of January 2019.

Results
Cystine concentration in 10 patients was decreased from the range of 4–13 to 2.3–8.5 nmol ½ cystine/mg proteins; eight patients had high activity of chitotriosidase (170–574 μmol/l/h), and then the activity range decreased to 90–371.5 μmol/l/h under treatment. One patient had high thyroid-stimulating hormone level (32 UI/ml), which was normalized to 12 UI/ml in the follow-up, and three patients had a high borderline thyroid-stimulating hormone level (20, 21, and 25.9 UI/ml) in the follow-up stage. Levels of free carnitine and acylcarnitine were normal at the two points of evaluation.

Conclusion
The current study draws attention to the importance of a follow-up policy, especially in Arabic countries, which have a high rate of consanguineous marriages.

Keywords:
chitotriosidase, cysteamine, follow-up, nephropathic cystinosis, thyroid-stimulating hormone

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Introduction

Cystinosis is the main cause of inherited renal Fanconi syndrome in children, which accounts for up to 20% of cases of hereditary tubular disorders (Liang *et al.*, 2020).

It is a multisystem disorder that is characterized by an accumulation of cystine crystals within all organs as a result of a genetic defect in cystinosin, which is responsible for cystine transport to the cytoplasm (Langman, 2019).

Cystinosis is an autosomal recessive metabolic disease that belongs to the family of lysosomal storage disorders and is inherited in an autosomal recessive manner (Pittendrigh *et al.*, 2017). Depending on kidney disease severity and the age at onset, the cystinosis phenotype is divided into three clinical forms. Infantile nephropathic cystinosis (NC) (MIM #219800) affects ~95% of patients and is characterized by the development of renal Fanconi syndrome, late-onset juvenile nephropathic type (MIM #219900) usually presents during childhood or at adolescence, and non-NC (MIM #219750) is a benign variant presenting with photophobia (Liang *et al.*, 2020).

As a result of cystine accumulation in different organs, endocrine complications were reported in affected individuals such as diabetes, hypothyroidism, pubertal delay, and male hypogonadism. Vascular

damage, muscles, and bones are also affected by the disease (Kasimer and Langman, 2021).

Chitotriosidase is a mammalian chitinase enzyme that hydrolyzes chitin in lysosome. It is considered as an inflammatory protein because it is secreted from activated macrophages (Chang *et al.*, 2020).

Previous studies indicated that cystine crystals are potent activators of human macrophages and that chitotriosidase activity is a useful marker for this activation and a promising clinical biomarker and therapeutic monitor for NC (Xaidara *et al.*, 2009; Elmonem *et al.*, 2014; Langman *et al.*, 2016).

Veys *et al.* (2020) hypothesized that a progressive accumulation of cystine leads to the release of inflammatory mediators by macrophages. These mediators could reflect the long-term adherence to cystine therapy. They found that plasma chitotriosidase activity was the only promising mediator for clinical use as an additional monitor for therapeutic monitoring of cystinosis, which resulted as a significant predictor for white blood cell (WBC) cystine levels in patients of all ages with cystinosis (Veys *et al.*, 2020).

The cornerstone of diagnostic routes is the estimation of cystine elevation in WBCs or polymorphonuclear (PMN) leukocyte cells (Chabli *et al.*, 2007; García-Villoria *et al.*, 2013). Molecular testing of the relatively small *CTNS* gene is also a well-established technique, and the presence of corneal cystine crystals by using slit-lamp examination is a clinically confirmatory option (Wilmer *et al.*, 2011; Biswas *et al.*, 2018).

Beyond supportive therapy, cysteamine is the mainstay treatment for these patients. It breaks lysosomal cystine into free cysteine and cysteamine–cysteine mixed disulfide and then it is released from lysosome. Different previous studies reported that cysteamine delays the disease's progression but does not cure it (Brodin-Sartorius *et al.*, 2012; Biswas *et al.*, 2018).

In recent years, our understanding of the follow-up and/or evaluation policies for treatable genetic diseases has increased substantially. In particular, we now understand the importance of maintaining control of WBC cystine concentrations life-long to achieve the best long-term outcomes. Concern for adherence to a follow-up and/or evaluation strategy remains a major challenge for the future, especially for adolescents and young adults.

Leukocyte-cystine measurement is a complicated test that is available in few university hospitals and research centers in the developed world. So, the aim of this study was to establish the biochemical diagnosis of NC through application of quantitative determination of cystine in PMN leukocytes by tandem mass spectrometry. Moreover, it aimed to evaluate other biochemical parameters that are affected by the disease to carry a short message to draw attention for following up.

Patients and methods

Material

A total of 15 Egyptian children from 13 unrelated families with infantile NC were enrolled from the cystinosis outpatient clinic of Department of Pediatrics, Cairo University Children's Hospital, over the period from June 2018 to January 2019. Moreover, 25 healthy normal participants of matching age and sex as controls were included.

Inclusion criteria of the patient group were based on the presence of corneal cystine crystals on slit-lamp examination or/and leukocyte-cystine content greater than 1 nmol half-cystine/mg protein.

Absence of corneal cystine crystals and/or leukocyte-cystine content equal to 0.2 nmol half-cystine/mg protein are the exclusion criteria of the patient group.

This study was approved by the Research Ethics Committee of the NRC according to the World Association Declaration of Helsinki, and written informed consent was obtained from all patients' legal guardians.

Venous blood samples were collected from patients and controls for biochemical evaluation, which included quantification of cystine level in PMN leukocyte, chitotriosidase activity in plasma, free carnitine and acylcarnitine profile in dried blood spot, and thyroid hormone profiles in serum. The initial samples for biochemical follow-up were taken in the beginning of June 2018, and the samples for follow-up were taken in the beginning of January 2019.

Method

Biochemical analysis

PMN cystine assay:

(1) Overall, 5–7 ml of venous blood was collected into a lithium heparin vacutainer. Plasma was obtained through centrifugation (2500 rpm for 5 min) for chitotriosidase assay and stored at 4°C till analysis.
(2) After the separation of plasma, PMN leukocytes were isolated and stored at –20°C in 150 µl of

N-ethylmaleimide and 50 μl of 12% sulfosalicylic acid till analysis (García-Villoria *et al.*, 2013). Protein was measured in the supernatant according to Lowry's method (Lowry *et al.*, 1951). Cystine was measured in pellet using an Acquity UPLC Waters Xevo TQD triple quadrupole mass spectrometer (Waters, UK) according to García-Villoria *et al.* (2013).

Chitotriosidase activity:

(1) Chitotriosidase activity was measured by incubating plasma with 4-methylumbelliferyl-β-D-NN, N'triacetylchitotriose (4 MU-chitotrioside) as substrate in citrate/phosphate buffer pH 5.2, at 37°C. The result was expressed in umol/l/h (Hollak *et al.*, 1994).

Free carnitine and acylcarnitine profile assay:

(1) Overall, 50 μl of venous blood from patients and controls was blotted on to Whatman 903 neonatal protein saver cards. The cards were dried at room temperature for at least 24 h. The samples were put in a gas-impermeable zipper bag, containing one to two desiccant sachets and stored at −20°C till analysis. Free carnitine and acylcarnitine profile was done by an Acquity UPLC Waters Xevo TQD triple quad mass spectrometer using masschrom amino acids and acylcarnitine from dried blood LC-MS/MS kit, Chromsystems, according to instructions of the manufacturer.

Measurement of thyroid hormone profile:

(1) Overall, 3 ml of venous blood was collected into BD vacutainer plus plastic serum tube. Blood was centrifuged (2500 rpm for 5 min) to obtain serum, which was stored at 4°C till analysis. Free triiodothyronine, free thyroxine, and thyroid-stimulating hormone (TSH) were measured using a microplate enzyme immunoassay (Chemux BioScience Inc., United State, California).

Statistical analysis

Data were statistically described in terms of mean ± SD, range, and frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was carried out using analysis of variance and regression testing. Two-sided *P* values less than 0.05 were considered statistically significant.

Results

The mean age of the patients was 11.6 years (7–17 years); there were nine males and six females with a male:female ratio of 1.5:1. All patients were on cysteamine treatment ranging from 20 to 50 mg/kg/day. Consanguinity was found in ten patients of 13 (77%) families. Three patients had end-stage renal disease and one patient underwent renal transplantation. Chronic kidney disease was reported in the rest of patients ranging from stages I to III.

The 25 controls were of matched mean age [12.6 years (7–19 years)] and sex (16 males and nine females, with male:female = 1.7:1).

The following tables and figure showed the biochemical results of 15 patients with cystinosis:

PMN leukocyte-cystine assay was done in all patients at the initial time. Their range was from 3.6 to 13 nmol ½ cystine/mg proteins (mean 6.2 ± 2.7 nmol ½ cystine/mg proteins), and then at the time of follow-up, cystine assay was done in 10 patients only, and their range was from 4 to 13 nmol ½ cystine/mg proteins (mean 4.1 ± 2.9 nmol ½ cystine/mg proteins), which reduced to a range from 2.3 to 8.5 nmol ½ cystine/mg proteins (4.6 ± 2.1) under treatment (Table 1).

Chitotriosidase activity was ranged from 22 to 574 μmol/l/h, with a mean of 162.6 ± 157.6 μmol/l/h in all patients. Of 15 patients, eight (53.3%) showed high level of chitotriosidase activity, with a range from 145 to 574 μmol/l/h (mean 265 ± 153.5 μmol/l/h) compared with normal participants (4–80 nmol/l/h, mean 24 nmol/l/h), and then the range of activity was reduced to 90–371.5 μmol/l/h (187.6 ± 106.7 μmol/l/h) under treatment (Table 2 and Fig. 1).

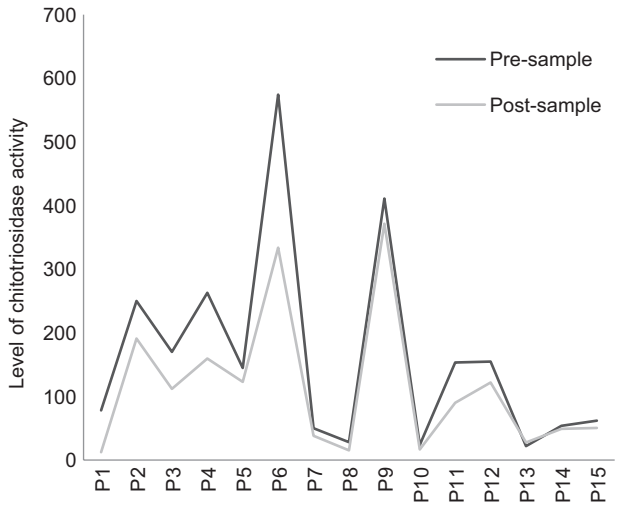
For thyroid profile, the concentrations of triiodothyronine and free thyroxine were in the normal range at the two points of follow-up (Table 2).

Table 1 Levels of cystine and chitotriosidase activity in the samples of 15 patients with cystinosis before and after treatment

Patients' serial number	(Cystine level in PMN cell) nmol ½ cystine/mg protein	Chitotriosidase activity (NR: 4-80 μmol/l/h)
P1	5.6/3.8	78/12.3
P2	**10/7.6**	**250/191**
P3	3.5/-	170/112
P4	**8/5.6**	**263/159.6**
P5	5.7/-	145/122.8
P6	**13/8.5**	**574/334**
P7	5.3/-	50/38
P8	4.2/2.6	28/15.6
P9	**9.5/6.6**	**411/371.5**
P10	5.6/4.1	24/16.6
P11	3.6/-	153.2/90
P12	5/2.8	155/122
P13	4/2.3	22/27.6
P14	3.7/-	54/49
P15	6.5/4.2	62/50.8
P		0.342

PMN, polymorphonuclear. Significance of bold values *P*<0.001.

Figure 1



Effect of treatment on chitotriosidase activity in 15 patients with cystinosis.

A total of 11 (73%) patients were within the normal range of TSH. One patient had hypothyroidism with high level of TSH (32 UI/ml), which normalized to 12 UI/ml in the follow-up step under supportive treatment. Three (20%) patients had a borderline of high TSH level (20, 21, and 25.9 UI/ml) in the follow-up step (Table 2).

Concentrations of carnitine and acylcarnitine were normal at the two times of evaluation, with mean values of 12.4 ± 6.55 and 16 ± 6 μmol/l/h, respectively, at the first time point and 27.8 ± 1.8 and 33.5 ± 10.3 μmol/l/h, respectively, at the second time point compared with normal (19.5 ± 11 and 29.5 ± 14.7 μnol/l/h, respectively) (Table 3).

Discussion

Nephropathic infantile cystinosis (NC) as a clinical structure is a progressive dysfunction of multiple organs resulting from cystine accumulation in the lysosomes of cell owing to genetic mutations in the CTNS gene. It is the most common and affects ~95% of patients. It is a severe yet potentially treatable form of cystinosis. So, early initiation of cysteamine treatment has a considerable effect on the long-term prognosis (Liang *et al.*, 2020).

Cystine assay in leukocyte is the fundamental route for diagnosis and therapeutic monitoring of the disease. Most laboratories have turned to measuring cystine in mixed leukocyte enriched with PMN, because these cells preferentially accumulate cystine in the blood. Consequently, it increases the sensitivity of cystine detection and differentiates between heterozygote and normal value (Levtchenko *et al.*, 2004; Bäumner and Weber, 2018).

Under cysteamine treatment, PMN cystine was depleted from a range of 4–13 nmol ½ cystine/mg proteins to a range of 2.3–8.5 nmol ½ cystine/mg proteins; according to these values, the clinicians should adjust the dose of treatment for further reduction of cystine level. Two points of evaluation were done in 10 patients only, because five patients were missed in the second point of evaluation, mostly attributed to financial reasons and poor blood samples.

Table 2 Concentrations of triiodothyronine, free thyroxine, and thyroid-stimulating hormone in the samples of 15 patients with cystinosis before and after treatment

Thyroid profile normal controls	Free T3 (2.2-4.1 pg/dl)	Free T4 (0.8-2.2 ng/dl)	TSH (up to 20 UI/ml)
Patients			
P1	2.9/3.1	1/1.4	5.4/6.4
P2	2/2.3	0.8/0.9	6.8/7.6
P3	1.9/2.4	1/1.7	5.4/6.3
P4	2.8/2.4	0.6/1.1	5.9/6.9
P5	1.7/2.1	1.2/1.4	5.8/7.2
P6	1.4/1.9	1/0.9	**32/12**
P7	1.7/1.9	0.7/0.9	4.3/5.2
P8	2.1/2	0.8/1.3	8.7/6.4
P9	1.5/2.1	1.3/0.8	6.3/5.2
P10	2.7/1.7	1/1.4	**5.3/25.9**
P11	1.9/2.3	0.9/1.2	5.6/7
P12	0.9/1.9	1.4/1.9	**12/20**
P13	2.5/3.1	1.6/1.33	**9.9/21**
P14	2.4/2.8	0.9/1.2	5.7/6.4
P15	1.4/1.8	1.4/1.8	8.8/9.8
P	0.187	0.689	0.21

T3, triiodothyronine; T4, thyroxine; TSH, thyroid-stimulating hormone. Significance of bold values $P<0.001$.

Table 3 Concentrations of free carnitine and acylcarnitine in the samples of 15 patients with cystinosis before and after treatment

Patients' serial number normal controls	Free carnitine 5-70 μmol/l	Acylcarnitine 10-161 μmol/l
Patients serial		
P1	9.5/13	27/33
P2	6/15	22/35.7
P3	12/16	23.4/28
P4	21/25	40/45
P5	7.8/12.5	22.6/31.5
P6	24/22.8	42/51
P7	10.6/14	26/32
P8	14.4/17	34.5/32.2
P9	13.4/16	36/33
P10	22.6/31	47.7/50
P11	20/16	35/41.8
P12	6.8/12.5	17.8/22
P13	6.3/9.8	19/32
P14	6.4/8.5	11/15
P15	5.5/10	14.3/20
P	0.74	0.23

Cystine measurements are sophisticated enough to limit its use to a few university hospitals and research centers mainly in the developed world. This is further complicated by the sample sensitivity to storage and transportation conditions; therefore, so far, most developing nations are still lacking the assay.

According to the current results, our laboratory succeeded to measure WBC cystine; therefore, instead of sending the samples abroad for diagnosis or scientific research in Arabic societies, we plan to cooperate with them, and this is one of our targets.

Chitotriosidase is currently an established screening marker, a severity marker, and/or a therapeutic monitor for over 40 different diseases, inherited and acquired (Elmonem *et al.*, 2014).

Xaidara *et al.* (2009), suggested that serial estimation of chitotriosidase activity could be used as a useful therapeutic monitor for cystinosis. Moreover, Elmonem *et al.* (2014), and Langman *et al.* (2016), concluded that chitotriosidase activity is a useful and promising clinical biomarker and a therapeutic monitor for NC.

Recently, Veys *et al.* (2020), hypothesized that chitotriosidase activity could mirror clinical disease severity, response to cysteamine treatment, and thus be used for disease monitoring. Finally, they concluded that chitotriosidase activity can serve as a novel alternative biomarker for the therapeutic monitoring of NC (Veys *et al.*, 2020).

This agrees with our finding, whereas eight (53.3%) patients of 15 showed high level of chitotriosidase activity at the initial step of evaluation. Their level were reduced under treatment of cysteamine in the follow-up step but were not normalized.

Moreover, the results showed that a high level of cystine corresponds to a high level of chitotriosidase as seen in patients P2, P4, P6, and P9. According to the clinical data, the patients P4, P6, and P9 had end-stage renal disease and underwent a regular hemodialysis. This agrees with the conclusion of Veys that chitotriosidase is correlated significantly with WBC cystine and reflects the inflammation related to chronic kidney disease in cystinosis (Table 1).

Because cystine measurement is not available in many countries, chitotriosidase can replace the role of WBC cystine in the follow-up process.

Endocrinologic system is one of the systems that are affected in multiple aspects owing to cystine accumulation. Hypothyroidism is an important and common disorder. Cystine crystals cause fibrosis and atrophy of thyroid gland. This leads to decrease in the capacity of gland and causes clinically increased TSH concentration, compensating for the decrease in thyroid reserve followed by overt hypothyroidism. Although early compliant cysteamine treatment improves body growth and can avoid thyroid hormone replacement, eventually 70% of patients need thyroid replacement therapy (Chan *et al.*, 1970; Kimonis *et al.*, 1995).

Patient 6 had hypothyroidism at the first step in the evaluation, and then it normalized in the follow-up after adjustment of the levothyroxine dose. The same patient showed higher concentrations of cystine (13 nmol ½ cystine/mg protein) and chitotriosidase activity (574 μmol/l/h) at the initial time of evaluation than the other studied patients, which reflects the effects of cystine accumulation inside cells. Concentration of TSH in three patients (P10, P12, and P13) was normal in the first step and then their levels were elevated to a borderline in the follow-up. Consequently, attention must be paid to adjusting the dose of levothyroxine and cysteamine to avoid any complications.

One of the most important benefits of follow-up is a prediction of any complications before happening and prevent occurrence.

Inside the mitochondria, carnitine regulates the transport of long-chain fatty acids for β-oxidation catabolism to produce energy. This process is essential for skeletal muscle. Overall, 97% of free carnitine is reabsorbed by kidney tubules (Cherqui *et al.*, 2017). Cystinosis patients with renal Fanconi syndrome fail to reabsorb carnitine (Gahl *et al.*, 1988; Hohenfellner *et al.*, 2019).

L-carnitine supplementation is considered in the treatment of children with cystinosis and renal tubular Fanconi syndrome. In the present study, levels of free carnitine and acylcarnitine were within normal range compared with normal participants (Table 3).

Extrarenal complications in patients with NC were recorded in many studies in infantile and adult states. Optimizing the cysteamine and supportive therapies may attenuate these complications. Better communication among pediatric, adult nephrologists, patients, and laboratories, along with regular follow-ups, is needed to improve the outcome of patients with cystinosis. Although the study is small for evaluation of patients with cystinosis, we used it as a first step for further evaluation in the near future to setup a follow-up system.

The current study succeeded to measure WBC cystine as a complicated test, and also it carries a small message

to patients, clinicians, and laboratories to draw their attention for a follow-up policy. In addition, it shows the sensitivity of the biomarkers to predict the state of the disease.

Wilmer *et al.* (2011), described a follow-up map for patients with cystinosis at every stage of age. We need to teach the patients and their families the huge value of following up and its outcome in the future to set this policy side by side with diagnosis, especially in countries with a high rate of prenatal consanguinity, such as the Arabic population (Al-Ghanim, 2020).

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Conflicts of interest
There are no conflicts of interest.

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