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Serum Chitotriosidase Level as a Novel Biomarker for Therapeutic Monitoring of Nephropathic Cystinosis among the Iraqi children

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Abstract

Cystinosis is a rare autosomal recessive lysosomal storage disease with high morbidity and mortality. It is caused by mutations in the CTNS gene that encodes the cystine transporter, cystinosin, which leads to lysosomal cystine accumulation. It is the major cause of inherited Fanconi syndrome, and should be suspected in young children with failure to thrive and signs of renal proximal tubular damage. Infants diagnosis can be missed since not all signs of renal Fanconi syndrome are presented during the first months of life. Elevated leukocytes cystine content is the cornerstone for the diagnosis of cystinosis. Whereas, chitotriosidase (CHIT1 or chitinase-1) is primarily produced by activated macrophages. Hence, inflammatory conditions including cystinosis could be associated with increased plasma chitotriosidase activity. This study is aimed to estimate serum chitotriosidase level, as a marker for screening as well as for therapeutic monitor for cystinosis disease, as the measurement of leukocyte cystine content is not available for Iraqi children with cystinosis.

The present study is a case-control study that included samples of 30 children with nephropathic cystinosis, compared to 25 healthy control children from those attending at The Genetic Rare Diseases Center / AL-Emamain AL-Kadhimain Teaching Hospital, Baghdad-Iraq. Cystinotic children included in the study were those aged equal to less than 10 years, and diagnosed to have cystinosis according to clinical symptoms of the disease and eye examination; demonstrating corneal cystine crystals. Blood specimens were obtained for separating white blood cell (WBC) for measuring their cystine content, within afew hours, by applying high-performance liquid chromatography (HPLC). While aliquots of serum were utilized for analysis of chitotriosidase (ELISA), creatinine and calcium concentrations. Also, urine specimens were collected using urine cups from each participant for measuring glucose in the urine.

The results reported that cystinotic patients had leukocyte-cystine content ranged between (0.5 to 6.2 nmol 1/2 cystine/mg protein), compared to age-matched healthy children which ranged between (0.07 to 0.32 nmol 1/2 cystine/mg protein). The patients also had abnormally higher levels of serum chitotriosidase ranged between (90 to 565 nmol/hr/ml) while for the control group ranged between (35 to 153 nmol/hr/ml). Besides a significant associated with leukocyte-cystine content for cystinotic patients ($r=0.957$, $p<0.001$).

Estimation of serum chitotriosidase activity might aid in monitoring the therapeutic benefits of cysteamine therapy, as well as the prognosis of the disease when WBC cystine assessment is not available.

Keywords: Cystinosis, Chitotriosidase.

مستوى الكايتوترايوسيديز في المصل كمؤشر حيوي جديد للرصد العلاجي لداء السيستيني الكلوي لدى الأطفال العراقيين

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الخلاصة

داءالسيستيني؛ هو مرض وراثي متنحي يسبب ارتفاع معدل الوفيات والمراضة. السبب الرئيسي لهذا المرض هو طفرات في جين (CTNS)المسؤول عن تشفير ناقل السيستين، السيستينوسين، يؤدي الى تراكم السيستين داخل اللايسوسوم. إنه السبب الرئيسي لمتلازمة فانكوني الموروثة ويجب الاشتباه به عند الأطفال الصغار الذين يعانون من فشل النمو وعلامات تلف الأنبوب الكلوي القريب. يمكن تفويت التشخيص الرضع، لأنه لا توجد جميع علامات متلازمة فانكوني الكلوية خلال الأشهر الأولى من الحياة. ارتفاع محتوى السيستين في خلايا الدم البيضاء هو حجر الاساس في التشخيص. نظرًا لأن الكايتوترايوسيديز (كيتينيز-١) يتم إنتاجه بشكل أساسي عن طريق البلاعم النشطة. وبالتالي، يمكن أن تترافق الحالات الالتهابية بما في ذلك داء السيستيني مع زيادة نشاط إنزيم كيتوتريوزيداز في البلازما. تهدف هذه الدراسة إلى تقدير مستوى إنزيم الكايتوترايوسيديز في الدم كعلامة فحص ومراقب علاجي لمرض داء السيستيني، حيث أن قياس محتوى الكريات البيض السيستين غير متوفر للأطفال العراقيين المصابين بداء السيستيني.

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الدراسة الحالية عبارة عن دراسة الحالات والشواهد تضمنت عينات من ٣٠ طفلاً مصابًا بداء السيستيني الكلوي، مقارنة بـ ٢٥ طفلًا يتمتعون بصحة جيدة من أولئك الذين يحضرون في مركز الأمراض الوراثية النادرة / مستشفى الإمامين الكاظمين التعليمي، بغداد، العراق. كان الأطفال الذين يعانون من مرض السيستيني المشمولين في الدراسة هم أولئك الذين تبلغ أعمارهم ١٠ سنوات أو أقل وتم تشخيص إصابتهم بداء السيستيني وفقًا للأعراض السريرية للمرض وفحص العين، ظهور بلورات السيستين في القرنية. تم الحصول على عينات الدم لفصل خلايا الدم البيضاء لقياس محتواها من السيستين، في غضون ساعات قليلة، عن طريق تطبيق كروماتوجرافيا سائلة عالية الأداء (HPLC). بينما استخدمت قسامات من المصل لتحليل تراكيزالكيتوتريوسيديز، الكرياتينين والكالسيوم. كما تم جمع عينات البول باستخدام أكواب البول من كل مشارك لقياس نسبة الجلوكوز في البول.

بينت النتائج أن مرضى داء السيتيني كان لديهم محتوى الكريات البيض يتراوح بين (٠,٥ إلى ٦,٢ نانومول ٢/١ سيستين / ملغ بروتين)، مقارنة بالأطفال الأصحاء المتطابقين مع العمر والذين تراوحت أعمارهم بين (٠,٠٧ إلى ٠,٣٢ نانومول ٢/١ سيستين / ملغ بروتين). كان لدى المرضى أيضًا مستويات أعلى بشكل غير طبيعي من الكيتوتريوسيديز في الدم تراوحت بين (٩٠ الى ٥٦٥ نانومول / ساعة / مل) بينما تراوحت المجموعة الضابطة بين (٣٥ إلى ١٥٣ نانومول / ساعة / مل). إلى جانب ارتباط كبير بمحتوى الكريات البيض السيستين لمرضى داء السيستيني ($r=0,957$ $p<0,001$).

قد يساعد تقدير نشاط الكايتوترايوسيديز في المصل في مراقبة الفائدة العلاجية للعلاج بالسيستامين والتشخيص بالمرض عندما لا يتوفر تقييم السيستيني في كريات الدم البيضاء.

الكلمات المفتاحية: داء السيستيني،الكايتوترايوسيديز.

Introduction

Cystinosis is a rare autosomal recessive lysosomal storage disease with high morbidity and mortality. It is caused by mutations in the CTNS gene that encodes the cystine transporter, cystinosin, which leads to lysosomal cystine accumulation[1]. Three clinical forms of cystinosis can be distinguished depending on the age at presentation and the degree of disease severity. Infantile Nephropathic Form: Also known as renal Fanconi syndrome, the most frequent and most severe form of the disease. Patients are generally present before the age of 12 months with polyuria, polydipsia, and failure to thrive, caused by generalized proximal tubular damage[2].

Early symptoms of classical nephropathic cystinosis include renal tubular Fanconi syndrome, rickets, impaired growth, hypothyroidism, and photophobia[3]**.** However, cystine accumulation continued in non-renal organs, including the muscle, brain, bone marrow, liver, spleen, lymph nodes, cornea, conjunctiva, thyroid, pancreas, testes, and intestines[4]. Consequently, the clinical course of cystinosis changed from that of a largely renal disease to that of a multisystemic , including a distal vacuolar myopathy, decreased pulmonary function, swallowing impairment, deterioration of the central nervous system (CNS), endocrinopathies, vascular calcifications, retinal damage, and other ophthalmic complications[5]. Definitive diagnosis is based upon a high index of suspicion because of the clinical presentation including: polyuria, thirst, failure to thrive, growth retardation, vomiting, periods of dehydration, constipation, developmental delay, and rickets in some patients[6].

The patients are usually presented with hypokalemia, hypophosphatemia, metabolic acidosis, low serum uric acid, low carnitine, and, sometimes, hyponatremia. Proteinuria can reach grams per day, and consists of low molecular proteins, albumin, and high molecular weight proteins[7]supported by slit lamp examination of the corneas showing crystals, which are generally present by 16 months of age [8].

The detection of elevated intracellular cystine content is the cornerstone for the diagnosis. The methods for cystine determination differ depending on the cell type: mixed leukocyte preparation or polymorphonuclear (PMN) leukocytes[9].

Furthermore, several biochemical methods are currently used for cystine measurements, such as a cystine binding assay, amino acid chromatography or high performance liquid chromatography, making it difficult to compare the results of different laboratories[10]

Nationwide birth prevalence data concerning cystinosis are only reported in few populations, it is estimated at 1-9 per 1,000,000[11]. However, the prevalence of cystinosis in Iraq about is 163 patients according to data collected from The Genetic Rare Diseases Center/AL-Emamain AL-Kadhimain Teaching Hospital, Baghdad-Iraq. The incidence of cystinosis is estimated as 1 in 100,000 –200,000 live births per year[12].

Cysteamine orally is the only specific targeted therapy available for managing cystinosis, as it act as cystine depleting therapy and mostly needs to be combined with cysteamine eye drops when corneal disease is involved[13].

Subjects and Methods

The present study is a case-control study included thirty child were diagnosed to have cystinosis from those attending at The Genetic Rare Diseases Center / AL-Emamain AL-Kadhimain Teaching Hospital, Baghdad-Iraq. This study was approved by the Ethics Committee of the College of pharmacy/University of Baghdad. All participants were informed about the aim and the proposed benefits of the study before obtained their agreements. Twenty-five age & sex matching apparently healthy individuals were included to serve as a control group.

Cystinotic children included in the study were those aged equal or less than 10 years, and diagnosed to have cystinosis according to clinical symptoms of

disease and eye examination ; demonstrating corneal cystine crystals[14].

Sample collection and preparation

From each participant (patient and control subjects), venous blood sample were collected, but for cystinosis patients the blood venous blood samples were collected after at least 6 hours from taking treatment (**Cysteamine**). Six milliliters was withdrawn, about (2 ml) was transferred to a tube containing Lithium Heparin and stored at (2-8 ^{o}C, less than 24 hours) to be taken it to the laboratory (ASCO Learning Center in Al-harthia city) for separation of white blood cell (WBC)[15]. After that, the separated leukocytes were taken within less than 24 hours to be assessed at the Ministry of Science and Technology / Department of Environment and Water Laboratories for measuring their cystine content by applying high performance liquid chromatography[16] (HPLC system, Sykamn-Germany).

The other part (4 ml) of the blood sample was transferred to a plane tube for 30 minutes to clot and then centrifuged at (3000 rpm) for 5 minutes to obtain serum, which is used for measuring the level of creatinine[17]and calcium[18](By taking precaution during blood sampling since the use of tourniquet might affect serum calcium level). The remaining aliquot of serum was kept in eppendorff tubes and frozen at (-20^{o}C) for later analysis of serum chitotriosidase concentration using double sandwich Elisa kit[19]. Glumerular filteration rate GFR was measured according to Schwartz equation [20].

Also, urine specimens were collected using urine cups from each participant for measuring glucose in urine.Notebly that the laboratory cystine assay procedures were blinded for the analyzer (regarding the sample was for a control or a patient) of the participants, i.e. samples were assayed in a random order. Because, the lack of blinding could introduce bias into the assessment of subjective outcomes such as health-related quality of life and adverse events. Additionally , it's the first time to to report the measurement of WBC- cystine content for cystinotic patients in Iraq.

Statistical analysis

The analyses were conducted using the Statistical Package for the Social Science (SPSS, version 22, IBM, New York, USA). Descriptive statistics (means, standards deviations, frequencies and percentages) of the participants (both patient and control group) were calculated. Because the variables were not normally distributed (Cystine & Chitotriosidase), we used non-parametric tests including Mann-Whitney (between 2 groups) tests to measure the difference in multiple measures according to participating groups (patient vs control). Spearman correlation was used to measure the relationships among different measures in patient group. A p-value of less than 0.05 was considered to be significant statistically.

Results

Patients and control group demographics and disease characteristics

As shown in the Table (1), the participating patients aged between 1.5 and 10 years (18 and 120 months) with a mean age of (65.20 ± 34.27) months about 5.4 years. The thirty cystinotic patients, included (17 male) and (13 female) representing 56.7% and 43.3% respectively. While, the control group had a comparable age to patients group with a mean age of (62.32 ± 35.45) months about 5.2 years and ranged between one and 10 years old (12 and 120 months)**.** Twenty-five control group included 13 male participants (52.0%) and 12 (48.0%) female.

The patients group had lower weight, height, body mass index (Table-1), GFR, and serum calcium (Table-2) measures compared to the control group. On the other hand, the control children had significantly lower levels of serum creatinine, cystine, and chitotriosidase levels compared to the patients' group (Figure-1).

Table 1. Patient and control groups demographics

Characteristic	Patient group (Mean± SD)	Patient group Median	Control group (Mean± SD)	Control group Median
Age (months)	65.20 ± 34.27	66	62.32 ± 35.45	60
Gender (M/F)	(17/13)	-	(13/12)	-
Weight (Kg)	11.20 ± 3.00	10.5	22.28 ± 10.71	20
Height (cm)	85.84 ± 11.88	85.5	108.56 ± 18.98	112
BMI kg/m^2	14.68 ± 2.36	14.9	17.71 ± 2.96	17
Age at diagnosis (months)	25.60 ± 20.88	18	-	-
Disease duration (months)	39.53 ± 33.24	39	-	-
Treatment duration (months)	19.93 ± 28.89	7	-	-
Age at treatment started (months)	45.27 ± 31.04	36	-	-
Age at reached ESRD (month)	81.7 ± 28.26	81.5	-	-

N for patients=30, N for Control=25, SD=Standard deviations, M=Male, F=Female, BMI= body mass index, ESRD=End stage renal disease

Table 2. Studied biomarkers among patients and control

Characteristic	Patient group (Mean± SD)	Patient group Median	Control group (Mean± SD)	Control group Median
S. Creatinine (µmol/L)	179.76 ± 177.53	99	38.86 ± 14.16	37
GFR (mL/min/1.73 m^2)	44.09 ± 37.64	31.4	110.13 ± 12.16	109.5
WBC cystine(nmol/mg protein)	2.98 ±1.80	2.6	0.20 ± 0.06	0.18
S.Chitotriosidase(nmol/hr/ml)	308.30 ± 134.79	293	115.96 ± 32.44	127
S. Calcium (mmol/L)	1.80 ± 0.55	1.9	2.15 ± 0.20	2.1

GFR=Glomerular Filtration Rate, Normal S.Cr range is 26 to 62 µmoles/liter, , Normal cystine level (Mixed leukocytes test) is less than 0.2 nm/mg protein, Normal Chitotriosidase level is < 78.5 nmol/hr/ml, Normal S.Calcium range is 2.2-2.6 mmol/L.

The Difference between patients and control groups in multiple demographics and disease measures

In the Table (3), there were non-significant differences between control and patients' groups in age (p-value > 0.05). There was a significant difference in mean rank (because most variables were not normally distributed, thus, mean ranked replaced the mean) for weight, BMI, serum creatinine, GFR, cystine, and chitotriosidase level of whole (30) cystinosis patients when compared with control (25) mean rank (P-value = 0.0001 < 0.05) as illustrated in Figures (1).

While serum calcium measures also showed significant differences between control and patient groups but with (P-value = 0.004 < 0.05) as shown in Figure (1).

Table 3. The difference between patient and control groups.

	Control group (Mean rank) n=25	Patient group (Mean rank) n=30	P-value
Age (months)	27.18	28.68	.728
Weight (Kg)	39.02	18.82	.0001*
BMI (kg/m^2)	37.14	20.38	.0001*
S. Creatinine (µmole/L)	17.90	36.42	.0001*
eGFR (mL/min/1.73 m^2)	40.98	17.18	.0001*
S. Calcium (mmol/L)	34.72	22.40	.004*
WBC cystine level (nmol\mg)	13.00	40.50	.0001*
S.Chitotriosidase (nmol/hr/ml)	14.56	39.20	.0001*

*Significant (P-value <0.05) according to the Mann-Whitney test.

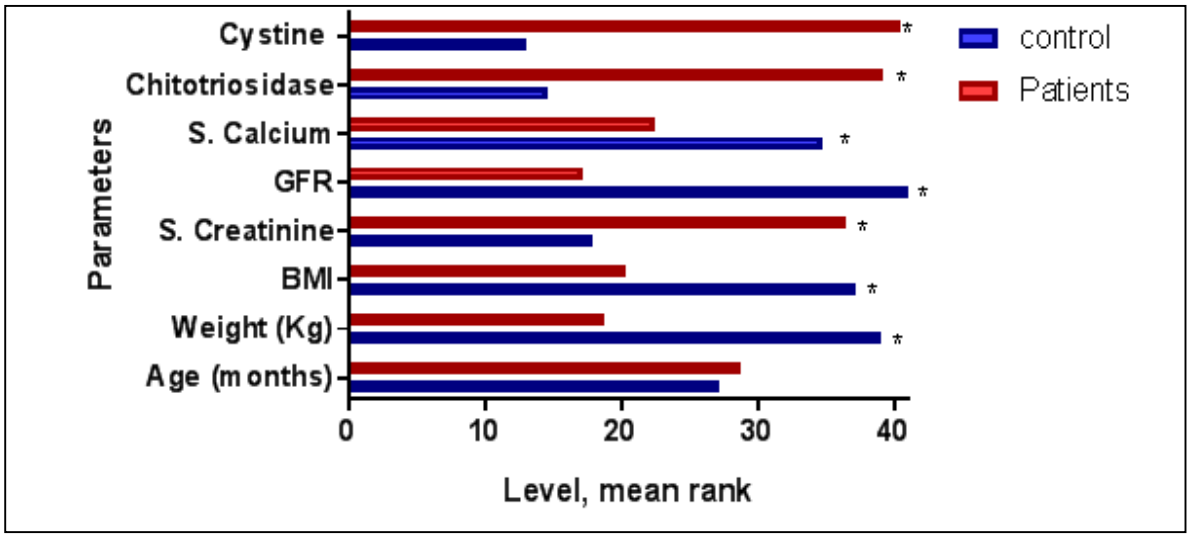

Figure 1.Comparison between different parameters of control and patients
***Significant (P-value <0.05) difference between mean rank**

Correlations of cystine & chitotriosidase levels with each other and with other measures:

As presented in Table (4) both cystine and chitotriosidase had significant positive correlations with each other's (r=0.957 P-value=0.000). Meanwhile, these two parameters (cystine and chitotriosidase) also had significant positive correlations with serum creatinine level (r=0.895, r=0.927 p-value=0.000).

On the other hand, cystine & chitotriosidase have significant negative correlations with serum calcium levels (r=-0.656, r= -0.612 p-value=0.000, respectively). Furthermore, cystine & chitotriosidase correlates negatively with GFR (r=-0.890, r= -0.917 p-value=0.000 respectively). Also there is significant negative correlations between cystine level & BMI (r= -.426 p-value=0.019) as mentioned in Table (4).

Table 4. Spearman's correlations among patient demographics and disease characteristics

Parameters		Cystine	Chitotriosidase
Age	Correlation Coefficient	.385*	.449*
	Sig.(2 tailed)	.036	.013
weight	Correlation Coefficient	.079	.200
	Sig. (2-tailed)	.676	.289
BMI	Correlation Coefficient	-.426*	-.281
	Sig. (2-tailed)	.019	.132
S. creatinine	Correlation Coefficient	.895**	.927**
	Sig. (2-tailed)	.000	.000
GFR	Correlation Coefficient	-.890**	-.917**
	Sig. (2-tailed)	.000	.000
S. calcium	Correlation Coefficient	-.656**	-.612**
	Sig. (2-tailed)	.000	.000
Cystine	Correlation Coefficient	1.000	.957**
	Sig. (2-tailed)	.	.000
Chitotriosidase	Correlation Coefficient	.957**	1.000
	Sig. (2-tailed)	.000	.

** Correlation is significant at the 0.01 level (2-tailed) according to Spearman's Correlation.
* Correlation is significant at the 0.05 level (2-tailed). Patient N=30

Discussion

As presented in Table (1), the measured children height and weight for those aged (≤10 years) for the cystinotic patients, as the (mean ±SD) height was (85.84 ±11.88) cm and the (mean ±SD) weight (11.20 ±3.00) kg, compared to that of healthy children of the same age (108.56 ±18.98) cm and (22.28 ±10.71) kg, respectively.

Furthermore, the results also indicated that GFR and serum creatinine levels were statistically different between the patient and the control groups as in Table (3). As the average glomerular filtration rate (GFR) of the patients was lower than that of the control group, while the average serum creatinine level was higher than of the control group owing to the progressive loss of the function of proximal tubular transporters in nephropathic cystinosis (NC) because of the accumulation of cystine, causing Fanconi syndrome. The glomerular involvement progress with an increase in plasma creatinine level and a decrease in the (GFR) if no specified treatment is administered, which evolves to advanced chronic kidney disease (CKD) that it is likely because of interstitial precipitates of cystine crystals[21].

The cystine WBC content of cystinosis patients was much higher than that of the control group. Since the cystinosis disease is resulted by (CTNS) gene mutations that encode the cystinosin (cystine transporter), which give rise to cystine accumulation in the lysosome. Cystine levels increase up to 100-fold in affected individuals compared with control subjects[22].

Meanwhile, at diagnosis of cystinotic patients were observed the serum chitotriosidase activity was elevated significantly over the age-matched healthy children and associated with leukocyte cystine level for patients. Therefore, in patients with nephropathic cystinosis, chitotriosidase is considered a therapeutic monitor and a promised clinical biomarker. Although the chitotriosidase enzyme is not specific for this disease, but it may aid in monitoring a patient's response to therapy[23]**.** Chitotriosidase is associated with activated phagocytes; its expression and release are induced by lysosomal stress. Thus, lysosomal stress can be present in cystinotic cells, as a result of cystine accumulation in cystinosis cells[24].

Meanwhile, serum calcium levels in cystinosis children were significantly lower than the control group despite that most of patients administered calcium supplements (Table-3 or Figure-1), this result is consistent with Theodoropoulos *et al.* study , since the natural history of nephropathic cystinosis includes tubular reabsorption defects beginning in the 1st year of life in which proximal tubular cells fail to reabsorb

minerals, electrolytes, and other small molecules, having both hypercalciuria and hyperphosphaturia as risk factors for nephrocalcinosis and patients with renal tubular Fanconi syndrome, excrete large amounts of calcium and phosphate[25]

There was a significant correlation between the age of patients and leukocyte cystine level, as the age of the patient increase the level of cystine increase (Table-4). Additionally, the patient's weight was increased with age thus requires the dose to be increased and it causes a burden to the patients which in turn influences the patients' compliance and in return the leukocyte cystine level. The adherence of patient to treatment is better in children ordinarily and head for fade in adolescents and adults[26].

Furthermore, the significant correlation between BMI and the leukocyte cystine levels due to the cystine crystals that precipitate throughout the body, including the bone leading to growth retardation[27]. The accumulation of cystine inside the lysosome destroys different tissues at different rates, possibly due to promoting apoptosis. One of the earliest organs to be damaged are the kidney, due to its potent proteolysis resulting in great amounts of cystine in the lysosome or due to its relative incapability to originate new differentiation of parenchymal cells[28]. Thus, our results revealed a significant effect of the cystine level on renal function (manifested as lowered GFR and elevated serum creatinine level) as leukocyte cystine level raised leading to a deterioration of the renal function.

Meanwhile, the present study demonstrated a relationship between the elevation of WBC cystine level and lowering serum level of calcium (Table-4), an interpretation of this result included cystine-loaded cells was associated with mobilization intracellular Ca^{2+} stores thus ER stress may influence the intracellular Ca^{2+} levels in cystinotic cells. The increased Ca^{2+} influx following CTNS knockdown may be a consequence of ER stress[29].

The elevation in serum chitotriosidase activity noticed in cystinosis patients may reflect a particular case of phagocyte activation concerned with cystine accumulation in the lysosome that causes excessive producing and/or releasing of the chitotriosidase. There is a linkage between cystine deposits and plasma chitotriosidase activity results from the noticing that the WBC cystine level and plasma chitotriosidase activity ran in parallel (the chitotriosidase level and WBC cystine level lowered concomitantly) (Table-4) and were both influenced by the regimen of doses[30].

Conclusion

In conclusion serum chitotriosidase activity estimation might aid in monitoring therapeutic benefit of cysteamine therapy, besides the prognosis of the disease when WBC- cystine assessment is not available, despite that chitotriosidase enzyme is not specific for this disease. However, it's believed to be a useful clinical screening test and a promising therapeutic monitor since a remarkable link between cystine accumulation and plasma chitotriosidase activity comes from the observation that plasma chitotriosidase activity and leukocyte cystine content ran in parallel and were both affected by the dosing regimen.

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