

Plasma citrate concentration: a possible biomarker for glaucoma in children

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ABSTRACT

Objectives The main aim of the present study was to examine a possible role of plasma and urine citrate levels as glaucoma indicators in school-aged children with glaucoma diagnosis.

Patients 34 school-aged children with a glaucoma diagnosis (mean age 15.69±1.86 years) were qualified for the study group and 34 patients with no ophthalmological ailments were qualified for the control group (mean age 16.1±1.98 years). Plasma and urine citrate levels in the study and the control group (Kruskal-Wallis test) were compared.

Results Plasma citrate levels in the study (16.33±4.51 mg/L) and the control group (19.11±3.66 mg/L) were different; the statistical significance (p=0.0036). Plasma citrate concentrations were significantly lower in the study group in comparison with the control group. There were no statistically important differences between the study group (291.12±259.13 mg/24 hours; 275.82±217.57 mg/g) and the control group (434.88±357.66 mg/24 hours; 329.81±383.27 mg/g) including urine citrate level (p=0.052) and urine citrate to creatinine ratio (p=0.4667).

Conclusion Plasma citrate concentration might be considered as glaucoma biomarker in paediatric population.

INTRODUCTION

Glaucoma is a group of chronic, progressive optic neuropathies which are marked with atrophy of the optic nerve and destruction of retinal ganglion cells (RGCs) and their axons.^{1 2} The process of the optic nerve atrophy and ganglion cells damage lead to irreversible changes in visual field and even to blindness.¹ In the majority of cases, the disease can be controlled by treatment decreasing intraocular pressure. Therefore, proper screening procedures giving early recognition of glaucoma are sufficient for counteracting the progression of the disease and the effective treatment.

According to the American Academy of Ophthalmology, proper glaucoma screening should consist mainly of the intraocular pressure (IOP) measurements, the visual field testing, assessing the optic nerve head (ONH)

What is already known on this topic?

- ▶ Glaucoma is one of the leading causes of blindness around the world.
- ▶ Targeting mitochondrial function to treat glaucoma is a promising possibility.
- ▶ Plasma citrate concentrations are lower in adults with glaucoma and are being considered for use as a biomarker.

What this study hopes to add?

- ▶ Plasma citrate concentrations are significantly lower in children with glaucoma.
- ▶ Plasma citrate concentrations may be suitable as a biomarker for glaucoma for children with this disease.

and retinal nerve fibre layer (RNFL). The credibility and repetitiveness of the results of the visual field testing, IOP measurements or ONH and RNFL scans are essential in diagnosing glaucoma in children.^{1 2} However, obtaining credible and repeatable results of mentioned measurements in children is often a challenging task for ophthalmologists.^{1 2} Therefore, identifying a reliable indicator for glaucoma appears to be in great demand.^{3 4}

In the recent years, several genetic and non-genetic biomarkers for glaucoma were described in specialist literature.^{3 4} The genetic studies showed differences in causes of glaucoma inheritance in adults and in children. Autosomal dominant inheritance is observed in children, whereas multiple genetic and environmental factors are observed in adults.⁴ The reasonable assumption appears that types of non-genetic biomarkers may vary in different age groups. However, non-genetic molecular markers were mainly tested only in adult patients.^{5–40}

Citrate are one type of organic molecules considered as glaucoma biomarker.⁴⁰ The study in adult population showed low plasma citrate level in patients with glaucoma



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diagnosis, whereas the test in children has not been performed yet. When preparing the test for children, a researcher should take into account idiosyncrasy of the certain examinations in paediatric population.⁴¹ Therefore, it is worth mentioning that citrate level did not remain constant through all the childhood period. Moreover, citrate level depends on gender.

The main aim of the present study was to examine a possible role of plasma and urine citrate levels as glaucoma indicators in school-aged children with glaucoma diagnosis. The research was also to reject the hypothesis that citrate plasma level as a biomarker in adults with glaucoma cannot be a biomarker in children with glaucoma, taking into account differences of glaucoma profile in adults and in children.

METHODS

The current study was the result of cooperation between two departments of Medical University of Białystok: the Department of Paediatric Ophthalmology and Strabismus and the Department of Pediatrics and Nephrology. Thirty-four patients with glaucoma hospitalised for observation in the Department of Paediatric Ophthalmology and Strabismus were qualified for the study group, and 34 sex-matched, age-matched and body mass index (BMI)-matched children/adolescents were qualified for the control group.

Inclusion criterion for the study group was glaucoma diagnosis. The glaucoma diagnosis and decision on treatment were the result of long-standing watchful waiting in all groups of children. The grounds of making glaucoma diagnosis were not only the increased values of IOP in daily measurements but also progressive changes in the visual field testing and/or progressive atrophy of the optic nerve assessed by the means of OCT. The treatment process was started and modified due to the increased values of IOP in daily measurements and/or progressive changes in the visual field testing, progressive atrophy of the optic nerve. Pachymetry was performed in all patients to verify IOP values.

The exclusion criteria were as follows: history of cerebral damage, non-glaucomatous optic disc neuropathies, active ocular inflammatory disease, head injury, abnormalities in dipstick urinalysis, diseases affecting urinary citrate excretion, intake of drugs affecting kidney function, mitochondrial disease, restrictive diet, drugs abuse, alcohol intake, tobacco consumption and incomplete 24-hour urine samples determined by creatine excretion rates.

The patients in the control group were the laboratory norms described by the Department of Pediatric Nephrology. The patients enrolled into the control group were patients with primary nocturnal enuresis who had stayed in the Department of Pediatric Nephrology and were healthy children of hospital staff families. The study and its testing procedures were approved by the

university ethics committee and were in accordance with the Declaration of Helsinki.

Citrate analysis

Fasting-blood samples were sampled in the morning. Initially, to prepare plasma for citrate analysis, the plasma was deprived of protein with the use of 2 M sulfosalicylic acid (in the ratio 0.1 mL sulfosalicylic acid:1 mL plasma). Afterwards, plasma was spun and kept in refrigerator at -80°C until examining. Alternatively, to receive protein-deprived plasma, the 0.05 mL of 1 M Tris-HCl buffer (pH 8.2) was added to alkalise the sample (at minimum 8.0 pH level).

The 24-hour urine collection was stored in plastic containers at temperatures $<4^{\circ}\text{C}$ to avoid bacterial colonisation. The 0.5 mL urine sample for citrate analysis was extracted from 24-hour urine collection and diluted with 0.5 mL demineralised water. Alkalisiation of urine sample at minimum 8.0 pH level was performed with the use of the 0.05 mL of 0.25 M Tris-HCl buffer (pH 8.2), according to the producer's kit instruction for citrate analysis.

Urinary and plasma citrate levels were assessed with the use of an enzymatic method using a commercial set (R-Biopharm, Darmstadt, Germany). Twenty-four-hour citrate excretion was expressed in absolute values, adjusted for urinary creatine per kilogram of body weight, related to the 1.73 m^2 of body surface area and as urinary concentration. Urinary and plasma creatine was measured by the Cobas-Integra 800 analyzer and Roche reagents (Roche, Indianapolis, Indiana, USA).

Statistical analysis

Statistical data were processed using STATISTICA V.10 (StatSoft). The χ^2 distributions, Kruskal-Wallis test, were processed for statistical analysis. χ^2 distribution was executed to confirm no statistically important differences in percentage of boys and girls between the study group and the control group. Kruskal-Wallis test was accomplished to determine possible age and BMI differences between the study and the control group. Citrate values obtained from urine and plasma were compared in the study group and the control group (Kruskal-Wallis test). Diagrams were prepared to visualise statistically important differences of citrate plasma levels between the test group and the control group.

RESULTS

A total of 34 school-aged children with the glaucoma diagnosis were recruited. The mean age in the study group was 15.69 ± 1.86 years old. BMI in the study group was equal to $20.18 \pm 3.96\text{ kg/m}^2$ (44 ± 32.22 percentiles). Boys ($n=21$) made up 62% and girls ($n=13$) 38% of the study group. Thirty-three patients had pharmacological treatment, whereas in one patient antiglaucomatous treatment was initiated after hospitalisation. One medication was used in 18 patients (17 patients—two eyes; 1 patient—one eye). Two medications were used in 10 patients (9 patients—two eyes; 1 patient—one eye).

Table 1 Citrate concentrations in the test and the control group (Kruskal-Wallis test)

Citrate levels	Test group (n=34), mean \pm SD	Control group (n=34), mean \pm SD	Significance of difference, (df=1)
Plasma citrate level (mg/L)	16.33 \pm 4.51	19.11 \pm 3.66	p=0.0036*
Urine citrate level (mg/24 hours)	291.12 \pm 259.13	434.88 \pm 357.66	p=0.052
Urine citrate (mg)/creatinine (g) ratio	275.82 \pm 217.57	329.81 \pm 383.27	p=0.4667

*Difference was significant at the p<0.05 level.
n, number of patients.

Three medications were used in five patients (four patients—two eyes; one patient—one eye). The monotherapy of brinzolamidum (4 patients—two eyes), dorzolamidum (3 patients—two eyes), latanoprostum (10 patients—two eyes), travoprostum (1 patient—two eyes) and bimatoprostum (1 patient—one eye) was used. Various combination of two or three medications involving brinzolamidum (2 patients—two eyes), dorzolamidum (9 patients—two eyes; 2 patients—one eye), timololum (12 patients—two eyes; 2 patients—one eye), latanoprostum (6 patients—two eyes), travoprostum (1 patient—two eyes) and bimatoprostum (1 patient—one eye) were used in politherapy. Best-corrected visual acuity was equal to 1.0 in all eyes in study group.

Mean age in the control group was 16.1 \pm 1.98 years. Boys (n=24) made up 71% and girls (n=10) made up 29% of the control group. BMI in the control group was equal to 19.95 \pm 3.8kg/m² (44 \pm 30.71 percentiles). No ocular ailments were noted while enrolling in the control group.

χ^2 distribution showed no statistically important differences in percentage of boys and girls between the study group and the control group. Kruskal-Wallis test revealed no statistically important age and BMI differences between the study group and the control group.

Citrate levels in plasma and urine varied in school-children with glaucoma diagnosis in comparison with their healthy peers (Kruskal-Wallis test). The plasma and urine citrate levels were lower in the study group, as compared with the control group (table 1). However, the statistically significant result was citrate concentration obtained only from plasma (p=0.0036). The relevance of urine citrate level (p=0.052) and urine citrate to creatine ratio (p=0.4667) differences between the study group (291.12 \pm 259.13 mg/24 hours; 275.82 \pm 217.57 mg/g) and the control group (434.88 \pm 357.66 mg/24 hours; 329.81 \pm 383.27 mg/g) was not statistically important. Diagrams highlight statistically important differences of plasma citrate levels between the study group (16.33 \pm 4.51 mg/L) and the control group (19.11 \pm 3.66 mg/L) (figure 1).

DISCUSSION

As one of the leading causes of blindness around the world, glaucoma is continually an urgent unsolved problem.¹ In the paediatric population in the USA, the glaucoma incidence rate amounts to 2.29 per 100 000

patients under 20 years of age.² Even though the rate mentioned above is not so high, it is highly expected to develop knowledge in the field of glaucoma—its pathological mechanisms, innovative diagnostic methods and new treatments, while the process of making proper diagnosis seems to be challenging and the long-term consequences of glaucoma might be serious.^{2 42}

Impaired mitochondrial function is regarded to be a possible indicator of glaucoma and may contribute to its pathogenesis.⁴² Mitochondria generate ATP, which is necessary for proper functioning of nerves, including the optic nerve. During the oxidative stress, mitochondrial function is being impaired—reactive oxygen species production prevails over ATP generation. ATP deficiency and oxidative stress contribute to dysfunction of mitochondria in RGCs and lead to RGCs apoptosis which is regarded to be the pathological feature of glaucoma. The role of a modern antiglaucomatous medication is to ensure neuroprotection and to meet antioxidative needs.

In the light of recent studies on mitochondrial dysfunction in glaucoma pathogenesis, the role of citrate as a glaucoma biomarker seems to be a relevant issue.^{40 43} Citrate are synthesised in mitochondria from acetyl coenzyme A and oxaloacetate by citrate synthase and then citrate become a substrate in the tricarboxylic acid (TCA) cycle. The TCA oxidation process provides the major source of cellular ATP production. Moreover, citrate

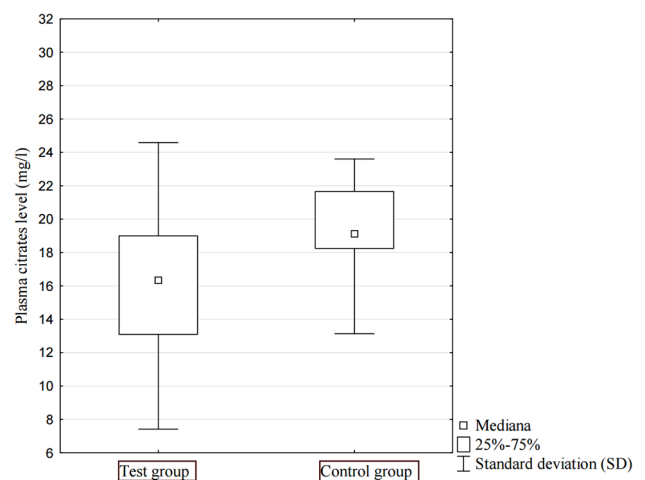


Figure 1 Whiskers diagrams highlight plasma citrate level differences between test and control group.



are also a key regulator of energy production while they inhibit and accelerate enzymes significant in the processes of glycolysis, Krebs cycle, gluconeogenesis and fatty acid synthesis. On the other hand, citrate contribute to gluconeogenesis and lipid synthesis which absorbs ATP energy.

Currently, the multidimensional role of citrate is being highlighted.⁴³ Citrate are signalling molecules in inflammation processes and in insulin secretion, and citrate are metabolites involved in tumorigenesis and an iron recruiter in the beginnings of the non-alcoholic fatty liver disease. The altered transport of citrate influences the reduction of histone acetylation and the development of neurological disorders. Moreover, fluctuations in the citrate levels are considered as a useful diagnostic tool partially as a biomarker.^{40 43}

Plasma citrate level as potential biomarker for glaucoma was described for the first time by Fraenkl *et al* who accidentally found low level of citrate in patients with glaucoma.⁴⁰ Outcomes of this study in paediatric population correspond with the results achieved by the researches from the University Hospital in Basel. As recommended by Remer *et al*, in this study, urine citrate and creatine concentrations in 24-hour urine collection were examined, which is a more suitable and precise test for paediatric population.⁴⁴ However, the results in children similar to those obtained by Fraenkl *et al* were received in this study.⁴⁰ As far as the study group and the control group were concerned no statistical significance between the excretion of citrate and the relation between citrate and creatine was demonstrated. In view of the above, it is very unlikely that pathology of the kidneys may have influenced citrate level in plasma, according to Fraenkl *et al*.⁴⁰ Therefore, citrate might be considered as a universal biomarker in children as well as in adults.

The fact that during glaucoma treatment plasma citrate levels remain low might be explained in such a way that ophthalmological diseases are still treated only symptomatically. Targeting mitochondrial function to treat glaucoma is a promising possibility that has been recently raised by Gueven *et al*. Hypothetically, citrate levels in plasma may normalise when impaired function of mitochondria is repaired; however, further research is required.

It might be possible that this study has some limitations. First, the number of patients in the study groups was low. Second, the patients remained mainly during antiglaucomatous treatment. However, obtaining a decent sample without antiglaucomatous treatment in the paediatric population for the purpose of a study group might involve worldwide and multiannual research, because of the fact that incidence of glaucoma in children is extremely low and the process of making glaucoma diagnosis is a complex and time-consuming one.

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critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. BU, WA and AB-L: interpretation of data; revising critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Competing interests None declared.

Patient consent Detail has been removed from these case descriptions to ensure anonymity. However, the patient consent form approved by the University Ethics Committee has been signed by the guardian and the patient (>15 years old). The patient consent form was prepared in Polish. In this way, the patient consent form was understood by the guardian and the patient.

Ethics approval The University Ethics Committee.

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