Assessment of Cross-correlations Between Selected Macromolecules in Urine of Children with Idiopathic Hypercalciuria.

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Purpose: The aim of the study was assessment of four selected macromolecules level: osteopontin, calgranulin, uromodulin and bikunin in fresh morning urine sample in children with nephrolithiasis in the course of idiopathic hypercalciuria.

Materials and Methods: The study included 90 subjects aged from 12 months to 18 years. The study group comprised 57 subjects- children with urinary tract lithiasis in the course of idiopathic hypercalciuria and the control group - 33 healthy children with no history of urolithiasis. Determinations of osteopontin, calgranulin, uromodulin and bikunin levels in the first morning urine were performed.

Results: The study group had a significantly decreased osteopontin excretion and significantly increased bikunin excretion, and increased, however statistically nonsignificant, calgranulin excretion in comparison with the control group. Uromodulin excretion did not differ between groups. In both groups a statistically significant positive correlation was observed between uromodulin and bikunin levels.

Conclusion: Children with urinary tract lithiasis in the course of idiopathic hypercalciuria reveal a different distribution of the study proteins than a healthy population.

Keywords: idiopathic hypercalciuria; urolithiasis; inhibitor proteins; osteopontin; bikunin.

INTRODUCTION

Urinary tract lithiasis is one of the oldest and most common diseases[1, 2]. Its incidence in developed societies is increasing together with development of civilization, changes in lifestyle, diet rich in protein and perhaps global warming. It is estimated that for the life expectancy of 70 years, this pathology may in certain communities affect even 15% of the population[3]. Renal stones develop at every latitude independently of age, sex or ethnic group[2]. In Europe, about 2% of children are affected with urinary tract lithiasis[4,5]. In recent years, we have observed an increase in the disease incidence in this group of patients. There are also reports of a growing incidence among infants[6].

The disease is usually recurrent, which means that treatment and prevention concerns virtually the whole life of the affected subject. It is estimated that there is about 80% (between 50 and 100%, depending on the type of stone) chance of recurrence in untreated patients and 10-15% in patients undergoing treatment followed by further prevention[7]. Lithiasis, especially in the course of idiopathic hypercalciuria, may also be associated with reduced bone density[8]. In more than 70% of cases, there are factors predisposing for development of this disease[9]. In paediatric populations, metabolic disturbances leading to development of stones and the risk of recurrence do not seem to be age-dependent[10]. The most important and simplest imaging study in the diagnosis of kidney stone disease is ultrasonography. It is a highly sensitive test in the hands of a skilled ultrasonographer. It allows to visualize stones 2-3mm in diameter. A typical stone is seen as a hyperechogenic structure with posterior acoustic shadow and twinkle artefact in color Doppler ultrasound.

The most common type of lithiasis, equally in adults and children, is calcium oxalate lithiasis in the course of idiopathic hypercalciuria, which accounts for 70-80% of renal stones[1]. The term 'idiopathic hypercalciuria' is in use from 1953 when this definition was proposed by Albright to describe the association of elevated urinary calcium excretion with a normal serum calcium level in patients with calcium containing renal stones[11]. Although the disease has existed for a long time and is widespread, its pathophysiology remains unknown. There are many factors that contribute to deposit formation. On one hand, there are environmental factors. On the other hand, there are genetic factors, metabolic disorders, infections, defects in renal and urinary structure, undernutrition and drugs administered for other concomitant diseases[12,13]. It is also common knowledge that high body mass index in adults is relat-

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ed with a higher risk of lithiasis[14].
Stone formation is induced by an increased level of urine crystallisation promoters and reduced levels of its inhibitors. They are active at different stages of deposit formation – nucleation, aggregation and growth. The inhibitors include magnesium, citrates, zinc and organic compounds produced by renal tubular epithelial cells - glycosaminoglycans, as well as various proteins, for instance uromodulin (Tamm-Horsfall protein), osteopontin (uropontin), osteocalcin (nephrocalcin), calgranulin and bikunin[15]. According to some authors, crystal aggregation inhibitors reveal daily and annual patterns of activity (16). Crystallization promoters are those substances that may constitute a nucleus on which stone is formed. These may include bacteria, foreign bodies or desquamated epithelial cells[9].
Healthy people regularly excrete calcium oxalate crystals in urine. However, stones are formed only in a small part of the population[1]. Affected subjects are those with disturbed processes of promotion and inhibition of crystal growth. What is the role of macromolecules? Paradoxically, most of them may act as both promoters and inhibitors depending on circumstances (for example urine pH). We do not know the exact role of many proteins present in urine. There is a theory that normal level and structure of macromolecules may constitute protection against formation of large, intratubular precipitates of calcium salts.
The purpose of treatment, apart from removal of stone(s), is to prevent formation of subsequent deposits[17,18]. The aim of the study was assessment of four selected macromolecules level in fresh morning urine sample in children with nephrolithiasis in the course of idiopathic hypercalciuria. We examined the level of uromodulin, osteopontin, calgranulin and bikunin and compared their excretion in children with urolithiasis and in control group.

PATIENTS AND METHODS

Study population

The study included 90 subjects aged from 12 months to 18 years hospitalised in the Clinic of Paediatrics, Nephrology and Paediatric Allergology at the Military Institute of Medicine, Warsaw from June 2011 to June 2012. Study group were children with urolithiasis in the course of idiopathic hyperclaciuria, who were in continuous care of the Clinic and were diagnosed prior to the study. Control group were healthy children with no urinary stones in ultrasound examination, no hypercalciuria and no history of urolithiasis

Study design

In the study group, diagnosis of idiopathic hypercalciuria was based on tests performed in the Clinic during previous hospitalisations. The basic criterion was increased calcium excretion in urine with normal serum calcium, normal gasometry and the presence of stones in urinary tract at any time since the onset of the disease (which was the appearance of the first concrement). For most patients, before qualification to the study, parathyroid hormone level was also determined, and for all patients - creatinine, phosphorus, magnesium and uric acid in serum and in a 24-hour and 3-hour urine samples. The results of these tests showed no abnormalities, which was a decisive factor of qualifying the children to the study group of patients with idiopathic hypercalciuria.
Exclusion criteria comprised any acute febrile disease, urinary tract infection, renal disease other than lithiasis or defects in the urinary tract. In the control group, additional exclusion criteria were a positive medical history or a family history of urinary tract lithiasis.

Determination techniques

Determinations of serum creatinine, calcium, phosphorus and sodium and eGFR calculations were performed for all the children (study group and control group). In patients with hypercalciuria blood tests were done to control again, that it is its idiopathic form and in case of control group they were done to ascertain that there are no abnormalities in tests results. The study molecules, i.e. osteopontin, calgranulin, uromodulin and bikunin were determined in the first morning fasting urine sample.
All determinations were based on one sample of urine and blood. Blood was collected in the morning on empty stomach. Urine came from the first morning fasting sample collected in sterile conditions (as for urine culture). Immediately after sampling, urine was centrifuged and placed in test tubes adapted to deep freezing, then frozen at -80°C. Urine samples were collected after general urinalysis, which was performed to exclude urine infection. It was performed on a typical normocalcemic child diet without administration of medicines. Schwartz equation (19) was used to calculate estimated glomerular filtration rate – eGFR :
GFR (mL/min/1.73m2) = k x growth (cm)/serum creatinine (mg/dL)
where k = 0.413 (Schwartz modification, common for all age groups).
Ultrasound scanning examinations were performed in our Clinic, always by the same person, with the use of Logiq 5 Expert machine from MedCorp. Ultrasonography allowed to visualize stones up to 2 mm in diameter.
Serum calcium, phosphorus, magnesium, uric acid and creatinine were determined using a colorimetric method, while sodium levels were determined using the method of ion-selective potentiometry on Cobes Integra 800 autoanalyser from Roche.
Protein levels in the first morning urine sample was determined with an immunoenzymatic method ELISA with the use of commercial tests:
—bikunin level with Human Protein AMBP ELISA Kit catalogue number E0965h from EIAab®,
—uromodulin level with Human Uromodulin ELISA Kit catalogue E2280h from EIAab®,
—calgranulin level with Human Protein S100-A9 ELISA Kit catalogue number E1793h from EIAab®,
—osteopontin level with Quantikine Human Osteopontin Immunoassay, catalogue number DOST00 by R&D Systems®.

Statistical analysis

Calculations and analysis were performed with the use of Statistica 10.1 software with a medical pack (StatSoft Co). Initially, distribution of collected variables was performed with the use of the following tests: Kolmogorov-Smirnov test with the Lilliefors amendment and the Shapiro-Wilk test. For variables characterised with normal distribution, the Student's t-test of mean variables was used. For variables whose distribution did not meet normality criteria, Mann-Whitney-U non-parametric statistics was calculated. Depending on the

Table 1. Distribution of age and parameters assessed in serum of the study and control group

Variables[a]	Study group mean ± SD min-max median	Control group mean ± SD min-max median	*P*-value
Age (years)	11.05 ± 4.99 1.0-18.0 11.0	9.21 ± 4.66 1.0-17.0 9.0	.09
Creatinine serum - mg/dL	0.539 ± 0.18 0.2-0.9 0.5	0.455 ± 0.13 0.3-0.7 0.4	.02
eGFR: mL/min/1.73m^2	118.87 ± 25.88 80.0-189.0 114.5	126.69 ± 20.70 96.0-180.0 127.0	.14
Serum Ca - mg/dL	10.0 ± 0.37 9.1-10.9 10.0	9.96 ± 0.42 9.1-10.7 9.9	.64
Serum P - mg/dL	4.48 ± 0.75 3.0-6.2 4.45	4.73 ± 0.62 4.7 3.3-5.7	.09
Serum Na - mmol/L	138.8 ± 2.09 135.0-144.0 139.0	138.7 ± 1.68 135.0-142.0 139.0	.70
Serum Mg - mg/dL	2.07 ± 0.14 1.8-2.5 2.1	2.14 ± 0.15 1.9-2.3 2.2	.23

Abbreviations: eGFR, Glomerular Filtration; Ca, Calcium; P, Phosphorus; Na, Sodium; Mg, Magnesium
a Continuous variables were compared by the Student's t-test or Mann-Whitney-U test (dependent of the results of Kolmogorov-Smirnov and Shapiro-Wilk tests).

results of the previous analyses, the following calculations were performed: Pearson's parametric correlation coefficient for variables with Gaussian distribution, or its non-parametric equivalent - Spearman's correlation coefficient for non-Gaussian variables. Each time, statistical significance level of $P \leq .05$ was used.

RESULTS

The study group comprised 57 subjects (27 girls and 30 boys), children with urinary tract lithiasis who were found to have idiopathic hypercalciuria. 32 of them (16 girls and 16 boys) were patients with renal stones at the moment of examination, shown in ultrasound and the remaining 25 (11 girls and 14 boys) were patients with no stones in urinary tract at the moment of examination. The control group comprised 33 children (19 girls and 14 boys) admitted to the Clinic due to headaches, suspected allergy or episodes of fainting, who revealed no significant pathologies upon examination and who had no history of urolithiasis in patients and in their families, no hypercalciuria and no stones in performed ultrasound examination.

The study and control groups did not reveal statistically significant differences with regard to age, sex and blood parameters – calcium, magnesium, phosphorus, sodium and glomerular filtration expressed as eGFR. A significant difference with regard to serum creatinine is not of clinical importance, since both values are within standards for this age and there were no differences in calculated eGFR between groups (**Table 1**).

Next, excretion of osteopontin, calgranulin, uromodulin and bikunin was analysed. The study group revealed statistically significantly lower osteopontin excretion and statistically significantly higher bikunin excretion in comparison with the control group. Moreover, increased, however statistically nonsignificant, calgranulin excretion was observed in the study group as compared to the control group. Uromodulin excretion did not differ between groups (**Table 2**).

In order to find out if there is a cross-correlation between the levels of the studied proteins, they were examined separately in the study and control group with the use of the Spearman test. In both groups, a statistically significant positive correlation was observed between uromodulin and bikunin levels (Tables 3and 4).

DISCUSSION

Crystals that change into stones develop in urine, which contains a mixture of ions, salts, macromolecules and metabolites[20]. Already in the 70's of the previous century, Gill et al. showed an inhibitory effect of macromolecules from human urine on crystallisation of calcium oxalate[21].

Sheng et al. observed reduced calcium oxalate monohydrate (COM) crystal adhesion to urinary epithelium depending on the presence of protein carboxyl groups[22]. The findings showed that proteins that potentially protect against lithiasis may have a different composition in affected patients than in healthy subjects. Therefore, macromolecules inducing stone formation should be examined both with regard to their quantity and quality. As for now, it is difficult to decide which proteins, in the rich urine proteome, should be examined with regard to their possible association with lithiasis. Literature describes attempts of various correlations.

The present study attempts to compare the level of four selected macromolecules (uromodulin, osteopontin, calgranulin and bikunin) in fresh morning urine sample in children with nephrolithiasis in the course of idiopathic hypercalciuria and in healthy control group. These four proteins were chosen for two reasons- they were frequently described in literature and we had good experience with the tests in terms of obtaining reliable results.

No statistically significant difference between groups was observed with regard to uromodulin excretion, which is in line with most literature data. Uromodulin is the most important protein in urine of healthy people.

Table 2. Data on excretion of the studied proteins in the study and control group

Variablesa	Mean – study group	Mean – control group	*P*-value	SD – study group	SD – control group
Osteopontin ng/mL	2058.66	3590.20	.0005	1690.51	2305.48
Calgranulin pg/mL	268.80	120.15	.07	451.71	84.15
Uromodulin ng/mL	13.52	12.19	.39	7.74	5.37
Bikunin ng/mL	23.11	16.48	.0128	14.79	2.98

[a] Continuous variables were compared by the Student's *t*-test or Mann-Whitney-*U* test (dependent of the results of Kolmogorov-Smirnov and Shapiro-Wilk tests).

One of its numerous functions is affecting aggregation of calcium oxalate crystals. Numerous researchers have proved, however, that uromodulin activity depends on the composition, instead of the amount of protein in urine, and that it is able to both prevent and promote stone formation[23,24]. In a recently published paper, Viswanathan et al. have shown that this protein contains less sialic acid in patients with lithiasis, which leads to reduction of its negative charge[25]. This form of protein promotes aggregation of calcium oxalate monohydrate, whereas the same protein prevents aggregation in healthy subjects with normal content of sialic residues. Wikiera-Magott et al. also studied uromodulin level in urine of children with urinary tract lithiasis[26]. They did not observe differences in concentration of the excreted protein between the group with symptomatic lithiasis, group endangered with lithiasis and the control group. In the study by Baggio et al. children with lithiasis had increased uromodulin excretion[27]. Similarly, increased excretion of this protein, with its different composition at the same time, was observed by Jaggi et al. in urine of affected adults with high intensity of stone formation[28]. Glauser et al. assessed 24-hour uromodulin excretion by means of ELISA method and presented it in the form of uromodulin/creatinine ratio[29]. They observed a significantly lower excretion of this protein in urine in subjects with calcium deposits as compared with the healthy control group. Excretion was not correlated with age of the subjects, urine volume, dietary calcium supply or protein consumption. It was significantly correlated, however, with citrate excretion in both groups. So, those few publications presenting quantitative differences in uromodulin excretion did not have the same findings, which may indicate random nature of the differences.

Another examined protein was calgranulin. No statistically significant difference it its urine concentration was observed between the study and control group. The ability of calgranulin to inhibit crystallisation, aggregation and adhesion to urinary epithelium of calcium oxalate monohydrate crystals was revealed for instance by Momohara et al.[30]. The presence of calgranulin in CaOx deposits were also observed in the study by Mushtaq et al.[31]. This study, however, proved that the protein promoted crystal aggregation. Bergsland et al. observed, similarly to this study, that the concentration and composition of calgranulin differed in subjects with a family history of urinary tract lithiasis in comparison with a healthy population[32].

Another examined protein was bikunin. A statistically significant higher excretion of this protein in urine was observed in the affected children. Atmani and Khan described the ability of bikunin to inhibit nucleation and aggregation of calcium oxalate crystals[33]. There are also data proving that bikunin of subjects with lithiasis does not prevent crystallisation so well as in healthy subjects[20,34]. In a study by Medetognon-Benissan et al. strong inhibitory effect of bikunin on CaOx crystallisation was confirmed by in vitro studies[35]. On the other hand, a comparison of this protein in urine of adults with calcium oxalate lithiasis with urine of healthy subjects by means of the ELISA method, the authors confirmed that bikunin level was 50% lower in affected subjects. This observation is contrary to the findings of this paper, which may result from different sampling conditions or perhaps from a small count of the study group in the quoted paper (31 subjects vs 18 subjects in the control group). A higher bikunin level in urine, which we observed in the study subjects may reflect striving to prevent deposit formation, especially in relation with the findings concerning osteopontin and possible correlations between them.

The last examined protein was osteopontin. Its urine level was statistically significantly lower in patients with idiopathic hypercalciuria than in children of the control group. Okada et al. conducted studies which showed an important role of osteopontin in transforming calcium oxalate crystals into stones[36]. In their study on mice deprived of osteopontin, Wesson et al. revealed that during experimental induction of hyperoxaluria the animals revealed numerous deposits of calcium oxalate crystals in renal tubules[37]. Such deposits were not found in wild mice on the same diet. Osteopontin level in urine of patients with calcium oxalate lithiasis was

Table 3. Assessment of correlations between the studied proteins in the study group. Spearman's rank correlation.

Variables[a]	R - Spearman	*P*-value
Osteopontin & Calgranulin	-0.20	.13
Osteopontin & Uromodulin	0.12	.36
Osteopontin & Bikunin	-0.08	.55
Calgranulin & Uromodulin	-0.14	.29
Calgranulin & Bikunin	0.13	.33
Uromodulin & Bikunin	0.43	.00094

[a] Continuous variables correlations were compared by the Pearson's or Spearman's test (dependent of the results of Kolmogorov-Smirnov and Shapiro-Wilk tests).

Table 4. Assessment of correlations between the studied proteins in the control group. Spearman's rank correlation.

Variablesa	R - Spearman	P-value
Osteopontin & Calgranulin	-0.15	.41
Osteopontin & Uromodulin	-0.15	.42
Osteopontin & Bikunin	-0.26	.15
Calgranulin & Uromodulin	-0.26	.14
Calgranulin & Bikunin	0.04	.82
Uromodulin & Bikunin	0.46	.0074

[a] Continuous variables correlations were compared by the Pearson's or Spearman's test (dependent of the results of Kolmogorov-Smirnov and Shapiro-Wilk tests).

found to be reduced by some researchers and to be normal by others[38,39]. Similar results to the present study were described by Yasui et al. who observed a reduced osteopontin excretion in subjects with lithiasis[40]. The latter authors associated a lower osteopontin level in the morning urine of patients with lithiasis with its embedding into the deposit matrix.

Studying the role of osteopontin in urinary deposit formation, Mazzali et al. similarly to Wesson et al. revealed that mice genetically deprived of this protein and with experimentally induced hyperoxaluria had intratubular deposits of calcium oxalate[37,38]. Mice of the same genetically modified type did not accumulate crystals inside tubules if there were no excess oxalates. The authors concluded that this proved existence of other crystallisation inhibitors that compensated for the lack of osteopontin. According to this theory, this role could be played in the children we studied by bikunin and calgranulin.

De Yoreo et al. observed in vitro that osteopontin from urine of healthy subjects had higher abilities to inhibit formation of calcium oxalate monohydrate crystals than in patients with lithiasis[34]. The author found osteopontin to be the main component of organic matrix of stones. The role of osteopontin in formation of COM crystal was also discussed by Langdon et al.[41]. They proved that the ability to inhibit crystallisation depends on osteopontin phosphorylation. Summing up data on osteopontin it must be stated that its activity also seems to be dependent on differences in the molecule structure. This does not exclude, however, quantitative differences in its excretion between healthy and affected subjects. Recently association between polymorphisms in osteopontin gene and urolithiasis was described [42, 43].

Finally, the comparison between the healthy and affected subjects presented in the present study revealed reduced osteopontin and increased bikunin levels in urine of the affected subjects. This may confirm the thesis about mutual correlations between these proteins. It may be assumed that the increased bikunin level was to compensate reduced osteopontin excretion.

Recently, a new publication by Khan has appeared, which, based on huge amount of literature data, presents hypothesis about a participation of free oxygen species in stone formation[44]. According to this hypothesis, macromolecules related to lithiasis are produced as a response to inflammation. The author presents mutual correlations between osteopontin, uromodulin, bikunin and calgranulin, and also prostaglandin E2, α-1 microglobulin and fibronectin. Free radicals initially cause production of macromolecules - inhibitors of crystallisation preventing stone formation. In the course of time, however, reduced antioxidative protection or permanent oversaturation of urine with crystallising substances may lead to increased production of free radicals and gradual formation of stones. Similar theories are considered by Hong[45].

In our study a correlation between uromodulin and bikunin excretion was observed both in children with urolithiasis and in the control group. The higher was urine uromodulin, the higher was urine bikunin.

Limitations of the study

Unfortunately, in our patients with urinary tract lithiasis in the course of idiopathic hypercalciuria excretion of oxalates and citrates was not determined. It could be used for further group differentiation without changing diagnosis but possibly affecting interpretation of results, since it is common knowledge that citrates inhibit crystallisation and some patients with idiopathic hypercalciuria demonstrate concurrent excessive output of oxalates. Both these parameters may affect stone formation.

We studied relatively small group of children. In order to draw undisputed conclusions, all the findings of the present study should be confirmed on a much more extensive group of subjects.

CONCLUSIONS

The children affected with urolithiasis in the course of idiopathic hypercalciuria reveal a different distribution of the studied proteins that the healthy population. It is only to speculate if our results can have practical clinical value, but they give us a possibility to look at one aspect of complicated pathogenesis of idiopathic hypercalciuria. We cannot have an impact on proteins level in urine of our patients, but if we knew their value, may be in the future we would be able to predict how active in terms of forming new stones is the disease.

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The children's guardians and patients over 16 gave their written consent for the participation in the study.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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