



Original research article

Urinary exoglycosidases, reference values in healthy children

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ABSTRACT

Purpose: The purpose of the study was to determine the effect of age on lysosomal exoglycosidase activities: α -fucosidase, β -galactosidase, β -glucuronidase and α -mannosidase in healthy children and adolescents.

Material and methods: Urine samples were collected from 203 healthy children and adolescents (girls = 99, boys = 104), aged six months to 17.9 years. The activities of α -fucosidase, β -galactosidase, β -glucuronidase and α -mannosidase were determined by colorimetric method and expressed in pKat/ μ g of creatine (pKat/ μ g Cr.).

Results: Urinary α -fucosidase, β -galactosidase, β -glucuronidase and α -mannosidase activities (pKat/ μ g Cr.) were the highest in children below 3 years of age in comparison to the remaining age groups. There was a statistically significant negative correlation between urinary α -fucosidase, β -galactosidase, β -glucuronidase and α -mannosidase (pKat/ μ g Cr.) and age ($r = -0.36$; $r = -0.36$; $r = -0.35$; $r = -0.35$; at $p < 0.0001$, respectively). In addition, we constructed the reference values for urinary activity of α -fucosidase, β -galactosidase, β -glucuronidase and α -mannosidase (pKat/ μ g Cr.) in percentiles according to age in 3-year intervals.

Conclusions: Our study is the first to show reference values for urinary α -fucosidase, β -galactosidase, β -glucuronidase and α -mannosidase in children and adolescents.

1. Introduction

Golgi and endoplasmic reticulum exoglycosidases: α -fucosidase (FUC), β -galactosidase (GAL), β -glucuronidase (GLU) and α -mannosidase (MAN) [1] are involved in modification of oligosaccharide chains of glycoconjugates (i.e. glycoproteins, proteoglycans and glycolipids) in the endomembrane system and their degradation in lysosomes. Each of lysosomal exoglycosidases cleave single sugar residues from non-reducing ends (GAL, GLU, MAN) or within (FUC) oligosaccharide chains [2]. Localized within the lysosomal vesicle, some molecules of exoglycosidases are partially diffused while others are partially bound to the membrane creating a specific polyenzymatic system [3].

The inherited lack or decrease in the activity of all or particular exoglycosidase disturbs degradation of oligosaccharide chains of glycoconjugates in the human body, leading to accumulation of the oligosaccharide chains in lysosomes of particular tissues and organs. Storage of oligosaccharide chains cause an enlargement of lysosomes in cells of involved organs and induce disorders in their structure and functions. Storage of oligosaccharide chains in the nervous system leads

to impaired physical and mental development [4].

Lysosomal exoglycosidases are found not only in tissues but also in the body fluids: serum and urine [5,6], saliva [7], joint fluid [8], cerebrospinal fluid [9] and their activities depend on the state of health of the subject. In some diseases associated with inflammation and tissue damage, increased activity of lysosomal exoglycosidases [10] has been observed. In rheumatoid arthritis a significant increase in serum and synovial fluid FUC, GAL, GLU and MAN activity in patients with various forms of arthritis [8] compared to healthy subjects was reported. Salivary gland tumors significantly increase FUC, GAL, GLU and MAN activities in serum and saliva [11], colorectal neoplasms significantly increase serum FUC, GAL and GLU activities as well as GAL, FUC and MAN in urine [5,12–14]. Determination of GLU activity has been used in oncological diagnosis and in nephrology as a marker of renal parenchymal injury [15]. Significant increase in the activities of lysosomal exoglycosidases was observed in the urine of children with pelvic-urethral stricture and damaged kidney tubules [16] as well as in subjects with one active kidney [17].

Review of the literature showed that there are no publications

Abbreviations: FUC, α -fucosidase; GAL, β -galactosidase; GLU, β -glucuronidase; MAN, α -mannosidase

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Table 1
Characteristics of the study population (Q1- lower quartile, Q3- upper quartile).

| Variable | GIRLS n = 99 | | | BOYS n = 104 | | | P value |
|--------------------|--------------|---------|----------|--------------|---------|----------|---------|
| | Median | Q1 | Q3 | Median | Q1 | Q3 | |
| Age (years) | 9.10 | 7.09 | 11.09 | 9.09 | 7.05 | 12.07 | NS |
| Weight (kg) | 30.98 | 23.90 | 41.80 | 38.50 | 30.50 | 51.80 | NS |
| Height (cm) | 137.70 | 126.30 | 148.30 | 144.50 | 132.30 | 158.40 | NS |
| FUC (pKat/mL) | 74.35 | 64.19 | 96.93 | 74.35 | 59.11 | 93.54 | NS |
| GAL (pKat/mL) | 82.25 | 68.70 | 98.06 | 77.74 | 65.32 | 96.93 | NS |
| GLU (pKat/mL) | 75.48 | 60.80 | 92.41 | 72.09 | 57.42 | 99.75 | NS |
| MAN (pKat/mL) | 79.99 | 65.32 | 99.18 | 76.61 | 65.32 | 95.80 | NS |
| Creatinine (µg/mL) | 1028.238 | 608.450 | 1465.356 | 936.905 | 612.214 | 1227.363 | NS |
| FUC (pKat/µg Cr) | 0.080 | 0.055 | 0.114 | 0.084 | 0.061 | 0.130 | NS |
| GAL (pKat/µg Cr) | 0.087 | 0.058 | 0.119 | 0.090 | 0.062 | 0.138 | NS |
| GLU (pKat/µg Cr) | 0.081 | 0.054 | 0.115 | 0.085 | 0.059 | 0.135 | NS |
| MAN (pKat/µg Cr) | 0.086 | 0.061 | 0.116 | 0.088 | 0.062 | 0.136 | NS |

NS – not significant.

concerning FUC, GAL, GLU and MAN activities in urine and other body fluids of healthy children and adolescents. This is the reason why we have attempted to determine the range of FUC, GAL, GLU and MAN reference values for healthy children and adolescents in the urine, which may be useful in interpreting the results concerning lysosomal exoglycosidase activities in various children disease states.

Additionally, we aimed to establish relations between age, gender and urinary FUC, GAL, GLU and MAN activities.

2. Materials and methods

2.1. Participants

The study was carried out in 203 healthy children and adolescents (girls = 99, boys = 104), aged from six months to 17.9 years. Out of all participants in the study, 48.8% were girls and 51.2% were boys of similar age ($p > 0.05$). The study group was divided into six age groups in 3-year intervals: group I: 0.5–2.9 yrs (28 subjects); group II: 3–5.9 yrs (29 subjects); group III: 6–8.9 yrs (36 subjects); group IV: 9–11.9 yrs (35 subjects); group V: 12–14.9 yrs (43 subjects) and group VI: 15–17.9 yrs (32 subjects). Urine and necessary information came from the participants of the OLAF study [18] and from the healthy children of the staff of the University Children's Hospital in Bialystok. The study was conducted with the consent of children aged over 16 and parents of all children, with the consent of the Local Bioethics Commission, Medical University of Bialystok (01 September 2014; ANZ-06042-134-41724/14) while the OLAF study was approved by the Children's Memorial Health Institute Ethics Committee.

The medical past and present history of the study participants was taken from the parents. For all participants clinical records were taken and physical examinations were performed. Body weights and heights were measured using a balance beam and pediatric wall-mounted stadiometer. Inclusion criteria of study participants: healthy children and adolescents aged 0.5–18 years. Exclusion criteria: any signs of infection, chronic diseases and medications that may have an influence on renal function. After an overnight fasting, samples were taken from each patient to disposable polyethylene containers without preservatives. Urine samples were delivered to the laboratory within 4 h of collection, centrifuged at 4000 rpm for 20 min, poured into Eppendorf tubes and frozen at -80°C until the assay was performed.

2.2. Creatinine determination

Urinary creatinine concentrations were determined using kits ABX Pentra Enzymatic Creatinine CP, biochemical analyzer ABX Pentra 400, and expressed in µg/mL.

2.3. FUC, GAL, GLU and MAN activity determination

FUC, GAL and MAN activities in urine (pKat/mL) were determined using the method of Zwierz et al. [19] and GLU activity in urine (pKat/mL) with the method of Marciniak et al. [20] as modified by Szajda et al. [21].

The reaction mixture for determination of FUC, GAL, GLU and MAN activities was composed of 10 µl of urine and 30 µl of the appropriate substrate solution: 20 mM solution of 4-Nitrophenyl- α -L-fucopyranoside (for FUC), 4-Nitrophenyl- β -D-galactopyranoside (for GAL), 4-Nitrophenyl- β -D-glucuronide (for GLU), 4-Nitrophenyl- α -D-mannopyranoside (for MAN) (Sigma, St. Louis, MO, USA). Additionally 40 µl of 100 mM citrate-phosphate buffer, pH 4.3 (for FUC, GAL, and MAN) or 200 mM sodium acetate buffer pH 4.5 (for GLU) were added. The microplates were incubated for 60 min at 37°C with constant shaking. The enzymatic reactions were stopped by adding 200 µl of 200 mM borate buffer, pH 9.8.

The activity of lysosomal exoglycosidases corresponding to the amounts of released 4-nitrophenol, were measured at 405 nm, using the microplates reader EL_X800™ and computer program KC junior (Bio-Tek Instruments, Winooski, VT, USA).

The concentrations of urinary activities of FUC, GAL, GLU and MAN, were expressed in pKat/mL of urine and standardized by comparison to urinary creatinine concentrations and expressed in pKat/µg of creatinine (pKat/µg Cr.).

2.4. Statistical analysis

The data were analyzed with Statistica (version 12.0, StatSoft, Tulsa, OK, USA) program. Kolmogorov–Smirnov test was used to determine normality of variables. Discrete variables were expressed as counts (percentage) and continuous variables as median and quartiles, unless stated otherwise. Nonparametric tests were used including Mann–Whitney U test and Student's t -test for unmatched comparisons and ANOVA for multiple comparisons. Correlations between urinary FUC, GAL, MAN and GLU activities to creatinine ratio and other variables were evaluated by Pearson's or Spearman's test, as appropriate. $P < 0.05$ was considered statistically significant. Age-specific reference values for FUC, GAL, GLU and MAN activities to creatinine ratio were generated by the Lambda-Mu-Sigma (LMS) method using LMS Chart Maker software [22], which characterizes the age dependent distribution of a target parameter based on a quantile regression fit by three different components: the median (M), the variance (S) and the skewness of the distribution, which is evaluated by an exponential factor (L) from a Box-Cox transformation [23].

Centile curves for age were obtained as:

$$C_{100\alpha}(t) = M(t) [1 + L(t) S(t)Z\alpha]^{1/L(t)}$$

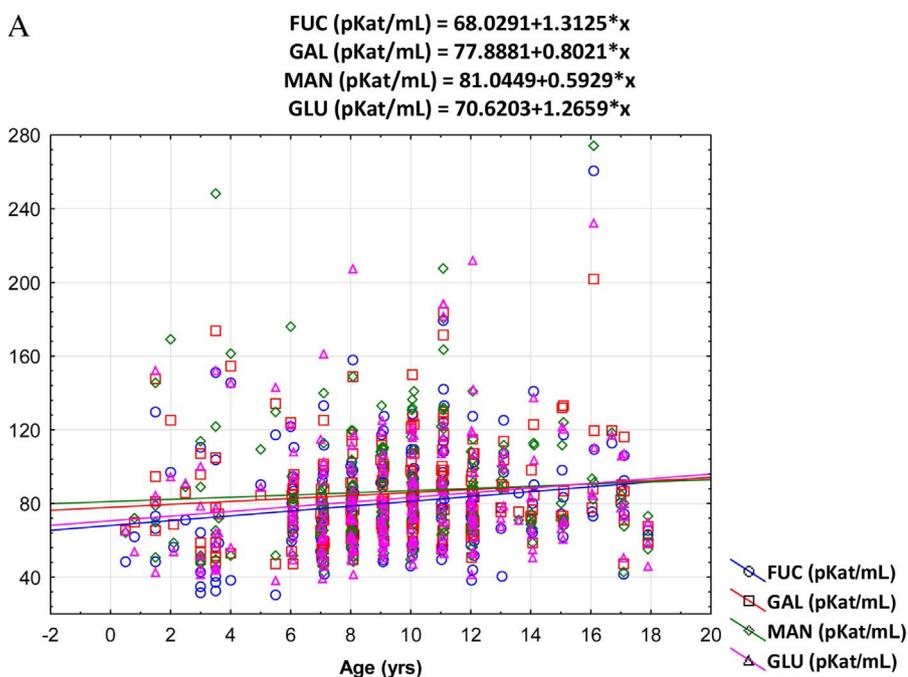


Fig. 1. Linear regression analysis showing the relationship between estimated urine exoglycosidases activity (A – pKat/mL; B – pKat/μg Cr.) and age.

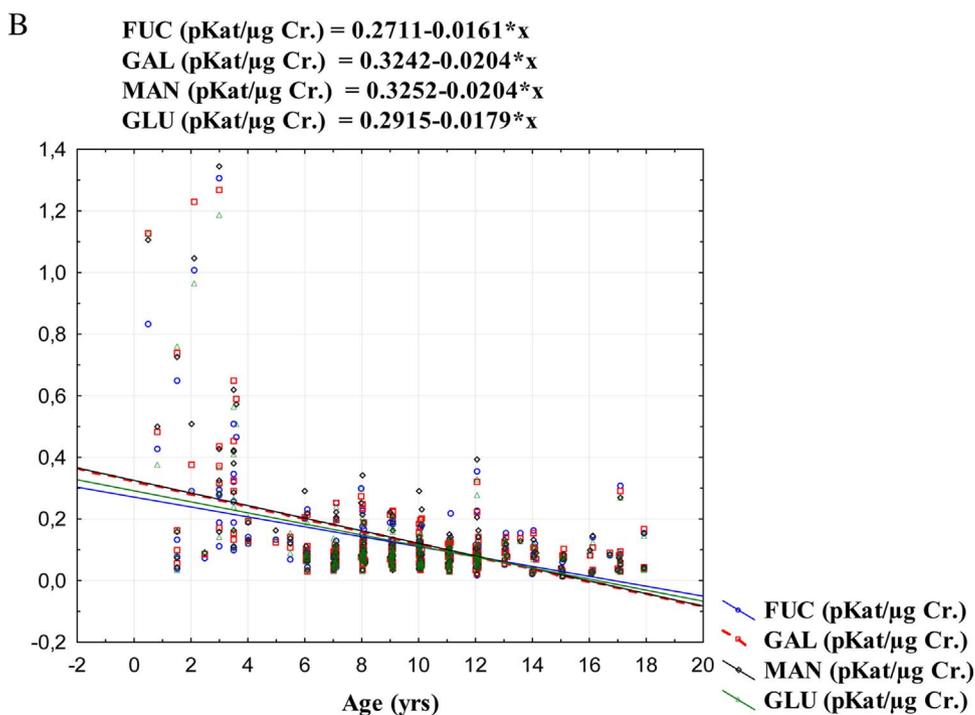


Table 2
 Urinary creatinine excretion (μg/mL) (Q1- lower quartile, Q3- upper quartile).

| Age (years) | Median | Q1 | Q3 |
|-------------|----------|---------|----------|
| 0.5–2.9 | 990.156 | 653.975 | 1385.986 |
| 3.0–5.9 | 1011.731 | 735.704 | 1436.935 |
| 6.0–8.9 | 993.425 | 594.066 | 1459.760 |
| 9.0–11.9 | 915.661 | 539.757 | 1247.063 |
| 12.0–14.9 | 970.965 | 594.343 | 1369.804 |
| 15.0–17.9 | 938.206 | 601.396 | 1260.004 |

ANOVA test: Chi squared = 27.93787 df = 5 p = 0.0000.

where Z_{α} is the normal equivalent deviate for tail area α and $C_{100\alpha}(t)$ is the centile corresponding to Z_{α} .

3. Results

Groups' characteristics is shown in Table 1. There were no statistically significant differences in medians of all estimated FUC, GAL, GLU and MAN activities (pKat/mL and pKat/μg Cr.) between girls and boys ($p > 0.05$). No significant differences in medians were also in the case of age, weight and height (Table 1). We observed statistically significant positive correlation between urinary FUC, GAL, GLU and MAN (pKat/mL) and age ($r = 0.17, p < 0.05$; $r = 0.16, p < 0.05$; $r = 0.19, p < 0.01$; $r = 0.16, p < 0.05$; respectively) (Fig. 1A).

Due to possible relations between enzyme activity in volume unit

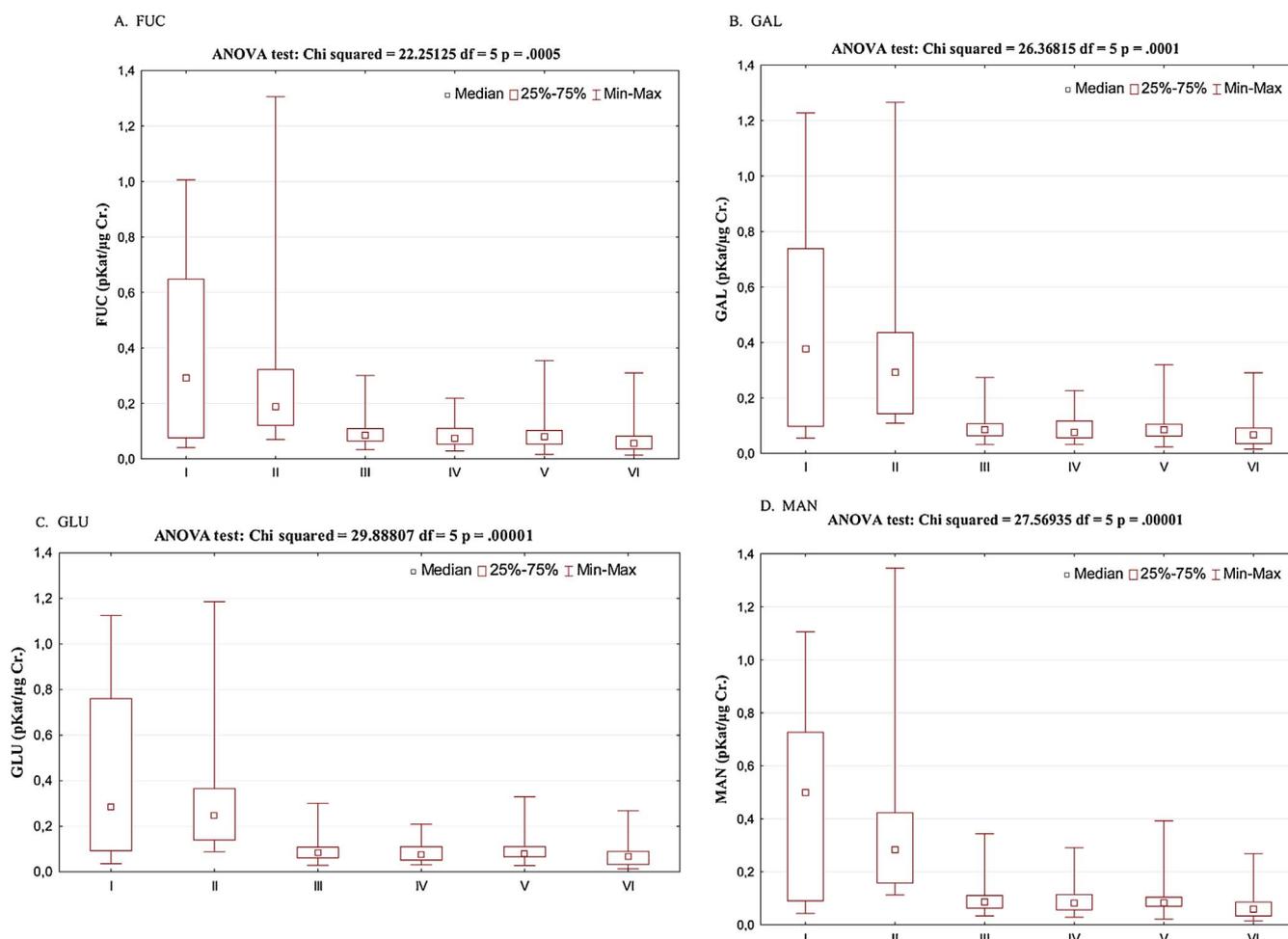


Fig. 2. Comparison of estimated urine exoglycosidases activity (pKat/ μ g Cr.) between six age groups: I 0.5–2.9 yrs, II 3.0–5.9 yrs, III 6.0–8.9 yrs, IV 9.0–11.9 yrs, V 12.0–14.9 yrs, VI 15.0–17.9 yrs; ANOVA analysis of variance.

and diuretic size, it is believed that the best way to eliminate the effect of diuresis on urinary enzyme activity is to refer it to urinary creatinine concentration. Reference to urinary creatinine concentration also excludes the effect of individual traits on enzyme activity [24]. In urine, creatinine concentration is directly dependent on muscle mass and excretion of kidney and is significantly reduced during fasting and acute as well as chronic renal failure [25].

The age-related changes in urinary creatinine excretion in the studied groups are shown in Table 2. The medians for urinary creatinine concentration differ with age, and this age-related changes were significant (ANOVA, $p < 0.001$). The median urinary creatinine concentration ranged from 990.156 μ g/mL at the age below 3 years to 938.206 μ g/mL at the age over 15 years.

Furthermore, we assessed urinary FUC, GAL, GLU and MAN (pKat/ μ g Cr.) in the particular age groups in 3-year intervals. Following adjustment for creatinine median activity in urine of FUC, GAL, GLU and MAN (pKat/ μ g Cr.) showed 3-year intervals differences but generally presented a significant trend toward decreasing values with the increased age (ANOVA, $p < 0.001$). The highest urinary activity of FUC, GAL, GLU and MAN were found in children below 3 years and the lowest in the oldest children over 15 years (ANOVA, $p < 0.001$) as shown in Fig. 2A–D. Fig. 1B presents statistically significant the negative correlation between urinary FUC, GAL, GLU and MAN (pKat/ μ g Cr.) and age ($r = -0.36$, $p < 0.0001$; $r = -0.36$, $p < 0.0001$; $r = -0.35$, $p < 0.0001$; $r = -0.35$, $p < 0.0001$, respectively). Statistically significant decrease in FUC, GAL, GLU and MAN urinary activities in the oldest groups in comparison to the youngest group was found (Table 3A–D).

4. Discussion

The most interesting findings of our study, concerning urinary activities of exoglycosidases such as FUC, GAL, GLU and MAN in healthy children are as follows: 1. Differences in patterns of urinary activity of FUC, GAL, GLU and MAN were not found based on gender (Table 1); 2. Urinary activity of FUC, GAL, GLU and MAN (pKat/ μ g Cr.) showed 3-year intervals differences with a significant trend toward decreasing values with increasing age (Fig. 2A–D, Table 3A–D); 3. The significant negative correlation between urinary FUC, GAL, GLU as well as MAN (pKat/ μ g Cr.) and age was found (Fig. 1B).

During the literature review, no data was found on the association between urinary activity of FUC, GAL, GLU as well as MAN and age in the pediatric population. This study provides normative ranges of urinary activity of FUC, GAL, GLU and MAN in the designated age groups of children.

The activities of lysosomal exoglycosidases in tissues and body fluids becomes a widespread subject of research, because they reflect the increasing catabolism rate of glycoconjugates in many types of diseases [1,5–9]. The most active of the lysosomal exoglycosidases is N-acetyl- β -D-hexosaminidase (HEX) (E.C.3.2.1.52) [1,26,27]. FUC, GAL, GLU and MAN are lysosomal exoglycosidases less investigated than HEX, therefore requiring careful research into the suitability of their activity in body fluids in the diagnosis of many diseases.

So far, there has been little known about the role of lysosomal exoglycosidases in children with kidney disease. Determination of lysosomal exoglycosidases activities has been used in laboratory diagnostics of children, e.g. pelvic and ureteric stenosis with damaged

Table 3
Estimated exoglycosidases (pKat/ μ g Cr.) reference values for children and adolescents by age.

| A) FUC | | | | | | | | | | |
|-------------|--------|-------|-------|----------|-------|-------|-------|-------|-------|-------|
| Age (years) | LMS | | | Centiles | | | | | | |
| | L | M | S | Third | Tenth | 25th | 50th | 75th | 90th | 97th |
| 0.5–2.9 | –0.189 | 0.194 | 0.820 | 0.050 | 0.074 | 0.114 | 0.194 | 0.348 | 0.627 | 1.205 |
| 3.0–5.9 | –0.252 | 0.152 | 0.680 | 0.050 | 0.069 | 0.098 | 0.152 | 0.247 | 0.407 | 0.713 |
| 6.0–8.9 | –0.279 | 0.104 | 0.544 | 0.042 | 0.055 | 0.073 | 0.104 | 0.154 | 0.227 | 0.350 |
| 9.0–11.9 | –0.233 | 0.079 | 0.493 | 0.034 | 0.044 | 0.057 | 0.079 | 0.112 | 0.157 | 0.226 |
| 12.0–14.9 | –0.139 | 0.068 | 0.537 | 0.026 | 0.035 | 0.048 | 0.068 | 0.099 | 0.141 | 0.203 |
| 15.0–17.9 | –0.069 | 0.061 | 0.626 | 0.019 | 0.028 | 0.040 | 0.061 | 0.094 | 0.140 | 0.211 |
| B) GAL | | | | | | | | | | |
| Age (years) | LMS | | | Centiles | | | | | | |
| | L | M | S | Third | Tenth | 25th | 50th | 75th | 90th | 97th |
| 0.5–2.9 | 0.652 | 0.466 | 0.832 | 0.059 | 0.075 | 0.232 | 0.466 | 0.753 | 1.048 | 1.372 |
| 3.0–5.9 | –0.219 | 0.278 | 0.659 | 0.093 | 0.128 | 0.182 | 0.278 | 0.445 | 0.710 | 1.185 |
| 6.0–8.9 | 0.006 | 0.098 | 0.503 | 0.038 | 0.051 | 0.070 | 0.098 | 0.138 | 0.188 | 0.253 |
| 9.0–11.9 | –0.559 | 0.083 | 0.462 | 0.041 | 0.050 | 0.062 | 0.083 | 0.118 | 0.172 | 0.275 |
| 12.0–14.9 | –0.158 | 0.077 | 0.491 | 0.032 | 0.042 | 0.056 | 0.077 | 0.108 | 0.150 | 0.210 |
| 15.0–17.9 | 0.713 | 0.065 | 0.536 | 0.010 | 0.025 | 0.043 | 0.065 | 0.090 | 0.114 | 0.140 |
| C) GLU | | | | | | | | | | |
| Age (years) | LMS | | | Centiles | | | | | | |
| | L | M | S | Third | Tenth | 25th | 50th | 75th | 90th | 97th |
| 0.5–2.9 | 0.322 | 0.403 | 0.826 | 0.046 | 0.110 | 0.218 | 0.403 | 0.673 | 1.002 | 1.420 |
| 3.0–5.9 | –0.286 | 0.247 | 0.668 | 0.084 | 0.114 | 0.161 | 0.247 | 0.400 | 0.661 | 1.176 |
| 6.0–8.9 | –0.017 | 0.095 | 0.519 | 0.036 | 0.049 | 0.067 | 0.095 | 0.135 | 0.186 | 0.255 |
| 9.0–11.9 | –0.452 | 0.078 | 0.442 | 0.038 | 0.047 | 0.059 | 0.078 | 0.107 | 0.150 | 0.222 |
| 12.0–14.9 | –0.795 | 0.076 | 0.426 | 0.041 | 0.048 | 0.059 | 0.076 | 0.106 | 0.157 | 0.275 |
| 15.0–17.9 | 0.911 | 0.061 | 0.487 | 0.008 | 0.024 | 0.041 | 0.061 | 0.082 | 0.101 | 0.120 |
| D) MAN | | | | | | | | | | |
| Age (years) | LMS | | | Centiles | | | | | | |
| | L | M | S | Third | Tenth | 25th | 50th | 75th | 90th | 97th |
| 0.5–2.9 | –0.152 | 0.236 | 0.958 | 0.048 | 0.076 | 0.127 | 0.236 | 0.467 | 0.922 | 1.949 |
| 3.0–5.9 | –0.208 | 0.176 | 0.780 | 0.049 | 0.071 | 0.107 | 0.176 | 0.308 | 0.543 | 1.021 |
| 6.0–8.9 | –0.246 | 0.112 | 0.597 | 0.041 | 0.055 | 0.076 | 0.112 | 0.172 | 0.262 | 0.419 |
| 9.0–11.9 | –0.220 | 0.084 | 0.506 | 0.035 | 0.045 | 0.060 | 0.084 | 0.119 | 0.169 | 0.244 |
| 12.0–14.9 | –0.117 | 0.075 | 0.521 | 0.029 | 0.039 | 0.053 | 0.075 | 0.108 | 0.151 | 0.214 |
| 15.0–17.9 | –0.019 | 0.065 | 0.597 | 0.021 | 0.030 | 0.043 | 0.065 | 0.097 | 0.141 | 0.203 |

kidney tubules [16] and single kidney [17] as well as adults: type 1 diabetes [28], thyroid carcinoma, kidney, colorectal adenocarcinoma pancreas [5,6,21,29–31] and alcohol abuse [32].

Lysosomal exoglycosidases are among the few enzymes whose activity can be determined in the urine, to provide important information on the functioning of the kidneys [24,33]. It is worthy of mention that lysosomal exoglycosidases are not denatured at the acidic pH of the urine and their optimum pH is 3.5–5.5 [3].

Glomeruli of healthy kidneys do not pass proteins with molecular weights greater than 68 kD [34]. Therefore, exoglycosidases (whose molecular weight is approximately 100 kDa) are not filtered through properly functioning filter membranes of the renal tubules. However, in physiological urine can be detected traces of lysosomal exoglycosidases [5,6,16,17,21,29], probably due to natural exfoliation of the renal tubular epithelium. Another reason for the presence of lysosomal exoglycosidases in physiological urine may be, that under normal metabolic conditions of the cells, lysosomal enzymes may be released outside the cell (in the case of urine to renal tubular urinary tract) [35].

The estimated markers: FUC, GAL, GLU and MAN are enzymes

which seemed to play a role in renal damage. Alterations in their activity have been described in our previous studies on children with congenital anomalies of the kidney and urinary tract [16,17]. It was also shown that increased lysosomal exoglycosidases activities might be used as an indicator of active renal disease and as an early warning of renal disorder [36,37].

Damage to the kidney parenchyma causes lysosomal breakdown followed by the excretion of lysosomal enzymes into the urine. The kidney is the primary source the majority of urinary enzymes, although if glomerular damage is present, some of the excreted enzyme activities originate from the serum. It is possible that some of the increased enzyme activities in the urine might be derived from the serum since all studied hydrolases are present in the serum.

Data on the FUC, GAL, GLU and MAN activities in serum or urine were published [5,16,17,29]. Our study shows that the activity of FUC, GAL, GLU and MAN in urine of children and adolescents is independent of sex (Table 1), but depends on age. By dividing children and adolescents into 6 subgroups at intervals of 3 years, we showed statistically significant ($p < 0.001$) age-related decrease in FUC, GAL, GLU and

MAN activities (pKat/ μ g Cr.) in urine.

Estimated activities of lysosomal exoglycosidases in urine was normalized to urinary creatinine concentrations in control for variations in urinary flow rates. Urinary creatinine excretion also revealed significant differences depending on age (ANOVA, $p < 0.001$) (Table 2). However, there are some suggestions that normalization of the urinary exoglycosidases activities to urinary creatinine concentrations may be inappropriate at times, when urinary creatinine excretion rate vary greatly, depending on the situation [38].

We found a positive correlation between age of children as well as adolescents and urinary activity of FUC, GAL, GLU and MAN (pKat/mL). Following adjustment for creatinine, urinary FUC, GAL, GLU and MAN activities presented a significant trend toward decreasing values with increasing age. The lower activity of lysosomal exoglycosidases and the higher creatinine excretion in adolescent seems to be the explanation for an age-related divergence in the exoglycosidases/creatinine ratio within these age ranges.

Dependence the urinary activities of FUC, GAL, GLU and MAN in children and adolescent on age, taking into consideration centile network in range of normal values 3rd, 10th, 25th, 50th, 75th, 90th, and 97th centiles are presented in Table 3A–D. Similar correlations as presented in Table 3 were demonstrated by investigating the activity of HEX and its isoenzymes in urine of healthy children and adolescents [39]. The relationship between FUC, GAL, GLU and MAN urinary activities and age, presented in Table 3, might be of practical importance in distinguishing between healthy and ill children and adolescents.

5. Conclusions

This study is the first such survey of reference values for FUC, GAL, GLU and MAN activities in urine of healthy children and adolescents, showing dependence on age, but non-gender. It allows the use of FUC, GAL, GLU and MAN activities assay to distinguish healthy from ill children.

Conflict of interest

The authors declare no conflict of interests.

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