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Urinary Collagen Cross-Links as Biochemical Markers of Growth: An Evaluation of Biological Variables

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Key Words
Urinary collection protocol · Pyridinoline · Deoxypyridinoline · Collagen cross-links · Height velocity

Abstract

Aim: To investigate the validity of urinary pyridinium cross-links (pyridinoline and deoxypyridinoline) as markers of growth in healthy children. ***Methods:*** Three pilot studies (P1–P3) were conducted to investigate the time of day, the minimal duration within a day, and how many times per week urine samples needed to be collected to obtain representative values of cross-link excretion in normal children 3–5 years of age. The results were used to design a 4-month longitudinal protocol to evaluate whether pyridinium cross-links could be used as markers of growth velocity. ***Results:*** Mean differences from 24-hour values were only between 1 and 4% for urinary cross-links (nmol/h) in overnight 12-hour collections. Three consecutive collections were required for weekly output estimates with a maximum error of 10% in >90% of the children. During the 4-month longitudinal study, the regression equation of height velocity on pyridinoline and deoxypyridinoline excretion explained approximately 60% of the variance in the subgroup of subjects who provided three complete urinary collections per observation period. No relationship was observed when the cases with fewer or incomplete collections were included in the analysis. Cross-link values collected at baseline were of no use to predict height velocity at 4 months. ***Conclusions:*** Urinary pyridinium cross-links correlate with the growth velocity in healthy children when using an appropriate urinary collection protocol. However, their predictive value in this population is negligible.

Introduction

The measurement of the pyridinium cross-links pyridinoline (Pyd) and deoxypyridinoline (Dpd), which are released during the breakdown of mature collagen, is now an established technique in monitoring bone resorption in adults. Pyd is the major cross-link in all connective tissues, whereas high amounts of Dpd are found in bone and dentin only (the ratio Pyd:Dpd is 3.5:1 in bone and only

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10:1 in human cartilage). Both cross-links are absent from the collagen of the skin, and their excretion in urine appears to be unaffected by changes in the renal function [1].

In children, in whom the cross-link output is about four to five times higher than in adults [2, 3], collagen cross-links may have a further application as biochemical markers of growth [4], as suggested by their sensitivity to both growth hormone (GH) treatment and GH treatment withdrawal in children with idiopathic short stature [5] and GH deficiency [6, 7].

Reference values of cross-link excretion in healthy children across ages and sexes [2, 3, 8–10] have been usually obtained by applying methodological assumptions validated in adults, but never tested in children – namely, the standardization of cross-link excretion by urine creatinine and the use of single morning spot samples as representative of the day. Nyctohermeral variation of cross-link excretion has been documented in adults [11] and pubertal girls [12], but no information is available for children. Similarly, the relationship between muscle mass and skeletal mass, relatively stable in adults, has been observed to change with age in children [13].

These and other methodological questions apply to the investigation of the relationship between cross-link excretion and growth velocity, which has been reported to be significant in children 4–18 years of age using single 24-hour urine samples and standardizing the results by body weight [14]. As previously suggested, longitudinal comparisons of the cross-link excretion may require multiple urine sampling to describe physiological changes over short periods of time. The between-day coefficient of variation reported in adults [15, 16] and children [8] is high and likely to be above the change expected. Full 24-hour urine collections would overcome diurnal variations of cross-link output, and several consecutive collections would decrease the standard error of repeated measures in longitudinal studies [15]. However, the difficulty in obtaining complete urine samples in normal children often imposes a trade-off between precision and viability that cannot be solved in the most favorable way with the existing data.

The aim of the present study was to investigate the validity of urinary pyridinium cross-links as markers of growth velocity in healthy children using a urine collection protocol developed for that purpose. Details on the within-day and day-to-day variations both within and between individuals are provided, as well as an evaluation of height as an alternative to creatinine for the standardization of results.

Methods

Study Design

Three pilot studies (P1–P3) were conducted to investigate the time of day, the minimal duration within a day, and how many times per week urine samples need to be collected in order to provide representative values for cross-link excretion in normal children. Pilot data were used to develop a longitudinal (LNG) protocol for the evaluation of cross-link output as a marker of growth velocity. Complete baseline urinary collections (n = 65) were analyzed for pyridinium cross-link to provide normal data for children 3–5 years of age (cross-sectional study; CSS).

Protocol Development

P1 was carried out in November 1992; spot urinary samples were collected in the morning and evening for 7 consecutive days. P2 included 8 children, 4 of whom had previously participated in P1. The latter were studied in winter, while the remaining 4 were studied in summer. Two of the children were siblings. Urine samples were collected overnight for 12 h between 20.00 h and 08.00 h and then during three 4-hour periods. P3 was carried out in January 1993. Timed overnight 12-hour urine samples (from 20.00 h to 08.00 h) were collected for 7 consecutive days. Anthropometrics were recorded at baseline in all pilot studies.

Evaluation of Cross-Link Output as Marker of Growth Velocity

To evaluate whether urinary cross-links could be used as markers of growth velocity, 108 children, 3–5 years of age, were enrolled in a longitudinal study conducted from January to May 1993. Urine was collected for 12 h (from 20.00 h to 08.00 h) on 3 consecutive days during each of 5 consecutive months. Anthropometry was performed at the beginning and at the end of the 4-month observation period, within the same week as urine was collected.

Subjects

For the three pilot studies (P1–P3), subjects were selected among friends and relatives of the investigators. For LNG and CSS, subjects were recruited from a public school in Rome. Normal growth (defined as height for age between the 10th and the 90th centiles of the reference population) and absence of any significant illness at the time of the study were criteria for inclusion. Ethical approval was granted by the Local Health Unit RM4, and informed consent was obtained from the parents of each child.

Anthropometry and Reference Data for Growth

In all studies, the height was measured to the nearest 0.1 mm according to Lohman et al. [17] by two observers with a Harpenden portable stadiometer. The within-observer technical error was 2.6 mm for observer A and 2.06 mm for observer B; the interobserver technical error was 2.67 mm, which is lower than that reported in the literature [18]. The weight was measured without shoes and in light clothes with a precision of 100 g.

Reference data for attained height and weight were taken from the National Centers of Health Statistics growth charts [19], and Z scores of weight for age, height for age, and weight for height were computed by means of software provided by the Centers for Disease Control (Anthro). Reference height increments were taken from Baumgartner et al. [20].

Table 1. Baseline physical characteristics of the subjects participating in the different studies (mean ± SEM)

Study acronym	Study design	n		Age months	Height cm	Weight for height (z score)	Height for age (z score)
		♂ + ♀	♀				
P1	longitudinal	10	5	53 ± 3	103 ± 3	0.72 ± 0.29	–0.46 ± 0.29
P2	cross-sectional	8	4	56 ± 8	106 ± 5	0.13 ± 0.57	–0.11 ± 0.33
P3	longitudinal	8	4	58 ± 4	107 ± 4	0.34 ± 0.41	–0.32 ± 0.48
CSS	cross-sectional	65	30	49 ± 1	103 ± 1	0.12 ± 0.13	0.31 ± 0.20
LNG	longitudinal	108	53	49 ± 5	104 ± 1	0.19 ± 0.11	0.27 ± 0.10

Collection, Processing, and Completeness of Urine Samples

Urinary collections were supervised by the children's parents who had been instructed personally by the investigators. The urine samples were kept at 4°C, frozen within 12 h of collection, and stored at –20°C until analysis. The storage time ranged from a few weeks in pilot studies to 1 year in the CSS, but storage at –20°C was unlikely to affect the cross-link concentrations, since freeze-thawing was avoided [21].

In P2, P3, LNG, and CSS, only 12-hour overnight samples collected when the children were kept dry at night, as reported by the parents, were analyzed. In order to assess the completeness of urine collections, the flow rate calculated from the observed volume and reported duration, standardized by the children's weight, was compared to reference values [22]. When the calculated flow rate differed by more than ±3 SD from the reference range, the sample was excluded from the analysis as a timed collection, although the calculated ratio of cross-links to creatinine was considered valid and used in the data analysis where appropriate.

Biochemical Analysis

During P1–P3 and in 62% of the samples from LNG and CSS, collagen cross-links were assayed in urine by reversed-phase HPLC with the manual extraction technique, as described by Black et al. [23]. The interassay coefficient of variation of a quality control urine was 5%. In 38% of the samples from LNG and CSS, cross-links were assayed by reversed-phase HPLC with fluorimetric detection after solid-phase extraction, as described by Pratt et al. [24]. The interassay coefficient of variation of a quality control urine was 3%. Samples from P3 were reanalyzed by the manual and automated techniques in order to calculate a correction factor that would allow comparability of data in CSS and LNG. In contrast to the manual method, the automated system made use of an internal standard which corrected for losses in recovery from the preliminary fractionation columns. Consequently, higher values were obtained using the automated system; the relationship between methods was linear for the whole range of cross-link excretion ($r^2 = 0.94$ for Pyd and $r^2 = 0.89$ for Dpd):

$$Pyd_{aut}(pmol/ml) = [1.72 \times Pyd_{man}(pmol/ml)] + 257$$

$$Dpd_{aut}(pmol/ml) = [1.46 \times Dpd_{man}(pmol/ml)] + 94$$

Statistical Analysis

Statistical analyses were performed with SPSS/PC+. Variable distribution was tested for normality by the Kolmogorov-Smirnov goodness-of-fit test. Mean values and standard deviations were calculated from normal distributions, median and centiles for non-normal distributions. Comparisons between normally distributed groups of data were performed by t test and by analysis of variance; post hoc comparisons were carried out with the Tukey HSD test. In non-normal distributions, the Kolmogorov-Smirnov test and the Kruskal-Wallis ANOVA were used for comparisons.

In within-subject designs, changes in biochemical markers over time were analyzed by repeated-measures analysis of variance (SPSS procedure Manova). Adjustment of the main effects with covariance analysis was performed when necessary. The null hypothesis of the absence of a time effect was tested with multivariate (Hotelling's trace, Wilk's lambda) as well as univariate procedures. In case of divergence of the two tests, the univariate approach was preferred. The assumptions of equal variance of transformed variables and zero covariance, which are required for the univariate analysis, were tested with the Mauchly test of sphericity. If the test was positive, the degrees of freedom of the analysis of variance table were corrected with the Huynh-Feldt epsilon and the significance level reevaluated [25, 26].

The relationship between normally distributed variables was analyzed by the Pearson correlation coefficient and by multiple regression analysis. Stepwise procedures were used, starting from the variable with the highest partial correlation and then testing the increase in the explained variance. Results are expressed as mean values ± SEM, unless otherwise noted. Significance was set at the $p < 0.05$ level.

Results

Subjects

Age and physical characteristics of children participating in all studies are shown in table 1.

Protocol Development

Within-Day Fluctuations of Cross-Link Excretion. In seven morning and seven evening collections performed in each of the 10 children participating in P1, the Pyd and Dpd output was higher in the morning than in the evening, both for the group and for every single child. Differences were 43 ± 3.2% for Pyd and 47 ± 4.4% for Dpd

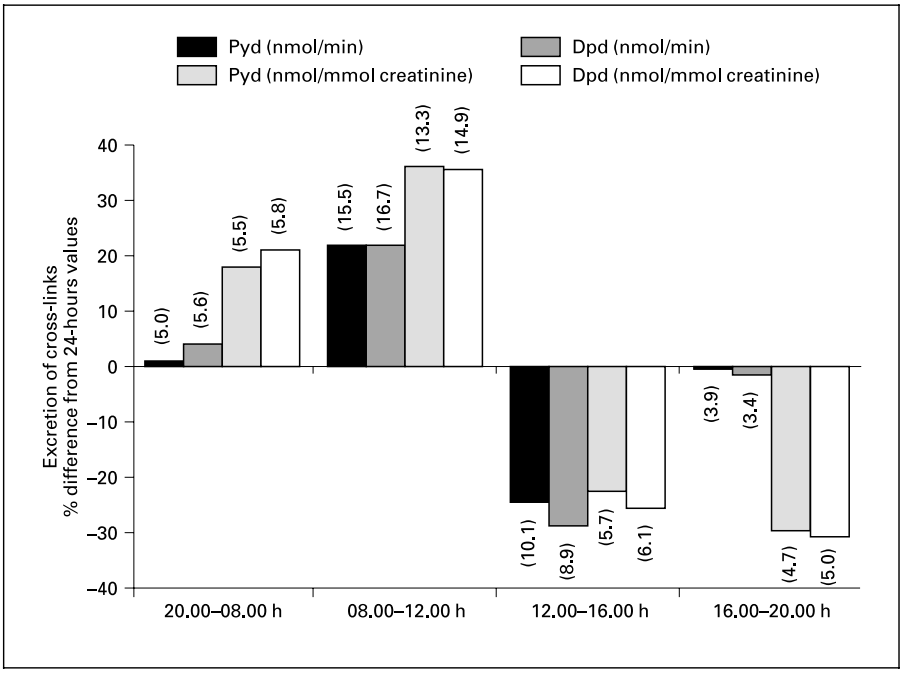

Fig. 1. Excretion of cross-links in split, timed urine collections from children participating in P2 (n = 8) as percent mean differences from 24-hour values. The results are expressed as mean values (bars) ± SEM (in parentheses).

($p < 0.001$). The Dpd/creatinine ratio in the morning showed a weak significant positive correlation with the Dpd/creatinine ratio in the evening ($r = 0.30$, $p = 0.01$), while no correlation was found between morning and evening in the Pyd/creatinine ratio.

In 24-hour split-timed collections (P2), the Pyd output (nmol/min) changed significantly during the day ($p < 0.05$) as well as the Pyd/creatinine ratio ($p < 0.001$). The largest difference in Pyd output was observed between evening and night, while all periods were significantly different regarding the Pyd/creatinine ratio. Neither the Dpd output nor the Dpd/creatinine ratio changed significantly during the day. As shown in figure 1, overnight collections (carried out between 20.00 h and 08.00 h) and evening collections (carried out between 16.00 h and 20.00 h) showed the least difference from the 24-hour values of cross-link excretion. Since the mean differences from 24-hour values were only between 1 and 4% for urinary cross-links (nmol/h) in overnight 12-hour collections, and since this time window was the most convenient for parents and children, the former collection protocol was used in P3.

Day-to-Day Fluctuations of Cross-Link Excretion. The extent of day-to-day fluctuations of cross-link output was analyzed on morning and evening spot samples (P1) and overnight, 12-hour timed collections (P3), all performed during 7 consecutive days. The cross-link/creatinine ratio in spot samples was less variable in the morning than in the evening, but the coefficients of variation between days for Pyd and Dpd were still 30 and 24%, respectively (versus 58 and 29% for Pyd and Dpd in the evening samples). The fluctuation between days was not statistically significant by univariate and multivariate repeated-measures Anova, suggesting a lack of trend during the week. In urine samples from P3, the coefficients of variation of Pyd and Dpd excretion were lower than in P1, but still 17% for Pyd and 19% for Dpd (expressed as both nmol/mmol creatinine and nmol/h). Repeated-measures analysis of variance failed to show a significant fluctuation between days.

Data on day-to-day fluctuations for 12-hour overnight urine samples were used to estimate the number of collections required to obtain cross-link excretion values representative for the whole week according to the equation $n = (cv\%)^2/(se\%)^2$ [27], where *cv* is the observed coefficient of variation between days and *se* the acceptable standard error. If ± 10% error is accepted, then one 12-hour collection is enough in children with 10% variation between days (lower range), and three are needed for most children (17% variation between days). Three collections are also required to reduce the error term affecting correlation analysis involving cross-link output, according to the equation by Liu et al. [28]:

$$n = [p^2/(1 - p^2)] \cdot \sigma^2_a/\sigma^2_r$$

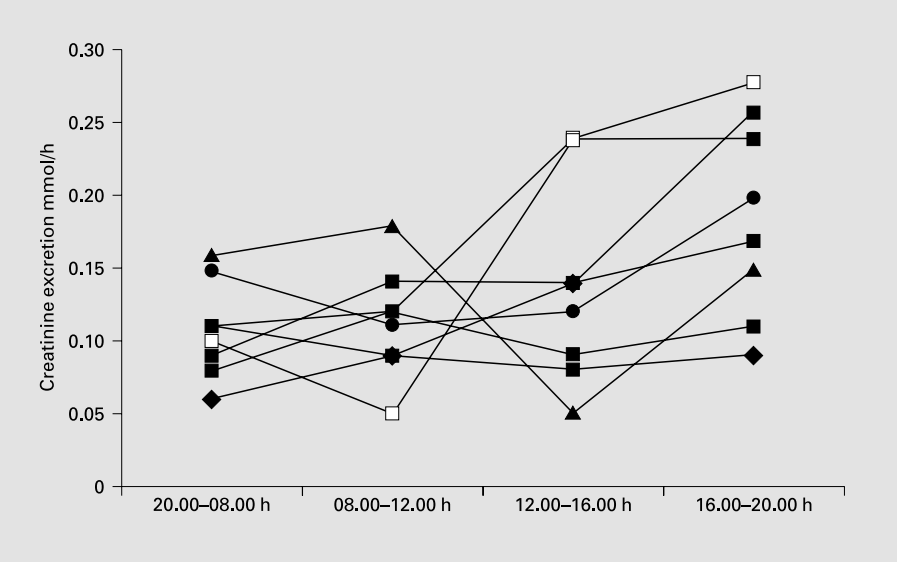

Fig. 2. Individual variation of creatinine excretion in split urine collections from children participating in P2 (n = 8). Repeated-measures analysis of variance showed significant variations in creatinine excretion during the day ($p < 0.05$).

Table 2. Urinary output of collagen cross-links in healthy children 3–5 years old (mean ± SEM)

Sex	n	Pyd nmol/h	Dpd nmol/h	Pyd nmol/h/m^3	Dpd nmol/h/m^3	Pyd/Dpd ratio
Male	35	38 ± 1.9	10 ± 0.5	33 ± 1.5	8 ± 0.3	3.8 ± 0.1
Female	30	35 ± 1.6	9 ± 0.5	32 ± 1.3	8 ± 0.4	3.9 ± 0.1

where σ^2_a/σ^2_r is the ratio of the between-individual to the within-individual variances, estimated from P3 to be 0.63 (data not shown), and p is the acceptable error term (0.9).

Evaluation of Collagen Cross-Links as Markers of Growth

Normalization of Results. Expression of urinary cross-links by urinary creatinine was not considered appropriate because repeated-measures Anova showed significant variations in creatinine excretion during the day in P2 ($p < 0.05$; fig. 2). Therefore, the cross-link concentration measured in urine samples was multiplied by the total volume and divided by the collection time to calculate hourly rates of excretion.

In order to compare subjects in the absence of direct skeletal mass measurements, the cross-link output was standardized by height only, choosing a suitable power, so that the effect of height on cross-link output could be entirely removed. This was calculated as the slope of the regression of $\log_e$Pyd on $\log_e$height [Pyd (nmol/h) = height^a (m) which is in fact equivalent to $\log_e$Pyd = a · ($\log_e$height)]. Since the slope of the regression ranged from 2.2 in CSS children to 3.7 in LNG at month 4 with an average value of 3, the cross-link excretion was standardized by dividing by the cube of height. This is in concordance with the approximately cubic relationship between height and skeletal mass described previously [Drinkwater, et al., personal commun.].

Collagen Cross-Links and Growth Velocity (LNG). Out of the 1,620 urine samples that were expected from the children (3 urine samples × 5 rounds × 108 children), 1,100 were collected (69%). As many as 78% of the collections performed by the parents were considered acceptable. The remaining 22% were discarded because undefined amounts were known to be lost (1%), the urinary flow rate was out of the normal range (9%), the duration of the collection had not been recorded (7%), or the urine had not been delivered to the laboratory on the collection day (5%). The full set of 15 complete 12-hour urine collections was obtained in only 8 children, while 28 children provided at least one complete collection per observation period and 76 at least one complete collection throughout the study. Complete urine samples provided at baseline (n = 65) were used to assess reference values of cross-link excretion in normal children 3–5 years of age (table 2).

Monthly variations in cross-link excretion rates were highly variable between subjects, ranging from 6 to 64% for Pyd (mean 22%) and from 6 to 63% for Dpd (mean 23%). Overall, the cross-link excretion was highest in Jan-

uary 1993 (10% above the mean) and lowest in March 1993 (10% below the mean), although the differences between months for either cross-link were not significant. The height velocity during the 4-month period (from January until May) was similar for boys and girls: 5.8 mm/month (SEM 0.2) and 5.6 mm/month (SEM 0.3), respectively.

The cross-link output measurements performed over the whole 4-month period were regressed against height velocity achieved within that time. When the 8 subjects with 15 complete urinary collections were analyzed, the correlation was significant, and the regression equation of height velocity on Pyd ($r = 0.73$; $p < 0.05$) or Dpd ($r = 0.78$; $p < 0.05$) explained approximately 60% of the variance. However, when fewer collections were used, or when values from incomplete collections were included, the correlation was no longer significant. The cross-link output values at baseline did not correlate with height increments at 4 months, regardless of the number of urine samples collected.

Discussion

Several studies have documented a nyctohemeral rhythm of excretion of cross-links in adult males [11] and females [12]. The total Dpd level increases during the night by 48% in postmenopausal women with and without osteoporosis [12, 29] and total Pyd and Dpd levels by 21 and 38%, respectively, in premenopausal women [29], peptide-bound forms of both cross-links accounting for most of the day-night variances. In healthy premenopausal women, pyridinium cross-links/creatinine ratios peak around 05.00 h, with confidence limits between 02.00 h and 09.00 h [30], to reach a nadir between 14.00 and 23.00 h [31].

Our study shows mean differences of 43 and 48% in Pyd/creatinine and Dpd/creatinine ratios between morning and evening spot samples. In timed urine collections, morning samples overestimate the 24-hour cross-link output by 25–30%, while afternoon collections lead to underestimates. Although the long overnight collection did not allow identification of the peak, these data suggest that a diurnal cycle in pyridinium cross-link excretion is also present in children. Consequently, if a 24-hour collection cannot be performed in a child, careful standardization of the collection period is essential, particularly in longitudinal studies. As a second-choice option, 12-hour overnight collections appear to be accurate when compared with 24-hour collections, but not practical enough in children incontinent at night or in subjects with anticipated low compliance with the research protocol (i.e., healthy), as in the present study. Alternatively, the validity of first-void urine samples for LNG studies deserves to be explored, as it has been for the cross-sectional assessment of Dpd excretion in children 4–10 years old [32].

The experiments on day-to-day variation demonstrate that children, as well as adults, have a highly variable cross-link excretion. In seven consecutive morning spot samples, the extent of the fluctuation was 30%, for Pyd/creatinine and 24% for Dpd/creatinine. This is in agreement with available data for healthy young women [16], but slightly above the values reported in children by others [2, 8]. The reasons for these discrepancies may be related to the subjects' health status, since Shaw et al. [8] recruited patients undergoing surgery and were, thus, not strictly healthy, or to the sample size considered which was much greater in the study by Marowska et al. [2]. The day-to-day coefficient of variation in cross-link excretion was considerably lower when urine was collected overnight for 12 h, but still 17% for Pyd and 19% for Dpd. These figures are close to the variation of 16–24% reported by McLaren et al. [11] and strongly suggest the need of multiple urine collections for a correct characterization of the subjects. Three collections per week would provide estimates of weekly cross-link excretion rates with a maximum error or 10% in >90% of the children, while an error of 10–30% could be expected in as many as 50% of the children if only one collection is available [33]. An example of how the collection protocol influences the results can be found in the present study: the cross-link output significantly correlated with height velocity only when the children providing three complete urine collections per observation period were considered.

Evidence to support that excretion of collagen cross-links parallels growth rates in children comes from the observation of a direct relationship between urinary cross-link output and height velocity. Using an appropriate urine collection protocol, either the Pyd or the Dpd output over 4 months accounted for as much as 60% of the variability in height velocity within the same period; this is higher than the variability of 36–42% reported by Rauch et al. [34] using single 24-hour urine collections and standardizing the results by creatinine. However, the value of either cross-link to predict the height velocity during the following 4 months was negligible. This result was not unexpected because normal growth is pulsatile, and a period of more accelerated growth may be followed by a period of decreased velocity, such that the excretion at a point in time cannot predict what would hap-

pen over a long period. Conversely, collagen cross-links have been proposed as helpful early markers of responsiveness to treatment in some growth deficiency states, such as GH deficiency. In GH-deficient children treated with GH, we observed that either the Pyd or the Dpd output during the 1st month can explain up to 67% of the variability in height velocity after 12 months of therapy [6]. There are several factors that may explain these differences in the ability of cross-link excretion to predict growth responses between health and disease. On one hand, the plateau of the pulses in pharmacological GH-induced growth may be shorter and less representative of a certain period of time than in normal growth. On the other hand, treatment with GH doubled the yearly growth rates from 4 cm/year to 9.8 cm/year, and the height was measured over a 6-month period, while in the present study the mean growth rate for boys was only 7.0 cm/year, and 4 months was the longest period considered. Since the measurement error of height increments will have a greater impact on the correlation with cross-link excretion for smaller changes in height, this will lead to a closer predictive relationship between cross-link output and height velocity in GH-treated children as compared with normal children under the same protocol.

The same reasoning would apply to malnourished children on refeeding, another situation of catch-up growth where urinary excretion of pyridinium cross-links is closely related to height velocity [33]. Actually, these bone resorption markers may be valuable to monitor the efficacy of different nutritional interventions in growing children, and the present study provides valuable data for the appropriate design of adequately controlled trials, where a group of normal children is to be included.

Finally, urinary metabolites are usually standardized with respect to creatinine in order to take into account the variable dilution of the urine and to correct for the size of the individual, as it is assumed that creatinine is an indicator of the skeletal mass. A second assumption is that there is a constant turnover of the creatinine contained in the muscle compartment. In children, however, both these assumptions may not hold. The creatinine turnover may be affected by changes in nutritional status [35], and the muscle mass increases at a variable rate during growth [36]. Furthermore, as demonstrated by the present study, creatinine itself has a diurnal rhythm that is highly variable between individuals. While the calculation of hourly excretion rates may avoid the problem of urinary dilution, differences in skeletal size should be accounted for using a surrogate of the skeletal mass. In the absence of direct measurements of bone mass, anthropometry can be used. Height, length of the skeletal segments, bone width, or more simply weight, are all associated with skeletal mass. The body weight is probably inadequate, as the weight-to-height relationship changes with age, gender, and nutritional status of the individual [37]. Other skeletal measures are cumbersome [33], while height could be simple and reproducible enough. The relationship between height and skeletal mass can, however, be affected as well by age and gender, with periods of bone size growth followed by periods of bone consolidation [38]. This implies that this standardization procedure could be appropriate only within relatively narrow age ranges and that comparisons across age groups may be inaccurate.

In conclusion, the urinary excretion of pyridinium cross-links parallels growth velocity in healthy children. When using an appropriate urine collection protocol, they can explain up to 60% of the variability in growth velocity over short periods of time, but their predictive value is negligible. 24-hour urine samples should be collected whenever possible due to the large daily variation observed in cross-link output, although 12-hour overnight timed collections appear a suitable trade-off between accuracy and viability. The characterization of a single individual requires multiple (at least three) 12-hour samples. In children incontinent at night or in populations in whom a low compliance with the research protocol is anticipated (i.e., healthy), as in the present study, the validity of first-void morning samples for longitudinal assessments should be investigated. Finally, although accurate measurements of skeletal mass would be of choice to compare the cross-link excretion between individuals of different body sizes, namely bone mass assessed by dual-energy X-ray absorptiometry, the cube of height may be used as a surrogate in small children. This methodological study provides valuable data to assess the effect of nutritional interventions on bone turnover and growth in preschool children in prospective, randomized, controlled trials, where the inclusion of a group of normal children applies.

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