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Longitudinal study of urinary hydroxy-pyridinium cross-links and growth in healthy infants: Higher values with breastfeeding and after daytime sleep

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Summary: Urinary pyridinoline and deoxypyridinoline crosslinks (crosslink) are excreted when bone is resorbed. The aims of this study in healthy infants were to determine whether crosslinks a) could predict growth velocity, b) are variable due to circadian rhythm, and c) differ in infants who were either breast-fed or formula-fed. In 78 healthy infants (48 male; 30 female) urine samples were collected and anthropometric measurements were taken at 2, 3, 4, 5, 6, 8, 10 and 12 months of age. In addition, a total of 25 samples were collected during the day (0700–2000) in 5 of the infants to determine circadian rhythm of crosslink excretion. Crosslink excretion decreased ($p < 0.001$) with age between 2 and 12 months. Pyridinoline excretion showed a significant, but weak correlation ($r \geq 0.21$; $p < 0.05$) with linear growth velocity and weight velocity in the subsequent month until 6 months of age, and no correlation thereafter. Infants studied for circadian rhythm showed a 63% greater ($p < 0.05$) rate of pyridinoline excretion after a nap as compared to the 13-hour mean value. In a subset of infants whose energy intake was exclusively from breast milk (BF, $n = 23$) or formula (FF, $n = 10$), crosslink excretion was greater in BF infants at 3 months of age ($p < 0.05$). The correlations between crosslink excretion and growth parameters indicate that crosslinks may be useful as a marker of growth in infant populations. However sources of variation in crosslink excretion, such as circadian rhythm and diet may limit their utility to predict growth in an individual infant. These factors should be considered in future studies examining markers of bone turnover in infants.

Introduction

Growth is routinely assessed in infants with anthropometric measurements such as weight, length and head circumference. This information can be useful for diagnosing a growth deficit due to poor bone development. During normal growth, bone is constantly modeling and remodeling. Biomarkers of bone metabolism have the potential to estimate growth velocity and hence could be a useful tool in predicting short-term increases in bone growth (Branca et al., 1992).

The hydroxy-pyridinium cross-linking amino acids (crosslinks), pyridinoline [PYD] and deoxypyridinoline [DPD], are formed during the maturation of collagen fibrils in bone (DPD) and cartilage (PYD and DPD) (Eyre et al., 1984). Free and peptide-bound crosslinks are released when bone is degraded and can be detected in the urine. Pyridinoline, though not found exclusively in bone, is derived predominantly from bone collagen (Robins et al., 1994). DPD has a more limited tissue distribution and its turnover in tissues other than bone is very slow (Robins et al., 1994). Pyridinoline and DPD are formed from a post-translational modification of collagen and cannot be reused during further collagen synthesis. Unlike the more traditional marker of bone resorption, hydroxyproline, pyridinium crosslinks are not influenced by their dietary intake (Colwell et al., 1993), they are not metabolized before excretion in the urine, and are more sensitive and specific (Calvo et al., 1996; Delmas et al., 1991).

Previous studies in adults have found an increase in crosslink excretion associated with diseases of high bone turnover, such as osteoporosis, primary hyperparathyroidism, and osteoarthritis (Delmas, et al., 1991; Seibel et al., 1996). In healthy infants and children, a decrease in crosslink excretion with increasing age has been reported in cross-sectional data (Blumsohn et al., 1994; Lieuw-A-Fa et al., 1995; Tsukahara et al., 1996; Pratt et al., 1996; Shaw et al., 1995). In

malnourished infants, crosslink excretion is a good estimate of growth velocity during catch-up growth (Branca et al., 1996). In a mixed longitudinal study examining bone mineral density in infants, the cross-sectional measurements of bone biomarkers reflected past, but not future bone growth (Lieuw-A-Fa et al., 1995). Hence, there are inconsistent findings in previous studies (Lieuw-A-Fa et al., 1995; Branca et al., 1992). In the present study we serially monitored crosslink excretion in healthy infants during their first year of life to determine whether crosslinks: a) could predict growth velocity, b) are variable due to circadian rhythm, and c) differ in infants who were either breast-fed or formula-fed.

Materials and methods

Subjects

Ninety-four healthy infants (54 males; 40 females) were recruited for this study and examined during their usual visits to the pediatrician's office. Seventy-eight of the infants (48 male; 30 female) provided more than 50% of the required urine samples. Fifteen infants completed the total study, i.e. provided urine samples at all time points. The individual number of infants at each time point is shown in Table 1. The age range of the infants was 2 to 12 months. Ninety-two percent of the infants were Caucasian, 5% were Asian and 2% were African-American. Medical and nutritional histories were obtained on all infants. A consent form was signed by the parent or guardian of each infant that was approved by the Institutional Review Board of Rutgers University.

Protocol

A urine sample was obtained by a trained nurse from each infant at 2–6, 8, 10 and 12 months of age. Samples were obtained during routine examination during daytime hours (1000–1700 h) and sequential collections were at approximately the same time of day (±1.5 hours) for a given infant. Infants who were ill and could not come to their exam, did not provide a urine sample. Urine was collected by placing a plastic urine bag (Mark Clark, Topeca, KS) over the area of urination and was left on ~1 hour while waiting for the physician and during their physical exam. Infants typically urinated at the end of their exam, during the vaccination. One mL of urine was removed from the bag with a transfer pipet into a storage tube, and the time of collection was recorded. Samples were stored at −20 °C for approximately 1 week until transported on dry ice to the laboratory. A trained nurse, measured infants for length, weight and head circumference, and the parent or guardian was questioned about the infant's food intake. Length was measured on a length board (Infant table, #109, Ritter), weight was recorded on electronic scales (Seca, Model #727) and head circumference was measured using a paper insertion tape. Analysis of circadian rhythm of crosslink excretion (from 0700–2000 h) was assessed in 5 infants (ages 4, 5, 6, 10 and 12 months). A trained technician remained in the home of each infant to perform these measurements and urine samples were collected every 2–5 hours in a newly replaced bag. In addition, to estimate day-to-day variation in crosslink excretion, 5 urine samples were collected/day (every 2–4 hours) from one infant (age 5 months) at approximately the same time on 3 consecutive days by a trained technician in our laboratory.

Determinations

For analysis of total PYD and DPD, a 0.5 mL aliquot of urine was hydrolyzed by adding an equal volume of 12 M HCl and applied to a CF1-cellulose (Whatman, Maidstone, Kent, U.K.) column for fractionation as described by Black et al (Black et al., 1988). The crosslinking amino acids were eluted with acetonitrile and water and centrifuged at 1000 rpm for 15 minutes to separate the water layer containing the crosslinks. The sample was heat-dried and mixed with 0.1 mol/L n-heptafluorobutyric acid solution and stored at −70 °C before analysis. Samples were subjected to high pressure liquid chromatography analysis, using a modified method described by Eyre et al (Eyre et al., 1984). Values were obtained by fluorescence detection and quantified by external standards (courtesy of S. Robins). The inter-assay reproducibility indicated by the coefficient of variation is 3.8% and 5.9% for PYD and DPD, respectively, as determined in 4 adult subjects on 3 consecutive days (Shapses et al., 1995).

Creatinine concentration was measured using a calorimetric assay (Sigma Diagnostics, St. Louis, MO. #555). Samples were assayed at a wavelength of 500 nm on a spectrophotometer. Urinary crosslinks are expressed relative to creatinine excretion.

Statistical Analysis

The statistical method included an analysis of variance using a general linear models procedure. Comparisons of anthropometric parameters and crosslinks between breast-fed and formula-fed groups were made by using a three-factor analysis of variance model with repeated measures over time, treating feeding practice, age and gender as main effects. Multiple-regression models were developed to determine factors relating to the rate of length and weight gain with pyridinium crosslink excretion with the following potential independent variables: feeding mode, gender, birth weight, weight, and time of day defined as morning or afternoon (1000–1230 and 1300–1800). Circadian rhythm over a three day period was examined in a repeated measures analysis, using day and

Table 1 Description of infants studied from 2 to 12 months

Age months	n	Gender n (Male/Female)	PYD/Creat nmol/mmol	DPD/Creat nmol/mmol	Length cm	Weight kg	HC cm
2	49	33/16	214.8 ± 95.8	42.1 ± 17.5	57.2 ± 2.5	5.5 ± 0.7	38.9 ± 1.4
3	49	34/15	211.1 ± 109.6	41.0 ± 17.7	60.6 ± 2.4	6.3 ± 0.8	40.7 ± 1.3
4	51	33/18	171.8 ± 115.3	37.6 ± 24.3	62.9 ± 2.5	6.9 ± 0.9	41.7 ± 1.4
5	31	18/13	178.8 ± 72.9	36.8 ± 17.3	64.9 ± 2.2	7.3 ± 0.7	42.6 ± 1.2
6	28	16/12	147.3 ± 82.9	29.8 ± 15.2	66.3 ± 4.3	8.0 ± 0.9	43.5 ± 1.2
8	26	15/11	142.7 ± 85.4	28.0 ± 13.6	69.5 ± 2.7	8.6 ± 0.9	44.9 ± 1.2
10	24	13/11	115.3 ± 52.4	25.9 ± 14.2	73.0 ± 2.4	9.4 ± 1.0	46.0 ± 1.1
12	15	9/6	105.7 ± 53.2	22.6 ± 7.4	76.6 ± 3.4	10.0 ± 1.2	46.7 ± 1.3

Values are the mean ± S.D.; Creat, creatinine; DPD, deoxypyridinoline; HC, head circumference; PYD, pyridinoline.

time as variables. Values were considered statistically significant if the two-sided p-value was less than 0.05. All computations were performed using the Statistical Analysis System (version 6.04).

Results

Seventy-eight of the 94 healthy infants provided at least 50% of the required urine samples, and 16 were eliminated from the analysis. Their mean birth length and weight were 51.9 ± 0.8 cm and 3.5 ± 0.3 kg, respectively. Values of anthropometric measurements and crosslink excretion are shown in Table 1. Crosslink excretion decreases with increasing age ($p < 0.0001$). This correlation between crosslinks and age was also significant ($p < 0.001$) when calculated for the 15 infants who completed the study at 12 months of age. There was no gender difference in crosslink excretion. There was a trend for crosslink excretion to increase with increasing length and weight at a given age that was significant at 2 and 8 months of age ($p < 0.05$). There was no correlation between crosslink excretion and head circumference. Crosslink excretion showed a weak correlation with linear growth velocity (cm/month) in the subsequent month from 2 to 6 months of age for PYD ($r = 0.21$; $p < 0.05$) and DPD ($r = 0.18$; $p < 0.06$). Additional analysis to determine if crosslink excretion reflected linear growth velocity from the *previous* months showed a weaker correlation ($r \leq 0.16$) that was only significant for PYD ($p < 0.05$). The correlation between future and *previous* weight velocity and crosslink excretion from birth to 6 months of age was significant for PYD ($r \geq 0.23$; $p \leq 0.01$) and DPD ($r \geq 0.21$; $p < 0.05$). No correlation between crosslinks and growth velocity was observed in our data set after 6 months of age.

Values for crosslink excretion (Fig. 1) and growth parameters (Table 2) are shown for infants whose energy intake was exclusively from breast-feeding (BF) or formula-feeding (FF, with cow milk based formula) for at least their first 4 months. Both groups of infants (BF and FF) were given their usual vitamin and mineral supplementation and may have received supplemental water throughout the study. After 4 months of age, food was introduced and infants were considered partially BF or partially FF. Although urinary excretion of PYD was not statistically different in BF and FF infants at 2 months of age, values were 44% greater in BF infants at 3 months of age ($p < 0.05$) and both PYD and DPD tended to be greater ($p < 0.08$) at 4 months of age (Fig. 1). Growth

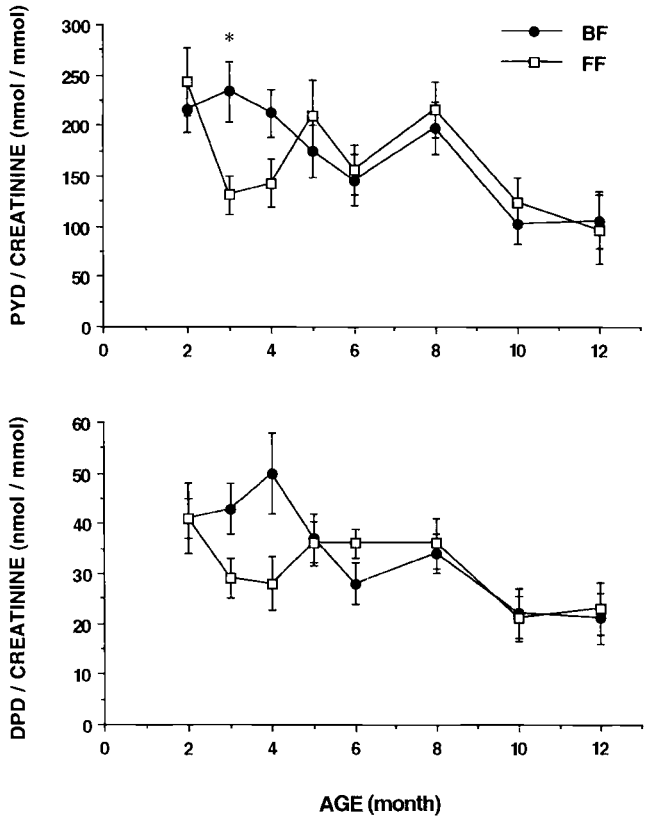

Fig. 1 Crosslink excretion in almost exclusively breast-fed (BF) and formula-fed (FF) infants, from 2 to 12 months of age (See Table 2 for sample size at each time point). PYD, pyridinoline; DPD, deoxypyridinoline. Values are expressed as the mean ± S.E.M. Differs from FF, * $p < 0.05$.

Table 2 Characteristics of breast-fed and formula-fed infants.

Age (n for BF; FF) months	BF Weight kg	FF Weight kg	BF Length cm	FF Length cm
2 (23; 10)	5.8 ± 0.8	5.5 ± 0.8	57.8 ± 2.9	57.1 ± 3.1
3 (23; 10)	6.3 ± 0.8	6.4 ± 0.7	60.6 ± 2.4	61.1 ± 3.0
4 (23; 10)	6.9 ± 0.9	7.0 ± 0.6	63.3 ± 2.0	62.5 ± 2.5
5 (16; 9)	7.0 ± 0.9	7.3 ± 0.7	64.1 ± 2.3	63.9 ± 2.1
6 (17; 9)	7.7 ± 0.9	8.4 ± 0.9	66.3 ± 1.4	66.3 ± 2.6
8 (8; 8)	8.9 ± 0.4	9.1 ± 0.9	69.7 ± 1.5	70.0 ± 2.6
10 (10; 6)	9.3 ± 1.0	10.1 ± 0.7	71.8 ± 1.5[1]	73.9 ± 2.2
12 (6; 6)	9.4 ± 0.9	10.5 ± 1.0	73.3 ± 2.2[1]	76.9 ± 2.6

Values are the mean ± S.D.; BF, breast-fed; FF, formula-fed.
[1] Differs from FF, $p < 0.05$.

velocity from months 3 to 4 and length at 5 months of age was marginally greater ($p < 0.09$) in BF than FF infants. Thereafter, length and linear growth velocity were not statistically different between groups until 10 months of age when FF infants showed greater values than BF infants ($p < 0.05$). Body weight did not vary significantly among the 2 groups, although FF infants tended to weigh approximately 10% heavier than BF infants after 6 months of age (Table 2).

Circadian Rhythm of PYD and DPD

There were no differences in the rate of crosslink excretion between morning and afternoon samples, as examined in all infants at any given age. In the five infants studied for partial circadian rhythm, crosslink excretion varied up to 3-fold. However, between infants there was no consistent pattern of crosslink excretion (Table 3). Because daytime sleep patterns are variable, data from those infants ($n = 3$) who slept at a regular time of day with length ≥ 1.5 hours were examined separately. Excretion of crosslinks in the infants was greatest in the urine voided after a period of sleep. The PYD value was 63 ± 25% greater ($p < 0.05$) than the mean for samples collected over the entire 13-hour sampling period (223.1 ± 58.7 nmol/mmol creatinine). After a period of sleep, DPD averaged 54 ± 21% greater ($p < 0.05$) than the 13-hour mean of 43.5 ± 12.3 nmol/mmol creatinine.

Day-to-day variation in crosslink excretion over different times of the day

Crosslink excretion was more consistent within an infant over a 3 day period (not shown) than values between infants (Table 3). Analysis of variance showed that there was an effect of time ($p < 0.01$), with higher values after napping (1200 h) compared to other times of the day. At ~0600 h, 1200 h (post-nap sample), 1500 h, 1700 h and 1900 h, the coefficient of variation for PYD was 27%, 16%, 7%, 21%, and 16%, respectively. For DPD, these values were very similar. The mean day-to-day coefficient of variation (includes all time points) was 17 ± 7% and 20 ± 12% for PYD and DPD, respectively.

Discussion

Traditional growth monitoring in infancy is used as a tool to promote and sustain good health, and to detect growth failure. Determining whether markers of bone turnover reflect standard parameters of growth and could be useful for estimating the rate of growth in healthy infants is not clear (Lieuw-A-Fa et al., 1995; Branca et al., 1992). In this study, we found that crosslink excretion decreased with increasing age and length. In addition, crosslinks are a weak, but significant predictor of growth (length and weight) velocity until 6 months of age. These data thus contribute to reference values of crosslink excretion in healthy infants, and show that there are sources of variability in crosslink excretion, due to circadian rhythm and diet. For example, infants show more than a 2-fold increase in crosslink excretion after daytime napping. In addition, values are approximately 44% higher in BF compared to FF infants at 3 months of age.

The level of crosslinks in infants 2 to 12 months (Table 1) is similar to that found in other infants (Lieuw-A-Fa et al., 1995). These levels of crosslinks are in a similar range as those found in children (4–10 years of age) (Rauch et al., 1994; Fujimoto et al. 1995), but are 4 to 9-fold greater than typical values in adults (Shapses et al., 1995). These data suggest that the majority of crosslink excretion in infancy represents new bone growth rather than bone remodeling, as occurs in adults. This is one basis for the hypothesis that the rate of bone resorption, as measured by crosslink excretion, correlates with longitudinal growth. A good correlation between crosslink excretion and growth velocity has been shown in 13 month old infants recovering from malnutrition (Branca et al., 1992). However, the healthy

Table 3 Circadian rhythm of urinary pyridinium crosslinks in 5 healthy infants.

Subject (age)	0700–0900 h		1000–1200 h		1300–1500 h		1600–1800 h		1900–2000 h	
	PYD	DPD	PYD	DPD	PYD	DPD	PYD	DPD	PYD	DPD
1. (4 mo)	263.9	48.0	478.1[1]	83.0[1]	233.2	43.4	178.4	45.1	295.1	69.0
2. (5 mo)	206.2	61.9	174.2	56.5	130.8	34.8	247.0	73.1	214.8	63.0
3. (6 mo)	170.8	29.7	166.4	28.1	372.8[1]	65.7[1]	162.5	32.5	127.3	28.4
4. (10 mo)	134.0	26.9	246.8[1]	50.3[1]	176.1	34.8	194.3	37.8	147.0	30.2
5. (12 mo)	133.0	20.6	216.6.	27.7	125.3	22.0	112.8	16.0	178.2	29.7
Mean	181.6	37.4	256.4	49.1	207.6	40.1	179.0	40.9	192.5	44.1
± S.D.	55.1	17.1	128.1	22.9	102.0	16.2	48.8	20.9	66.2	20.2

DPD, deoxypyridinoline; PYD, pyridinoline; Values for PYD and DPD are expressed as nmol/mmol creatinine.
[1] Samples collected after a 1.5–2 hour nap.

infants in the present study only showed a weak correlation between crosslink excretion and growth velocity until 6 months of age. In addition, others have not observed a relationship between bone markers and future growth velocity in healthy infants (Lieuw-A-Fa et al., 1995). Differences in findings may be due to greater accuracy of crosslinks in a 24-hour urine collection (Branca et al., 1992) as compared to spot urine collections used in the present study and by others (Lieuw-A-Fa et al., 1995). Also, bone remodeling during catch-up growth (Branca et al., 1992) compared to growth in healthy infants may explain differences between the studies.

Other markers of bone turnover have been found to correlate with height and provide an estimate of bone growth in cross-sectional studies (Blumsohn et al., 1994; Lieuw-A-Fa et al., 1995; Rauch et al., 1995; Bollen et al., 1994). Studies in children have used markers of bone formation including serum osteocalcin, carboxyterminal propeptide of type I procollagen (PICP), and aminoterminal propeptide of type III procollagen (Blumsohn et al., 1994; Saggese et al. 1994), and markers of bone resorption, N-telopeptides of type I collagen, galactosyl-hydroxylysine (Bollen et al., 1994; Rauch et al., 1995; Saggese et al. 1994). In infants, there is data examining two markers of bone formation, PICP and osteocalcin (Fleischer-Michaelson, et al., 1992; Lieuw-A-Fa et al., 1995) both showing to be a poor estimate of future growth velocity. It is however suggested that PICP may be a more sensitive indicator of previous and present bone metabolism than PYD (Lieuw-A-Fa, et al 1995). Another possible marker is urinary hydroxyproline. However, it does not specifically reflect bone resorption because it is also derived from nonskeletal tissues.

We found a higher rate of crosslink excretion in BF than FF infants at 3–4 months of age with a marginally greater growth velocity. In addition, others have found that serum osteocalcin is approximately 3 times higher in BF than FF infants at 2 and 6 months of age (Fleischer-Michaelson, et al., 1992). This may indicate that breastmilk contains factors that stimulate bone turnover and modeling. For example, there are bone-regulating hormones such as estradiol, calcitonin and insulin-like growth factor I in human milk, but not infant formulas (Kodolvsky et al., 1995). Furthermore, unique quantity, quality and/or bioavailability of nutrients in human milk (i.e., Vitamin D, calcium and phosphorus) may also regulate bone turnover differently between BF and FF infants (Lo and Kleinman 1996). It is possible that the higher rates of bone turnover may influence the quality of bone structure (Fleischer-Michaelson, et al., 1992; Mimouni et al., 1993). It should be noted, however, that length and linear growth velocity measurements were greater for the FF than BF infants from 10–12 months of age (Table 2). In addition, others have shown that bone mineral content is similar in BF and FF infants during the first year of life (Mimouni et al., 1993).

The circadian rhythm of the average crosslink excretion showed approximately a two-fold fluctuation from peak to nadir within an individual infant (Table 3). This is slightly greater than that found in adults (Blumsohn et. al., 1994; Schlemmer et. al., 1992) and in children (Fujimoto et. al., 1995; Saggese et al., 1994; Rauch et al., 1995). To our knowledge, there is no previous data examining circadian rhythm of crosslinks in infants. The practical problems of collecting urine samples from healthy infants during the night, precluded such measurements in this study. Rather, daytime sleep that is an important component of the circadian rhythm during the first 14 months (Glotzbach et al., 1994), was measured. Periods of sleep during daytime hours were associated with a markedly elevated rate of crosslink excretion ($p < 0.02$). Hence, a particular time of day for sample collection is not recommended, as in the adult (Blumsohn et al., 1994; Abiatti et al., 1993). Collecting the sample at a consistent time of the day, such as ~2 hours after a period of sleep, may improve the reliability of crosslinks as a marker of growth in infants.

In conclusion, this mixed longitudinal analysis shows that while crosslink excretion may be useful in a research setting for monitoring linear bone growth in infant populations; its utility as a diagnostic tool in the clinical setting to predict growth in an individual infant is not supported by this data. Variation in crosslink excretion due to diet and circadian rhythm limit their ability to be a simple predictor of growth. These factors should be considered in the design of future studies.

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