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Utility of Urine Calcium and Phosphate as Screening Tools for Vitamin D Deficiency in Children

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Abstract

Objective: Vitamin D deficiency is estimated to affect one billion people worldwide. In the United States, 9-12% of healthy children have vitamin D deficiency. Renal calcium and phosphate excretion in healthy people is a sensitive indicator of total body mineral balance. We explored the possibility of using urine calcium-to-creatinine (U_{ca}/U_{cr}) and urine phosphate-to-creatinine (U_{phos}/U_{cr}) as noninvasive biomarkers of vitamin D deficiency in at risk children.

Patients and methods: This was an observational study of children with one or more risk factors for vitamin D deficiency. Anthropometric data and a physical exam were obtained in all children and skin pigmentation, sunlight exposure, dietary history, and frequency of vitamin supplementation of both mother and child were determined by parent report. We measured serum levels of calcium, phosphate, magnesium, intact PTH, 25-hydroxyvitamin D, alkaline phosphatase, and creatinine.A random urine sample was collected for calcium, phosphate and creatinine.

Results: A total of 60 healthy children were recruited. Mean age of the subjects was 1.4 (range 0.5 to 2.9) years. Twenty percent of the children were regularly given vitamin D supplements. The prevalence of vitamin D deficiency (25-hydroxyvitamin D < 50 nmol/L) was 3.4%, vitamin D insufficiency (≥ 50 and < 80 nmol/L) was 28.6% and vitamin D sufficiency (≥ 80 nmol/L) was 68%. One subject had biochemical evidence of rickets. Linear regression analysis showed no correlation between 25-hydroxyvitamin D levels and random U_{ca}/U_{cr} or U_{phos}/U_{cr}.

Conclusion: Random urine calcium or phosphate levels offer little promise as screening tools for vitamin D deficiency in children.

Background

Vitamin D deficiency is extremely common worldwide and is estimated to affect one billion humans [1]. In children, vitamin D deficiency may lead to nutritional rickets, which is considered to be the most common non-communicable disease of children worldwide [1-3]. Rickets can cause significant morbidity, including delays in growth and motor development, failure to thrive, short stature, skeletal deformities such as tibial bowing and splaying of the anterior ribs, tetany, seizures, and enamel defects. Growing evidence of a role of vitamin D on the immune system suggests vitamin D deficiency may also have other long-term effects [4-6]. Nutritional rickets was once thought to be nearly eliminated from the developed world, but is currently increasing in incidence [7-12].

Vitamin D is synthesized in the body by the conversion of 7-dehydrocholesterol to vitamin D_3 by UV radiation in the skin [13]. Vitamin D_3 is also obtained through dietary sources as is the closely related vitamin D_2 [14]. Vitamin D is converted to 25-hydroxyvitamin D in the liver [13]. 25-hydroxyvitamin D is the storage form of vitamin D although the vitamin D receptor does bind it with reduced affinity [15]. 25-hydroxyvitamin D is converted to the active 1,25-dihydroxyvitamin D primarily in the kidney, by an enzyme tightly regulated by parathyroid hormone (PTH) and to a lesser extent by hypocalcemia, hypophosphatemia, insulin, growth hormone and other factors. Conversion also takes place in other tissues, but this does not play a major role in calcium homeostasis.1,25-dihydroxyvitamin D acts by binding the vitamin D receptor. The primary action of vitamin D is to increase calcium uptake in the small intestine [16]. It also plays a role in calcium reuptake in the kidney and has a direct effect on growth plate chondrocytes and osteoblasts mediated through insulin-like growth factor-I [17].

When vitamin D levels are low, calcium uptake in the gut is reduced. This reduction in calcium uptake results in increased secretion of parathyroid hormone, which increases calcium reuptake and decreases phosphate reuptake in the kidney, increases bone resorption, and increases conversion of 25-hydroxy- to 1,25-dihydroxyvitamin D [18]. These effects all serve to maintain blood calcium levels. It is the effect of PTH on renal calcium and phosphate handling that we sought to utilize for screening purposes.

Rickets is typically associated with 25-hydroxyvitamin D levels <25 nmol/L (10 ng/mL) and consensus remains that levels ≥ 80 nmol/L (32 ng/mL) defines sufficiency [5].

The risk factors for developing vitamin D deficiency in children are well described and include exclusively breast fed infants not given vitamin D supplementation, premature infants, immigrants, darkly pigmented racial groups, infants and children with poor weight gain, vegetarians, children with cow's milk protein allergy or lactose intolerant who are avoiding dairy, and those children with severe eczema [19-27]. The prevalence of vitamin D deficiency in children in the United States is reportedly 9-12%.A study in Boston showed 12% prevalence of vitamin D deficiency (defined as 25-hydroxyvitamin D level ≤ 50 nmol/L) among healthy infants and toddlers [28]. One third of those defined as deficient exhibited demineralization on X-rays, revealing

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the deleterious skeletal effects of this condition [28]. Kumar et al. [29], published data from a nationally representative sample of children aged 1 to 21 years in the National Health and Nutrition Examination Survey (NHANES) 2001–2004 (n = 6,275). They measured serum 25-hydroxyvitamin D levels and defined deficiency (<37 nmol/L) and insufficiency (37–72 nmol/L) and found a prevalence of vitamin D deficiency of 9%.

The high prevalence of vitamin D deficiency with its associated morbidity and mortality, coupled with the disease modifying potential of vitamin D replacement, has led to calls for screening of high risk children [30], although there is little consensus on what screening tests should be used. The gold standard biochemical biomarker of 25-hydroxyvitamin D is an expensive assay and not available in many parts of the world. In a large Nigerian study, $U_{ca}/U_{cr}$was found to be very low in rachitic children and statistically different when compared with the non-rachitic children [31]. These data suggested that urine calcium levels may offer utility as a screening tool by detecting vitamin D deficiency early in its course. Urine is easier to obtain than blood and assays are inexpensive and widely available. In a first step to test this hypothesis, we performed an prospective, observational study of infants and toddlers with one or more risk factors for vitamin D deficiency to determine if U_{ca}/U_{cr} or U_{phos}/U_{cr} correlated with 25-hydroxyvitamin D levels.

Patients and Methods

Study design

This was a prospective, observational, cross-sectional study of infants and children with one or more risk factors for vitamin D deficiency. The study was approved the IRB of the Nemours Children's Clinic in Jacksonville, FL.Signed parental permission was obtained for all subjects. The study was registered on Clinical Trials.gov (NCT01105689).

Subjects

Subjects were informed of the study by their primary physician and those interested in the study were scheduled for a study visit to verify eligibility and to review informed consent. Eligible subjects were any infant or child older than 6 months and less than 36 months of age with one or more of the following six risk factors: breast fed (defined as infants exclusively breast feed for 1 month or longer); dark skin pigmentation; history of premature birth (born at < 32 weeks gestational age); recent immigrant from developing country; weight-for-length < 10%; wrist and costochondral enlargement or genu varum. Children were excluded from study participation for any of the following: a history of known familial rickets (hypophosphatemic or vitamin D resistant), known parathyroid hormone problems, diuretic use, any chronic disease or use of medications known to affect vitamin D metabolism, renal insufficiency, known malabsorption, or rickets secondary to other syndromes ($e.g.$ Fanconi syndrome). Subjects with acute illnesses were scheduled so that they were off of all antibiotics and healthy for at least two weeks prior to the study visit.

Study visits

All subjects had a single study visit. The parent/guardian completed a written medical, sun exposure and dietary history questionnaire with the assistance of the lead investigator (MB). Vital signs, height, and weight were obtained. Center for Disease Control (CDC) 2000 growth charts were used for plotting weight and height percentiles, as weight/height < 10% was used as one of the eligibility criteria for the study. A physical exam was done to assess health status and document if there were any physical features suggestive of active rickets. A urine collection bag was placed in the diaper or underwear. Five-to-ten milliliters of whole blood were drawn and serum was obtained and then frozen at -80 C until analysis.

If the labs from the initial study visit suggested biochemical evidence of rickets [25-hydroxyvitamin D level < 50 nmol/L and alkaline phosphatase ≥ 420 U/L for (6 months-24 months) or ≥ 320 U/L (≥ 24 months)], the family was contacted and asked to return for a follow up visit. At this visit, X-rays (AP knees and wrists) were done to assess for radiographic signs of active rickets [32]. The subjects were then referred for full evaluation and treatment in the endocrinology clinic. Data from subjects with evidence of rickets were included in the analysis.

Subjects with isolated elevations in alkaline phosphatase but no other evidence of rickets were referred back to their primary care provider with a recommendation to repeat levels at a later date.

Assays

Serum assays were run by Quest-Nichols Institute, San Juan Capistrano, CA. Serum calcium, phosphate, magnesium, creatinine, alkaline phosphatase and intact PTH were determined using standard methods. 25-hydroxyvitamin D was measured by liquid chromatography tandem mass spectrometry (LC/MS/MS).Urine calcium, phosphate, and creatinine were assayed by colorimetric assays (QuantichromCreatinine, Calcium and Phosphate respectively) using kits (BioAssay Systems, Hayward, Ca).Manufacturer's instructions were followed. Urine calcium and creatinine assays were also run in the clinical laboratory (Baptist Medical Center, Jacksonville, FL), using ion specific electrode (calcium), and the Jaffe (picrate) rate method (creatinine).

For 25-hydroxyvitamin D levels, conversion from nmol/L to ng/mL is done by dividing by 2.496.

Statistical analysis

No assumption of normality was made for the analyses. On-parametric tests were performed for all analyses. Categorical variables were summarized using frequencies and percentages, while numerical variables were described using median, 5th and 95th percentiles. Height and weight SD scores were calculated using the CDC 2000 growth charts [33]. The vitamin D sufficient and vitamin D deficiency and insufficient groups were compared using the Mann-Whitney U test. Linear regression models were used to explore relationships between 25-hydroxyvitamin D levels and other analytes. These analyses were performed both on the entire cohort and again on those with vitamin D insufficiency or deficiency. Significance was assumed for p-values less than 0.05.

Results

We recruited 60 infants and toddlers for this study. For two of the subjects, we were not able to obtain paired urine and blood samples. Thus, we were able to analyze 58 infants and toddlers urine and serum samples. A summary of the subjects is included in Table 1. Table 2 details the risk factors for vitamin D deficiency as reported by their parent.

The ethnic distribution of the cohort was 38% Caucasian, 33% African-American, 15% Asian, and 14% other/mixed. Chi-square analysis did not show any significant differences between the vitamin

D sufficient or vitamin D insufficient/deficient groups as it related to sex (p = 0.4) or ethnicity (p = 0.7).

Being described as having sensitivity to sun as "never burned" was associated with a higher rate of vitamin D deficiency and insufficiency (p = 0.02), although parental described skin pigmentation (using a graded scale) did not associate with vitamin D status (p = 0.6). We also found that parental report of sun exposure of less than or more than 7 hours per week did not associate with vitamin D status (p= 0.9).

At the time of the study, 36% of mothers reported some breastfeeding and 28% were still giving formula. Forty-five percent of mothers were taking a vitamin supplement. Forty percent of the children were getting some cow's milk. Parent reported supplementation frequency of the child had an affect vitamin D status. When a parent reported use of supplements as "every day" or "most days" (20% of the cohort), no child was deficient or insufficient (p = 0.04).

We found 2 of 58 subjects (3.4%) had vitamin D deficiency (25-hydroxyvitamin D <50 nmol/L), while 28.6% were insufficient (≥50 nmol/L but <80 nmol/L) and 68% were sufficient (≥80 nmol/L). Characteristics of the sufficient versus the insufficient/deficient groups are shown in Table 3. The lower range of U_{ca}/U_{cr} values approached the lower limit of sensitivity of the assay in both groups.

Linear correlation between 25-hydroxyvitamin D was poor when we looked at random U_{ca}/U_{cr} in all subjects (r^2= 0.030, p= ns) and in those with vitamin D deficiency or insufficiency (r^2= 0.045, p= ns) (Figure 1). Similar results were seen for U_{phos}/U_{cr} (Figure 2) and U_{phos}/U_{ca} (data not shown).

The serum analytes alkaline phosphatase and PTH also showed poor correlation with 25-hydroxyvitamin D for the cohort as a whole. As expected, in children with vitamin D deficiency or insufficiency, PTH was negatively correlated with 25-hydroxyvitamin D (r^2= 0.470, p< 0.005) (Figure 3). We looked as well at the correlation between random U_{ca}/U_{cr} and PTH and found poor correlation in all subjects (r^2= 0.037, p= ns) and in those who were insufficient and vitamin D deficiency (r^2= 0.000, p = n s).

Number	60
Age (years)	1.2 (0.6,2.6)
Sex (boys:girls)	33:27
Race (Caucasion:AfricanAmerican:Asian:other/mixed)	23:20:9:8
Height or length SD score	-0.3 (-2.4,1.2)
WeightSD scores	-0.6 (-2.9,1.1)

Data are median (5th percentile, 95th percentile).

Table 1: Clinical Characteristics of the Study Subjects.

Risk factor	*Number*
breast fed	48
dark skin pigmentation	35
history of prematurity	6
weight-for-length < 10%	8
costochondral enlargement or genu valgus	2
recent immigrant from developing country	1

Table 2: Subject Risk Factors for vitamin D deficiency (n = 60).

	Vitamin D status		*P**
	deficient or insufficient	*sufficient*	
Number	20	39	
Age (years)	1.3 (0.6,2.5)	1.1 (0.8,2.8)	0.2
Sex (boys:girls)	13:7	19:20	0.4
Race (Caucasion:African American:Asian:other)	7:6:5:2	15:14:4:6	0.7
Height or length SD score	-0.1 (-1.6,1.0)	-0.5 (-1.7,1.0)	0.2
Weight SD score	0.0 (-2.4,1.1)	-0.9 (-2.7,0.4)	0.2
Serum (n= 59)			
25-hydroxyvitamin D (nmol/L)	62 (42,70)	105 (62,85)	<0.001
calcium (mmol/L)	2.5 (2.4, 2.6)	2.5 (2.5, 2.6)	0.4
phosphate (mmol/L)	1.6 (1.4,1.9)	1.6 (1.3,1.8)	0.4
magnesium (mmol/L)	0.9 (0.78,0.98)	0.86 (0.78,0.9)	0.5
alkaline phosphatase (IU/L)	245 (170,565)	223 (173,1258)	0.5
intact PTH (pmol/L)	2 (0.3,7.3)	1.2 (0.3,4.1)	0.3
Urine (n= 58)			
calcium-to-creatinine (mg/mg)	0.18 (0.07,0.53)	0.15 (0.04,0.42)	0.4
phosphate-to-creatinine (mg/mg)	1.23 (0.27,2.83)	0.99 (0.47,2.69)	0.8
calcium-phosphate (mg/mg)	7.0 (2, 20.97)	7.7 (2.89,33.51)	0.3

Vitamin D deficient is 25-hydroxyvitamin D <50 nmol/L, insufficient is ≥50 but <80, and sufficient is ≥80.

Data are median (5th percentile, 95th percentile)

*By Mann-Whitney *U* test or Chi-square analysis

Table 3. Comparison of Vitamin D Insufficient and Vitamin D Sufficient Subjects.

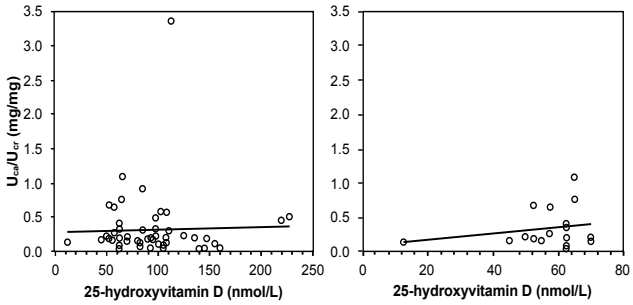

Figure 1: Correlation between U_{ca}/U_{cr} and 25-hydroxyvitamin D.*Line* shows the least-squares line. *Left panel*, data for all subjects. There is no correlation between U_{ca}/U_{cr} and 25-hydroxyvitamin D (n=58, r^2=0.030, p is ns). The outlier was a subject found to have idiopathic hypercalciuria.*Right panel*, subjects with 25-hydroxyvitamin D <80 nmol/L. There is no correlation (n=17, r^2=0.045, p is ns).

We had one child with biochemical rickets who was severely vitamin D deficient with a 25-hydroxyvitamin D of 12 nmol/L. His PTH was elevated at 16.1 pmol/L and his alkaline phosphatase was on the high end of the normal range at 381 IU/L. His bones did not show active rickets radiographically. Five percent of the children in the cohort also had transient hyperphosphatasemia [34]. This was confirmed in these cases by repeat alkaline phosphatase testing with the subjects primary physician. Two subjects had very high renal calcium excretion, that was normal when retested by their primary physician.

Figure 4 details the correlation between the clinical assays used for analysis of urine calcium and creatinine with the research assays. This was done to compare a more sensitive calcium assay (Quantichrom) which was validated for a range of 0.08 – 20 mg/dL vs. the assay in our

clinical lab with lower limit of the assay of 2 mg/dL. The correlation between the assays were good, however 23 samples were at or below the limit of detection for the clinical lab assay.

Discussion

The purpose of this study was to assess if random urine calcium and/or phosphate levels offer utility as a potential biomarkers of vitamin D deficiency. We have shown in this small sample of subjects that random urine minerals do not correlate well with 25-hydroxyvitamin D levels, due to the high variability of urine mineral levels in these children. Even in vitamin D sufficient children, the level of urine calcium occasionally approached the lower sensitivity limit of the assay. For the urine calcium assay utilized by the clinical lab, almost half of the study cohort had levels at or below the lower limit of detection. Hence, it seems from this small sample size, that low urine calcium levels offer little promise, in distinguishing vitamin D deficient children, using either a clinical assay or research assay with improved sensitivity.

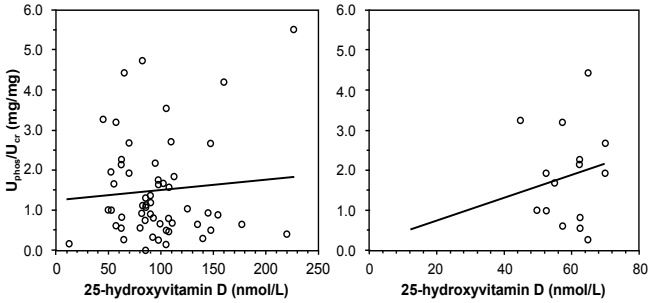

Figure 2: Correlation between U_{phos}/U_{cr} and 25-hydroxyvitamin D.*Line* shows the least-squares line. *Left panel*, data for all subjects. There is no correlation between U_{phos}/U_{cr} and 25-hydroxyvitamin D (n=58, r^2=0.006, p is ns). *Right panel*, subjects with 25-hydroxyvitamin D <80 nmol/L. There is no correlation (n=17, r^2=0.000, p is ns).

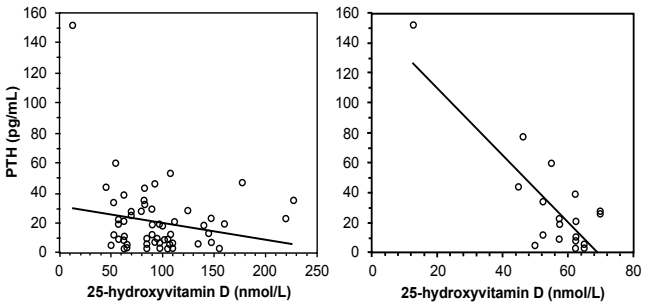

Figure 3: Correlation between PTH and 25-hydroxyvitamin D.*Circles* show the individual data points and the *line* shows the least-squares line. *Left panel*, in all subjects. There is no correlation between U_{ca}/U_{cr} and 25-hydroxyvitamin D (n=58, r^2=0.078, p is ns). *Right panel*, subjects with 25-hydroxyvitamin D <80 nmol/L.Here, there is significant correlation (n=17, r^2=0.469, p< 0.005). The outlier in both panels was the subject found to have biochemical rickets.

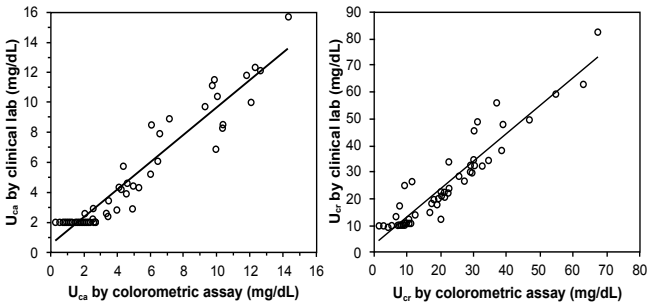

Figure 4: Comparison of assays. Highly sensitive colorimetric assays for urine calcium and creatinine were compared with assays done by the clinical lab. *Line* shows the least-squares line. *Left panel*, assays for urine calcium. There is good correlation between the assays (n=52, r^2=0.901, p< 0.0005), however, 23 samples were at or below the limit of detection in the clinical lab. *Right panel*, assays for urine creatinine. There is again good correlation (n=52, r^2=0.881, p< 0.0005), however, 9 samples were at or below the limit of detection in the clinical lab.

While Thacher et al. [31], showed that U_{ca}/U_{cr} levels were able to discriminate children with calcium deficient rickets and controls in Nigeria, this may be unique to his population in Nigeria where calcium deficient rickets is more common than vitamin D deficient rickets. We could speculate that when the diet is not replete in calcium the utility of U_{ca}/U_{cr} may increase and thus better discriminate rachitic and non-rachitic children.

In our study population, we found a prevalence of vitamin D deficiency of 3.4%; a figure much lower than was seen in the previous studies by Gordon et al. [28], where they reported 12% and NHANES data reported by Kumar et al. [29], where they reported 9%, using a more stringent cutoff. We expected a much higher prevalence of vitamin D deficiency in our population, especially since we targeted children who reported one or more risk factors for vitamin D deficiency. There was not a high rate of supplementation in our cohort, which could have explained our lower prevalence of vitamin D deficiency. The possible reasons for this discrepancy may include our location in the sunny Southeast. Another possibility may be due to the presence of 25-hydroxyvitamin D epimers that we could not differentiate from 25-hydroxyvitamin D in our assay. Singh et al. [35], reported that epimers of vitamin D may confound measurement of 25-hydroxyvitamin D in infants, who were highly represented in our sample. The most likely possibility, however, is selection bias in this self-selected study population; families already concerned about vitamin D status would be more likely to participate in such a study. We attempted to control for urine concentration by measuring urine creatinine, however, this may also reflect muscle mass and other variables which we did not quantify and impacted our results. Finally, these were random urine samples; we did not control for fasting state which may impact $U_{ca}/U_{cr.}$[36].

Five percent of the children in the cohort had transient hyperphosphatasemia which is similar to other published reports in a similar age group [34].

The economic burden of vitamin D deficiency is estimated to be large [37,38], while treatment is easy and inexpensive. At this time, serum measurement of vitamin D is the standard for diagnosis, but is costly and population-wide screening is not cost effective. More research is needed to identify cost effective ways to screen children at risk for vitamin D deficiency.

In conclusion, we have found poor correlation between random U_{ca}/U_{cr}, U_{phos}/U_{cr} and 25-hydroxyvitamin D in a small convenience sample. These random urine assays hence offer little promiseas screening tools for vitamin D deficiency in infants and toddlers. Confirmation of these findings is needed in larger groups of children to further support this hypothesis. We also showed that children who are regularly given vitamin D supplements are not likely to be deficient. The incidence of new cases of vitamin D deficiency and rickets remains high worldwide

due in part to poor adherence to supplementation guidelines. Low cost screening tools are still needed to combat this global epidemic.

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