Free Vitamin D: Relationship to Insulin Sensitivity and Vascular Health in Youth

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Objective To evaluate the relationship of free 25 hydroxy vitamin D [free 25(OH)D] or bioavailable vitamin D (BioD) concentrations to insulin sensitivity and cardiovascular disease risk markers in normal weight and overweight youth.

Study design Cross-sectional study of 79 adolescents 15.4 ± 0.2 years, 18 normal weight, 30 overweight, and 31 overweight with prediabetes who underwent peripheral arterial tonometry, dual-energy x-ray absorptiometry, and hyperinsulinemic-euglycemic clamp in subset (n = 71) for determination of reactive hyperemia index (RHI), body composition, and insulin sensitivity. 25(OH)D and vitamin D binding protein were measured; free 25(OH)D and BioD were calculated.

Results Across tertiles of free 25(OH)D concentrations (4.0 ± 0.2, 7.5 ± 0.3, and 17.0 ± 2.1 pg/mL, $P < .001$), the group in the lowest tertile had significantly higher percent body fat (37.8 ± 1.1, 35.2 ± 1.5 and 25.3 ± 2.1%, $P < .001$), lower insulin sensitivity (4.4 ± 0.4, 6.7 ± 1.2, and 8.2 ± 0.9 mg/kg fat-free mass/minute per μu/mL, $P = .03$), lower RHI (1.42 ± 0.06, 1.54 ± 0.06, and 1.77 ± 0.09, $P = .002$), higher high-sensitivity C-reactive protein (3.4 ± 0.6, 1.7 ± 0.3, and 1.6 ± 0.4 mg/L, $P = .015$) compared with the second and third tertiles, respectively. Free 25(OH)D levels were inversely related to percent body fat and high-sensitivity C-reactive protein, and positively related to RHI and insulin sensitivity. The relationships of free 25(OH)D to RHI and to insulin sensitivity were no longer significant after adjusting for %body fat. Similar relationships were observed for BioD.

Conclusions Youth with low free 25(OH)D or BioD concentrations have lower insulin sensitivity and worse endothelial function and inflammatory biomarkers compared with those with more sufficient 25(OH)D. However, the effects of vitamin D on these biomarkers may not be independent of the effect of adiposity. *(J Pediatr 2019;212:28-34)*.

Vitamin D deficiency is highly prevalent in children particularly in overweight and obese children, and in those of minority ethnic background.[1,2] In the National Health and Nutrition Examination Survey 2003-2006 data, one-half of all severely obese children were vitamin D deficient (defined as a 25 hydroxy vitamin D [25(OH)D] level <20 ng/mL).[1]

In children and adolescents, low serum of 25(OH)D concentrations have been associated with systolic blood pressure,[3] and markers of inflammation and oxidative stress,[4] measures of arterial stiffness,[5] and intima media thickness.[6] However, in a recent meta-analysis of 51 trials in adults, supplementation with vitamin D did not demonstrate a significant effect on cardiovascular risk or mortality.[7] The majority of vitamin D in the circulation is bound to vitamin D binding protein (VDBP) (~90%) and loosely to albumin (~10%), with less than 1% circulating as free and unbound [free 25 (OH)D].[8] The serum levels of VDBP can greatly influence the action of the hormone, as the bound fraction is unavailable for target cell action.[9] Recently, VDBP gene polymorphisms were noted to result in alterations in VDBP levels that could explain the racial differences observed in serum total 25(OH)D levels while having similar free vitamin D concentrations.[9] Thus, total serum 25(OH)D levels may inadequately predict vitamin D deficiency, when no other biochemical markers of deficiency are present.[10] Yet, most studies of cardiovascular risk in relation to vitamin D have not taken into account the variation in VDBP. One study suggested that VDBP levels are related to insulin resistance as reflected by the Homeostatic Model Assessment (HOMA) index.[11] In this study, we tested the hypothesis that youth with low levels of free 25(OH)D (or bioavailable vitamin D [BioD]) have evidence of insulin resistance and endothelial dysfunction. Specifically, we aimed to assess the relationship of total, bioavailable, and free 25(OH)D concentrations to endothelial function and cardiovascular disease risk markers in normal weight and overweight youth along the spectrum of glucose regulation.

25(OH)D	25 hydroxy vitamin D	IS_{FFM}	Insulin sensitivity per fat free mass
BioD	Bioavailable vitamin D		
FFM	Fat-free mass	LC-MS/MS	Liquid chromatography mass spectrometry
HbA1c	Glycosylated hemoglobin		
HDL	High-density lipoprotein	OGTT	Oral glucose tolerance test
hs-CRP	High-sensitivity C-reactive protein	PAT	Peripheral arterial tonometry
		PTH	Parathyroid hormone
IFG	Impaired fasting glucose	RHI	Reactive hyperemia index
IGT	Impaired glucose tolerance	VDBP	Vitamin D binding protein

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Supported by the USDA ARS 6250-5100-054 (to F.B.) and Thrasher Research Fund (to F.B.). The authors declare no conflicts of interest.

Portions of this study were presented at the Obesity Society Meeting, November 2015, Los Angeles, CA

https://doi.org/10.1016/j.jpeds.2019.04.057

Methods

Seventy-nine adolescents were evaluated in this cross-sectional study. Glucose tolerance status was defined according to American Diabetes Association criteria after a 2-hour oral glucose tolerance test (OGTT).[12] Normal glucose tolerance was thus defined as glycosylated hemoglobin (HbA1c) <5.7%, fasting glucose <100 mg/dL, and a 2-hour value less than 140 mg/dL during the OGTT; impaired fasting glucose (IFG) as a fasting glucose ≥100 mg/dL, impaired glucose tolerance (IGT) as a 2-hour value ≥140 mg/dL but <200 mg/dL. Prediabetes indicated presence of IFG, IGT, or combined IFG/IGT. Eighteen participants were of normal weight (age- and sex-specific body mass index [BMI] <85th percentile) with normal glucose tolerance. Thirty were overweight (BMI ≥85th percentile) with normal glucose tolerance, 31 were overweight with prediabetes. Participants were recruited through advertisement in the community and the medical center. One hundred participants were screened, and 79 underwent study evaluations. Patients were excluded if they were engaged in scheduled diet or physical activity program, had chronic medical conditions, were taking any medication including dietary supplements, or were smoking. Of the 79 participants studied, 71 agreed to participate in the hyperinsulinemic-euglycemic clamp portion of the study. Procedures were conducted at the metabolic research unit of the Children's Nutrition Research Center at Texas Children's Hospital, and approved by the Institutional Review Board of Baylor College of Medicine. Parental consent and child assent were obtained before any research procedures. Subsequently, participants were scheduled for study visits and presented in fasting state to the metabolic research unit. Anthropometrics and vital signs were obtained and study procedures were performed in this sequence: (1) endothelial function assessment using EndoPAT (Itamar Medical Ltd, Atlanta, Georgia); (2) fasting blood draw and oral glucose tolerance test; (3) body composition assessment; and (4) admission to the inpatient unit for euglycemic clamp procedure the next morning.

Participants underwent a physical examination with determination of pubertal Tanner stage by a pediatric endocrinologist according to the method of Tanner.[13] Height was measured using a wall-mounted Harpenden stadiometer (Holtin Limited, Crymych, United Kingdom). The average of 3 measurements was recorded. Weight was obtained by a digital scale without shoes and in light clothing (Health-O-Meter; McCook, Illinois). BMI was calculated; BMI percentile and z score were defined based on age and sex norms. Blood pressure was measured with an appropriate size cuff using an automated device (Welch-Allyn; Skaneateles, New York) while resting in the morning. The average of 7 measurements taken 10 minutes apart was recorded. Body composition was determined by Hologic dual-energy x-ray absorptiometry to determine measurements of total fat mass, fat-free mass (FFM), and percentage of body fat.

Assessment of endothelial function was performed by peripheral arterial tonometry (PAT) using EndoPAT, in resting state, as the first study evaluation, in a quiet, dimly lit, and temperature- regulated room as previously reported.[14] Briefly, the index fingers are placed into pneumatic probes. Pulse wave is recorded from both fingers before, during and after occlusion of the test arm with a blood pressure cuff inflated to suprasystolic blood pressure cuff for 5 minutes. The reactive hyperemia index (RHI) is calculated as the ratio of the average of the PAT signal starting 1 minute after cuff deflation divided by the average amplitude of the PAT signal of 3.5- minute period before cuff inflation and normalized to the signal from the contralateral unoccluded digit, using operator independent software.

A 2-hour OGTT (1.75 g/kg glucola, maximum 75 g) was performed after a 10- to 12-hour overnight fast. Blood samples were obtained at −15, 0, 15, 30, 60, 90, and 120 minutes for determination of glucose and insulin.

After 10-12 hours of overnight fast, insulin-stimulated glucose metabolism was measured with a 3-hour hyperinsulinemic-euglycemic clamp in a representative subsample of 71 youth of similar age, sex, and ethnic distribution as the total group (of 79 subjects) with 17 normal weight, 26 overweight, and 28 overweight with prediabetes. After baseline fasting blood samples were collected (4 samples every 10 minutes), intravenous crystalline insulin (Humulin, Lilly, Indianapolis, Indiana) was infused at a constant rate of 40 mU/m^2/minute in participants with a BMI <85% and 80 mU/m^2/minute for participants with a BMI ≥85%, as described previously.[14,15] Plasma glucose was measured every 5 minutes using a YSI glucose analyzer (Yellow Springs Instruments, Yellow Springs, Ohio), and a variable rate of dextrose 20% was administered to maintain plasma glucose at 100 mg/dL. Blood was sampled every 10-15 minutes for determination of insulin concentration.

Plasma glucose was measured by the glucose oxidase method with the use of a YSI glucose analyzer (Yellow Springs Instruments). Plasma insulin, intact parathyroid hormone (PTH), and total 25(OH)D levels were measured by electrochemiluminescence assay on the Elecsys 2010 (Roche Diagnostics Corporation, Indianapolis, Indiana). This assay has a strong correlation with liquid chromatography mass spectrometry (LC-MS/MS) with r = 0.85, and detects both 25(OH) vitamin D_3 and 25(OH) vitamin D_2 with high specificity (100% and 92% cross-reactivity, respectively). VDBP was measured using enzyme-linked immunosorbent monoclonal assay (R&D Systems, Minneapolis, Minnesota). The intrassay coefficient of variation (CV) was 6.2% and interassay CV was 7.4% in our laboratory. HbA1c by Roche Tina Quant turbidimetric immunoinhibition and lipid panel by enzymatic methods were measured at Labcorp (Burlington, North Carolina). High-sensitivity C-reactive protein (hs-CRP) was measured by nephelometry at Esoterix Inc (Calabasas Hills, California).

Rate of insulin-stimulated glucose disposal (Rd) is calculated under steady state condition during the last 30 minutes of the hyperinsulinemic-euglycemic clamp as equivalent to the rate of glucose infusion. Insulin sensitivity is calculated

Table I. Subject characteristics across tertiles of free 25(OH)D levels

Subject characteristics	First	Second	Third	*P* value
Age (years)	14.7 ± 0.4	15.7 ± 0.4	16.0 ± 0.3	.05*
Black/Hispanic/white	2/20/4	7/15/5	14/7/5	.003
Season	9/7/4/6	6/8/4/9	6/9/7/4	Ns
Summer/fall/winter/spring				
Sex	10/16	9/18	15/11	Ns
Male/female				
Tanner stage	5/21	3/24	0/26	.07
II-III/IV-V				
Normal weight/overweight-NGT/ overweight-prediabetes	0/16/10	4/9/14	14/5/7	<.001
BMI (kg/m^2)	31.9 ± 1.0	30.5 ± 1.3	26.5 ± 1.2	Ns
BMI percent (%)	96.8 ± 0.7	90.6 ± 3.1	77.7 ± 4.8	.004*
BMI z core	2.01 ± 0.1	1.74 ± 0.2	1.1 ± 0.2	<.001*,†
Fat mass (kg)	33.1 ± 2.0	30.1 ± 2.3	19.9 ± 2.1	.016*
FFM (kg)	51.6 ± 2.0	50.9 ± 2.4	53.7 ± 2.1	Ns
Percent body fat (%)	37.8 ± 1.1	35.2 ± 1.5	25.3 ± 2.1	<.001*,†
Vitamin D and calcium homeostasis measures				
Vitamin D binding protein (ug/mL)	263.2 ± 12.6	168.2 ± 15.1	108.1 ± 8.6	<.001*,†,‡
Total 25(OH)D (ng/mL)	13.1 ± 1.0	15.6 ± 1.4	21.0 ± 1.9	.001*,†
Bioavailable vitamin D (ng/mL)	1.62 ± 0.09	3.03 ± 0.12	7.22 ± 0.9	<.001*,†,‡
Free 25(OH)D (pg/mL)	4.0 ± 0.2	7.5 ± 0.3	17.0 ± 2.1	<.001*,†,‡
Calcium (mg/dL)	9.4 ± 0.1	9.5 ± 0.1	9.4 ± 0.06	Ns
Magnesium (mg/dL)	1.9 ± 0.03	1.9 ± 0.03	1.9 ± 0.03	Ns
Phosphorus (mg/dL)	4.6 ± 0.2	4.3 ± 0.2	4.0 ± 0.1	Ns
PTH (pg/mL)	38.5 ± 2.7	33.6 ± 2.0	36.4 ± 2.1	Ns
Metabolic and CVD markers				
HbA1c (%)	5.6 ± 0.09	5.6 ± 0.07	5.6 ± 0.06	Ns
Fasting glucose (mg/dL)	96.4 ± 2.2	96.4 ± 2.3	93.7 ± 1.3	Ns
Fasting insulin (μu/ml)	30.9 ± 3.1	28.9 ± 4.0	16.4 ± 1.03	.003*,†
Total cholesterol (mg/dL)	151.1 ± 5.0	156.0 ± 5.3	151.0 ± 7.3	Ns
Non-HDL cholesterol (mg/dl)	107.7 ± 5.3	111.0 ± 4.9	101.0 ± 6.7	Ns
Triglycerides (mg/dL)	113.6 ± 12.0	96.1 ± 9.5	73.5 ± 6.0	.015*
HDL (mg/dL)	43.4 ± 1.7	45.0 ± 2.0	49.6 ± 2.7	Ns
Systolic blood pressure (mm Hg)	106.7 ± 1.9	107.2 ± 2.1	107.0 ± 1.8	Ns
Diastolic blood pressure (mm Hg)	67.0 ± 1.2	65.6 ± 1.4	64.2 ± 1.5	Ns
RHI	1.42 ± 0.06	1.54 ± 0.06	1.77 ± 0.09	.003*,†
hs-CRP (mg/L)	3.4 ± 0.6	1.7 ± 0.3	1.6 ± 0.4	.02*
Leptin (ng/mL)	29.6 ± 6.7	27.3 ± 3.9	12.5 ± 2.3	.02*

CVD, cardiovascular disease; *Ns*, non significant. *P* value represents *P* value for χ^2 analysis for categorical variables or the ANOVA (or Kruskal-Wallis) model for continuous variables. Post hoc analysis: *$P < .05$, first vs third tertile; †$P < .05$, second vs third tertile; and ‡$P < .05$, first vs second tertile.

as Rd divided by the average insulin concentration during the last 30 minutes of the clamp and expressed per total body weight (kg) and per metabolically active FFM. Non-high-density lipoprotein (HDL) cholesterol was calculated by subtracting HDL from total cholesterol.[16] Free 25(OH)D was calculated using the mathematical equations of Bikle et al, using measured values of 25(OH)D, VDBP, and albumin.[8] The calculated value from this equation [free 25(OH)D = total 25(OH)D/1 + (6 × 10^3 × albumin) + (7 × 10^8 × VDBP)] was demonstrated to highly correlate (r = 0.925) with measured free 25(OH)D by centrifugal ultrafiltration.[8] BioD refers to the vitamin D bound to albumin as well as free 25(OH)D. BioD was calculated as [Bio D = (Kalb*[albumin] + 1)*free 25(OH)D = (6*10^5*albumin +1)*free 25(OH)D].[9]

Statistical assumptions of normality and variance were checked and the appropriate parametric or nonparametric test was applied. Differences in continuous variables among tertiles of free 25(OH)D groups were determined by ANOVA with post-hoc correction (Bonferroni or Games-Howell based on Levene's test of homogeneity of variance). General linear models (GLM) were applied to examine the effects of free vitamin D tertiles (or total vitamin D sufficiency vs insufficiency) and covariates (race, sex, % body fat, and HbA1c or glycemic status category: normal glucose tolerance vs impaired) on the primary outcome (RHI). Pearson or Spearman correlation tests were used to examine bivariate relationships. We used logarithmic RHI when evaluated as dependent variables in the GLM. All analyses were performed using SPSS (IBM v 24, IBM statistics, Armonk, New York). Data are presented as mean ± SE, and a *P* value of ≤ .05 was considered statistically significant. Our sample size estimate indicated that a total sample size of 60 participants will be needed to detect a difference in RHI among 3 groups with an estimated effect size of 0.5, given $\alpha = 0.05$ and power of 0.8.

Results

Seventy-nine pubertal youth (34 male and 45 female; 42 Hispanic, 23 black, and 14 white), 15.4 ± 0.2 years in age, underwent study evaluations. Subjects' characteristics are detailed

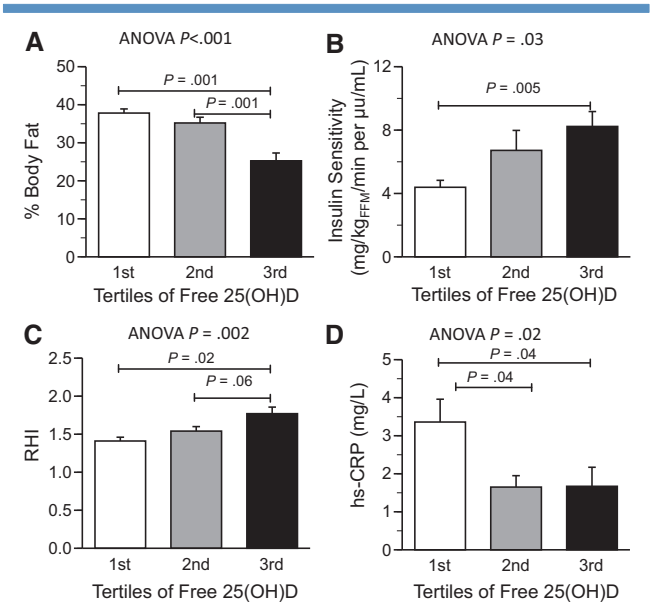

Figure 1. Cardiometabolic risk markers by tertile of free 25 (OH) vitamin D. A, Percent body fat, B, insulin sensitivity, C, reactive hyperemia index (RHI), and D, high sensitivity CRP (hs-CRP).

in **Table I**. Of the 79 subjects, only 17 met the criteria for vitamin D sufficiency, using a cut-off of 20 ng/mL. To evaluate the effect of free 25(OH)D or BioD on outcomes of interest, the subjects were examined according to tertiles of free 25(OH)D (**Table I**), or tertiles of BioD concentrations (**Table II**; available at www.jpeds.com). The 3 groups did not differ in terms of sex, Tanner stage, or season of study. They were all pubertal (Tanner stage II-V). The tertiles of free 25(OH)D differed in race-ethnicity distribution. The group in the lowest tertile of free 25(OH)D had significantly higher BMI percentile, BMI z score, fat mass (kg), and percent body fat (**Figure 1**, A) compared with the second and third (highest) tertiles. The lowest tertile group consisted of overweight individuals with and without prediabetes. Insulin sensitivity data was available in 71 subjects (22 in first tertile of free 25(OH)D, 24 in second tertile, and 25 in the third tertile). The lowest free 25(OH)D tertile had higher fasting insulin, and lower insulin sensitivity per fat free mass (IS_{FFM}) (**Figure 1**, B) compared with the second and third tertile. The difference in insulin sensitivity among tertiles of free 25(OH)D persisted after adjusting for race ($P = .03$) but was no longer significant after further adjusting for percent body fat ($P = .1$) (**Figure 2**; available at www.jpeds.com). Insulin sensitivity was positively related to total ($r = 0.36$, $P = .003$), BioD ($r = 0.32$, $P = .006$), and free 25(OH)D levels ($r = 0.31$, $P = .009$) (**Table III**), with no relationship between insulin sensitivity and VDBP levels. VDBP levels were positively related to percent body fat ($r = 0.27$, $P = .016$) and not to FFM. There was an inverse relationship between free 25(OH)D and BioD with fat mass ($r = -0.41$, $P < .001$) but no relationship between free or BioD with FFM.

RHI was significantly higher in the third free 25(OH)D tertile compared with the other 2 groups and tended to be higher in the third vs the second tertile ($P = .06$ in post-hoc analyses) (**Figure 1**, C). The contribution of free 25(OH)D tertiles to the variance in RHI remained significant after adjusting for race ($P = .02$), but was no longer significant after further adjusting for percent body fat ($P = .2$). Hs-CRP was lower in the second and third tertiles compared with the first tertile of free 25(OH)D (**Figure 1**, D). The difference in hs-CRP among tertiles remained significant after adjusting for race ($P = .02$) and was not significant after adjusting for race and percent body fat ($P = .05$) (**Figure 2**). Triglycerides were highest in the lowest tertile of 25(OH)D compared with the other 2 groups. Total, low-density lipoprotein-cholesterol, non-HDL cholesterol levels, and blood pressure were not significantly different among the 3 tertiles (**Table I**). There was a positive linear relationship between free 25(OH)D levels and RHI ($r = 0.35$, $P = .002$) (**Table III**) and an inverse relationship between free 25(OH)D and hs-CRP ($r = -0.32$, $P = .004$). The relationship of free 25(OH)D with RHI and hs-CRP remained significant after adjusting for race ($r = 0.23$, $P = .047$ and $r = -0.23$, $P = .043$, respectively) but lost significance after additional adjustment for percent body fat (**Table III**). In GLM, with RHI as the dependent variable and sex, race, percent body fat and glycemic status (normal glucose tolerance vs prediabetes as covariates), percent body fat ($P = .07$) and not 25(OH)D contributed to the variance in RHI ($R^2 = 0.22$, $P = .005$).

Given that the majority of youth were vitamin D deficient, we further compared 2 groups with respect to total 25(OH)D levels. RHI was significantly lower in the group with total 25(OH)D less than 20 ng/mL compared with those with vitamin D levels greater than 20 ng/mL (1.50 ± 0.04 vs 1.84 ± 0.13, $P < .013$). This difference persisted after adjustment for percent body fat ($P = .046$). Total 25(OH)D was positively related to IS_{FFM} (as above) and to RHI ($r = 0.24$, $P = .036$) (**Table III**). In GLM, with RHI as the dependent variable and sex, race, %body fat, and glycemic status as covariates, both vitamin D status (sufficient vs deficient) weakly ($P = .06$) and percent body fat ($P = .03$) contributed to the variance in RHI ($R^2 = 0.24$, $P = .001$).

Free 25(OH)D correlated positively with total 25(OH)D ($r = 0.63$, $P < .001$) and inversely with VDBP ($r = -0.53$, $P < .001$).The group in the lowest tertile of free 25(OH)D had higher VDBP and lower serum 25(OH)D and bioavailable 25(OH)D than the second and third (highest) tertile. Calcium, phosphorus, and magnesium were similar among the three groups. PTH levels were within the normal range and not significantly different across the free 25(OH)D tertiles (**Table I**).

Discussion

Our findings of endothelial dysfunction as measured by RHI in the lowest tertiles of free 25(OH)D and in association

Table III. Relationship of total, bioavailable, and free 25(OH)D to RHI, insulin sensitivity, and hs-CRP (unadjusted and adjusted for race and percentage body fat)

	RHI				Insulin sensitivity (mg/kg/min per μu/mL)				hs-CRP (mg/L)			
	Unadjusted		Adjusted		Unadjusted		Adjusted		Unadjusted		Adjusted	
Vitamin D measures	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Total 25(OH)D (ng/mL)	0.24	.036	0.2	.09	0.36	.003	0.28	.02	−0.18	.1	−0.12	-
Bioavailable 25(OH)D (ng/mL)	0.35	.002	0.12	-	0.32	.006	0.03	-	−0.33	.003	−0.13	-
Free 25(OH)D (pg/mL)	0.35	.002	0.14	-	0.31	.009	0.02	-	−0.32	.004	−0.13	-

with total 25(OH)D deficiency vs sufficiency are consistent with findings of a relationship of low vitamin D concentrations with measures of vascular stiffness,[5] intima media thickness,[6] and markers of inflammation[4] in youth. This is also consistent with adult studies suggesting that vitamin D plays an important role in cardiovascular health and is an independent risk factor for cardiovascular disease[17-19] and mortality.[20] Juonala et al demonstrated that childhood vitamin D insufficiency was associated with increased endothelial dysfunction in adulthood,[21] suggesting the potential detrimental effects of vitamin D insufficiency throughout the lifespan. However, other studies in youth showed a positive relationship between bioavailable and free 25(OH)D and augmentation index, a measure of vascular stiffness, but not with flow mediated dilation in postmenarcheal female adolescents, particularly in black female adolescents.[22] The authors attributed this unexpected positive relationship to augmentation index to indicate an effect of vitamin D on wave reflection and not arterial stiffness per se. Our study which included male and female subjects with a wide range of BMI and detailed evaluation of body composition adds to the limited and conflicting data in the literature, and suggests that the effect of vitamin D on insulin sensitivity and measures of vascular function may not be independent of body adiposity.

Most studies use serum 25(OH)D as the measure of vitamin D status, but like many other hormones, vitamin D activity is influenced by the transport proteins.[9] We found that VDBP was related to body fat content with no direct relationship between VDBP with insulin sensitivity, in contrast to another study.[11] The difference could be related to the use of insulin resistance indices in that study vs in vivo measurement of insulin sensitivity by the hyperinsulinemic-euglycemic clamp in our study. The relationship between low total and free 25(OH)D and measures of adiposity is consistent with the decreased bioavailability of vitamin D in obese individuals related to greater fat stores.[23] However, this may not detract from the importance of vitamin D in vascular health, given the consistent findings of an association between circulating vitamin D with markers of vascular dysfunction.[6,24] The results from vitamin D supplementation studies on vascular measures have been inconsistent and interpretation of the findings is hampered by the heterogeneity of the subject populations, duration of therapy and the use of different doses of vitamin D.[25-27] Nevertheless, in a randomized controlled trial, daily 2000 IU vitamin D supplementation led to a slowing of the progression of aortic stiffness (as measured by carotid femoral pulse wave velocity) in black youth.[28] The lack of consistency among studies points to the need for a more unified approach, particularly in dosing. Several studies support the use of larger doses of vitamin D in obese individuals[23] and in pubertal children regardless of race, particularly in the winter months[29] to attain sufficient circulating vitamin D concentrations. Our findings also support the importance of consideration of body composition in the design and interpretation of the results of vitamin D studies.

Our findings do not support the superiority of utilizing free 25(OH)D as a biomarker of vitamin D status rather than total 25(OH)D, as both vary in the same direction and inversely to VDBP levels. Similarly, a large study that compared racial differences in vitamin D metabolism with direct measure of free vitamin D, found that free 25(OH)D reflected total 25(OH)D concentration.[30] Also, total and free 25(OH)D behave similarly in response to vitamin D supplementation,[31] independent of race.[32] Nevertheless, determination of free 25(OH)D may still be useful in examining racial differences in vitamin D metabolism or in conditions of altered binding proteins, as suggested by Aloia et al.[31]

Our study's strengths include examining the relationship of free 25(OH)D levels to insulin sensitivity measured by the hyperinsulinemic-euglycemic clamp and to a direct measure of endothelial function, a surrogate marker of early subclinical atherosclerosis.[33] Our main limitation is the cross-sectional nature of this study, which prevents us from implying causal relationships. Another limitation of the study is the lack of dietary history from the participants. Although none were on vitamin D supplementation or supplements of any kind, we do not have estimates of dietary intake of these nutrients. Our youth were largely sedentary. However, we did not measure physical activity which was related to improvement of vitamin D concentrations in relation to exercise interventions in adults[34] and youth.[35] These improvements were largely related to change in body composition and decrease in fat mass[35] and abdominal fat[34] in response to exercise. The glycemic status of our youth did not significantly contribute to the variance in RHI. This may be related to the absence of more profound

hyperglycemia in youth with recent onset impaired glucose tolerance status. We used the monoclonal antibody DBP assay.[9] This assay has since been reported to be affected by genotype and the use of the polyclonal assay is advocated.[30] LC-MS/MS is the "gold standard" assay for vitamin D measurement. Nevertheless, the electrochemiluminescence assay shows strong correlation to LC-MS/MS derived measures. We did not find significant differences in PTH among the tertiles of free 25(OH)D, likely because the majority of the participants (78%) were in the vitamin D deficient/insufficient range. Although vitamin D concentrations are known to have seasonal variation, the season of study did not differ among the groups evaluated. The Hispanic youth were more likely to have free 25(OH)D in the lowest tertile. Adjustment for race differences among tertiles did not affect our main findings in this study. Nevertheless, it is important to take race into account in the assessment of vitamin D metabolism.

Studies are needed to determine if vitamin D supplementation in youth with 25(OH)D deficiency/insufficiency will improve insulin sensitivity and/or endothelial function, particularly in obese youth. These studies will be important to guide clinical recommendations. ■

We thank the nurses and staff of the Metabolic Research Unit at the Children's Nutrition Research Center, Baylor College of Medicine, and Adam Gillum and Maurice Puyau for help with illustration. We are thankful to the study volunteers and their parents.

Submitted for publication Dec 27, 2018; last revision received Apr 23, 2019; accepted Apr 28, 2019.

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References

1. Turer CB, Lin H, Flores G. Prevalence of vitamin D deficiency among overweight and obese US children. Pediatrics 2013;131:e152-61.
2. Smotkin-Tangorra M, Purushothaman R, Gupta A, Nejati G, Anhalt H, Ten S. Prevalence of vitamin D insufficiency in obese children and adolescents. JPEM 2007;20:817-23.
3. Reis JP, von Muhlen D, Miller ER III, Michos ED, Appel LJ. Vitamin D status and cardiometabolic risk factors in the United States adolescent population. Pediatrics 2009;124:e371-9.
4. Codoner-Franch P, Tavarez-Alonso S, Simo-Jorda R, Laporta-Martin P, Carratala-Calvo A, Alonso-Iglesias E. Vitamin D status is linked to biomarkers of oxidative stress, inflammation, and endothelial activation in obese children. J Pediatr 2012;161:848-54.
5. Jha P, Dolan LM, Khoury PR, Urbina EM, Kimball TR, Shah AS. Low serum vitamin D levels are associated with increased arterial stiffness in youth with type 2 diabetes. Diabetes Care 2015;38:1551-7.
6. Bacha F, Arslanian SA. Race or vitamin D: a determinant of intima media thickness in obese adolescents? Pediatr Diabetes 2017;18:619-21.
7. Elamin MB, Abu Elnour NO, Elamin KB, Fatourechi MM, Alkatib AA, Almandoz JP, et al. Vitamin D and cardiovascular outcomes: a systematic review and meta-analysis. J Clin Endocrinol Metab 2011;96:1931-42.
8. Bikle DD, Gee E, Halloran B, Kowalski MA, Ryzen E, Haddad JG. Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein. J Clin Endocrinol Metab 1986;63:954-9.
9. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, et al. Vitamin D–binding protein and vitamin D status of black Americans and white Americans. N Engl J Med 2013;369:1991-2000.
10. Aloia J, Mikhail M, Dhaliwal R, Shieh A, Usera G, Stolberg A, et al. Free 25(OH)D and the vitamin D paradox in African Americans. J Clin Endocrinol Metab 2015;100:3356-63.
11. Ashraf AP, Huisingh C, Alvarez JA, Wang X, Gower BA. Insulin resistance indices are inversely associated with vitamin D binding protein concentrations. J Clin Endocrinol Metab 2014;99:178-83.
12. American Diabetes A. Standards of medical care in diabetes—2010. Diabetes Care 2010;33(Suppl 1):S11-61.
13. Travers SH, Jeffers BW, Bloch CA, Hill JO, Eckel RH. Gender and Tanner stage differences in body composition and insulin sensitivity in early pubertal children. J Clin Endocrinol Metab 1995;80:172-8.
14. Bartz SK, Caldas MC, Tomsa A, Krishnamurthy R, Bacha F. Urine albumin-to-creatinine ratio: a marker of early endothelial dysfunction in youth. J Clin Endocrinol Metab 2015;100:3393-9.
15. Bacha F, Saad R, Gungor N, Arslanian SA. Adiponectin in youth: relationship to visceral adiposity, insulin sensitivity, and beta-cell function. Diabetes Care 2004;27:547-52.
16. Frost PH, Havel RJ. Rationale for use of non-high-density lipoprotein cholesterol rather than low-density lipoprotein cholesterol as a tool for lipoprotein cholesterol screening and assessment of risk and therapy. Am J Cardiol 1998;81:26B-31B.
17. Artaza JN, Mehrotra R, Norris KC. Vitamin D and the cardiovascular system. CJASN 2009;4:1515-22.
18. Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, et al. Vitamin D deficiency and risk of cardiovascular disease. Circulation 2008;117:503-11.
19. Gunta SS, Thadhani RI, Mak RH. The effect of vitamin D status on risk factors for cardiovascular disease. Nat Rev Nephrol 2013;9:337-47.
20. Fiscella K, Franks P. Vitamin D, race, and cardiovascular mortality: findings from a national US sample. Ann Fam Med 2010;8:11-8.
21. Juonala M, Voipio A, Pahkala K, Viikari JS, Mikkila V, Kahonen M, et al. Childhood 25-OH vitamin D levels and carotid intima-media thickness in adulthood: the cardiovascular risk in young Finns study. J Clin Endocrinol Metab 2015;100:1469-76.
22. Ashraf AP, Alvarez JA, Dudenbostel T, Calhoun D, Griffin R, Wang X, et al. Associations between vascular health indices and serum total, free and bioavailable 25-hydroxyvitamin D in adolescents. PloS One 2014;9:e114689.
23. Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. Am J Clin Nutr 2000;72:690-3.
24. Al Mheid I, Patel R, Murrow J, Morris A, Rahman A, Fike L, et al. Vitamin D status is associated with arterial stiffness and vascular dysfunction in healthy humans. J Am Coll Cardiol 2011;58:186-92.
25. Jamka M, Wozniewicz M, Walkowiak J, Bogdanski P, Jeszka J, Stelmach-Mardas M. The effect of vitamin D supplementation on selected inflammatory biomarkers in obese and overweight subjects: a systematic review with meta-analysis. Eur J Nutr 2016;55:2163-76.
26. Shah S, Wilson DM, Bachrach LK. Large doses of vitamin D fail to increase 25-hydroxyvitamin D levels or to alter cardiovascular risk factors in obese adolescents: a pilot study. J Adolesc Health 2015;57:19-23.
27. Javed A, Vella A, Balagopal PB, Fischer PR, Weaver AL, Piccinini F, et al. Cholecalciferol supplementation does not influence beta-cell function and insulin action in obese adolescents: a prospective double-blind randomized trial. J Nutr 2015;145:284-90.
28. Dong Y, Stallmann-Jorgensen IS, Pollock NK, Harris RA, Keeton D, Huang Y, et al. A 16-week randomized clinical trial of 2000 international units daily vitamin D3 supplementation in black youth: 25-hydroxyvitamin D, adiposity, and arterial stiffness. J Clin Endocrinol Metab 2010;95:4584-91.
29. Rajakumar K, Moore CG, Yabes J, Olabopo F, Haralam MA, Comer D, et al. Estimations of dietary vitamin D requirements in black and white children. Pediatr Res 2016;80:14-20.
30. Nielson CM, Jones KS, Chun RF, Jacobs JM, Wang Y, Hewison M, et al. Free 25-hydroxyvitamin D: impact of vitamin D binding protein assays

on racial-genotypic associations. J Clin Endocrinol Metab 2016;101: 2226-34.
31. Aloia J, Dhaliwal R, Mikhail M, Shieh A, Stolberg A, Ragolia L, et al. Free 25(OH)D and calcium absorption, PTH, and markers of bone turnover. J Clin Endocrinol Metab 2015;100:4140-5.
32. Alzaman NS, Dawson-Hughes B, Nelson J, D'Alessio D, Pittas AG. Vitamin D status of black and white Americans and changes in vitamin D metabolites after varied doses of vitamin D supplementation. Am J Clin Nutr 2016;104:205-14.
33. Bonetti PO, Pumper GM, Higano ST, Holmes DR Jr, Kuvin JT, Lerman A. Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. J Am Coll Cardiol 2004;44:2137-41.
34. Gangloff A, Bergeron J, Pelletier-Beaumont E, Nazare JA, Smith J, Borel AL, et al. Effect of adipose tissue volume loss on circulating 25-hydroxyvitamin D levels: results from a 1-year lifestyle intervention in viscerally obese men. Int J Obes 2015;39:1638-43.
35. Hossain MJ, Levinson A, George D, Canas J, Kumar S, Balagopal PB. Vitamin D status and cardiovascular risk in obesity: effect of physical activity in nonvitamin D supplemented adolescents. Metab Syndr Relat Disord 2018;16:197-203.

Appendix

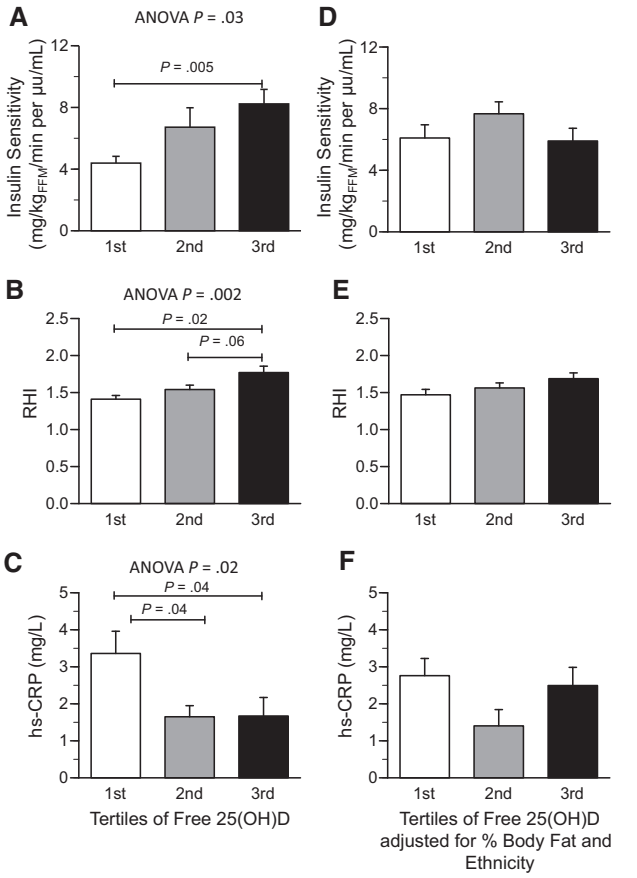

Figure 2. **A, D,** Insulin sensitivity and **B, E,** cardiometabolic risk markers by tertile of free 25(OH)D before (A, B, C) and after adjustment for race and percent body fat (D, E, F).

Table II. Subject characteristics across tertiles of bioavailable 25(OH)D

Subject characteristics	First	Second	Third	*P* value
Age (y)	15.1 ± 0.5	15.1 ± 0.4	16.1 ± 0.3	Ns
Sex	8/18	11/16	15/11	Ns
Male/female				
Percent body fat (%)	37.5 ± 1.3	35.5 ± 1.4	25.2 ± 2	<.001*,†
Vitamin D measures				
VDBP (ug/mL)	271 ± 13.5	163.1 ± 11.9	108.7 ± 8.8	<.001*,†,‡
Total 25(OH)D (ng/mL)	13.8 ± 1.2	15.3 ± 1.3	20.6 ± 1.9	.001*,†
Bioavailable vitamin D (ng/mL)	1.6 ± 0.08	3.1 ± 0.1	6.0 ± 0.3	<.001*,†,‡
Free 25(OH)D (pg/mL)	4.0 ± 0.2	7.6 ± 0.3	14.2 ± 10.7	<.001*,†,‡
Metabolic and CVD Markers				
HbA1c (%)	5.6 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	Ns
Fasting glucose (mg/dL)	95.8 ± 2.3	97 ± 2.2	93.6 ± 1.3	Ns
Fasting insulin (μu/mL)	30 ± 3.1	29.8 ± 4	16.4 ± 1.8	.003*,†
Insulin sensitivity (μu/ml per mg/kg $_{FFM}$/min)	4.5 ± 0.5	5.1 ± 0.9	8.4 ± 0.9	.002*,†
Total cholesterol (mg/dL)	146.8 ± 4.5	157.7 ± 4.9	153 ± 7.8	Ns
Non-HDL cholesterol (mg/dL)	102.2 ± 5.9	109.6 ± 5.7	106.2 ± 8.4	Ns
Triglycerides (mg/dL)	111.5 ± 12.2	95.7 ± 9.2	76.1 ± 6.8	.04*
HDL (mg/dL)	42.8 ± 1.7	45.3 ± 2.1	50 ± 2.7	Ns
Systolic blood pressure (mm Hg)	106.5 ± 2	107.1 ± 2.1	107.3 ± 1.8	Ns
Diastolic blood pressure (mm Hg)	66.5 ± 1.3	65.9 ± 1.4	64.7 ± 1.5	Ns
RHI	1.45 ± 0.06	1.49 ± 0.06	1.78 ± 0.09	.003*,†
hs-CRP (mg/L)	3.3 ± 0.6	1.8 ± 0.3	1.5 ± 0.4	.017*
Leptin (ng/mL)	29.2 ± 6.5	27.6 ± 4	12.4 ± 2.3	.02*

CVD, cardiovascular disease; *Ns*, non significant. Post hoc analysis: *$P < .05$, first vs third tertile; †$P < .05$, second vs third tertile; and ‡$P < .05$, first vs second tertile.