Bioavailable Vitamin D in Obese Children: The Role of Insulin Resistance

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Context: Studies examining vitamin D levels in association with childhood obesity usually do not consider the effect of insulin on vitamin D–binding protein and do not calculate the unbound, bioavailable vitamin D.

Objective: This study aimed to evaluate in a group of children 1) the concentrations of both total 25-hydroxyvitamin D and bioavailable fraction, and 2) the potential role of insulin resistance in modulating the concentrations of bioavailable vitamin D.

Design, Setting, and Patients or Other Participants: This was a cross-sectional study at a University Pediatric Department in which 63 obese children and 21 lean controls were enrolled.

Main Outcome Measures: Total 25-hydroxyvitamin D and vitamin D–binding protein were measured, two single-nucleotide polymorphisms in the coding region of the vitamin D–binding protein (rs4588 and rs7041) were studied, and the vitamin D bioavailable fraction was calculated.

Results: Obese children showed total 25-hydroxyvitamin D levels lower compared with nonobese children (21.3 $\pm$ 6.7 ng/mL vs 29.6 $\pm$ 11.7 ng/mL; $P = .0004$). Bioavailable 25-hydroxyvitamin D levels were not different among the two groups (3.1 $\pm$ 1.6 ng/mL vs 2.6 $\pm$ 1.2 ng/mL; $P > .05$). Insulin-resistant children showed higher bioavailable levels of 25-hydroxyvitamin D compared with non-insulin-resistant children (3.4 $\pm$ 1.4 ng/mL vs 2.0 $\pm$ 0.9 ng/mL; $P = .013$) and an inverse correlation between insulin resistance and vitamin D–binding protein was found (r:= -0.40; $P = .024$).

Conclusions: Obese children present levels of bioavailable 25-hydroxyvitamin D similar to those of normal-weight children due to reduced concentration of vitamin D–binding protein. The insulin resistance could play a role in this reduced concentration. (***J Clin Endocrinol Metab*** **100: 3949–3955, 2015)**

In less than one generation, child obesity prevalence has increased substantially in most high-income countries and seems to be increasing rapidly also in low-income and middle-income countries (1). Vitamin D deficiency is another increasingly prevalent public health concern in many countries and observational studies have reported an increased risk of vitamin D deficiency in those who are obese (2). The nature of this association has been defined in a recent bidirectional Mendelian randomization analysis providing evidence for obesity as a causal factor in the development of vitamin D deficiency but not for vitamin D deficiency as a causal factor in the development of obesity (3). Sequestration of vitamin D by adipose tissue is a conceivable mechanism explaining this finding (4). The results on the role of these reduced levels of vitamin D in the constellation of obesity-related comorbidities such as hypertension, dyslipidemia, insulin resistance, and metabolic syndrome are contrasting (5).

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Abbreviations: BMI, body mass index; BP, blood pressure; CV, coefficient of variation; DBP, vitamin D–binding protein; HOMA-IR, homeostasis model assessment of insulin resistance; WHtR, waist-to-height ratio.

Because levels of 25-hydroxyvitamin D are also consistently low in childhood obesity, obese children are frequently classified as being vitamin D deficient (6–8). The Institute of Medicine recommended 25-hydroxyvitamin D levels as reliable biomarker for assessment of vitamin D status and considered values lower than 20 ng/mL as inadequate or not sufficient and values greater than 20 ng/mL as adequate or sufficient (9). There remains, however, considerable controversy regarding the choice of cutoff for vitamin D sufficiency with the Endocrine Society recommending a cutoff of 30 ng/mL (10).

Prohormone 25-hydroxyvitamin D is a lipophilic molecule that is transported in the circulation mainly bound to vitamin D–binding protein, whereas a small amount is free in the plasma (11). The concentration of unbound 25-hydroxyvitamin D is not only dependent on the concentration of vitamin D–binding protein and of alternative serum binding proteins such as albumin, but is also influenced by variations in vitamin D–binding specific affinity (12).

These variations are due to changes in the vitamin D–binding protein amino acid sequence, which, in turn, reflect single nucleotide polymorphisms (rs7041 and rs4588) of the vitamin D–binding protein gene (12). Bioavailable 25-hydroxyvitamin D refers to all the circulating 25-hydroxyvitamin D that is not bound to vitamin D–binding, in other words the free portion plus the portion bound to albumin. It represents approximately 10% of all circulating 25-hydroxyvitamin D (13, 14).

Recently, Powe et al (15), investigating both Black and White Americans have suggested that measurement of the bioavailable 25-hydroxyvitamin D may provide a better assessment of sufficiency compared with total 25-hydroxyvitamin D. The same Authors have also shown that bioavailable 25-hydroxyvitamin D levels are better correlated with measures of mineral metabolism than total 25-hydroxyvitamin D in patients receiving hemodialysis (14). This is in agreement with the free hormone hypothesis stating that protein-bound hormones are relatively inactive whereas hormones not bound to binding proteins are available to exert biological activity (16, 17).

Considering that past studies examining 25-hydroxyvitamin D levels in association with childhood obesity have not concurrently measured levels of vitamin D–binding protein and calculated bioavailable 25-hydroxyvitamin D fraction, the aim of the present study was to evaluate in a group of both obese and lean children 1) the concentrations of both total 25-hydroxyvitamin D and of the bioavailable fraction 2) the association between total or bioavailable 25-hydroxyvitamin D and the metabolic syndrome, and 3) the potential role of insulin resistance in modulating the concentrations of bioavailable 25-hydroxyvitamin D.

Materials and Methods

Sixty-three Caucasian obese children and adolescents (age ranging from 4–15 y) consecutively referred to the Department of Woman, Child and General and Specialized Surgery of the Second University of Naples (Italy) from January 2014 to March 2014 have been enrolled onto this study, as well as 21 sex- and age-matched lean controls who consulted, in the same period, the Department for presumed diseases and were found to be normal.

Procedures followed were in accordance with the Helsinki Declaration of Principles 1975 as revised in 1983. The ethical committee of the Second University of Study of Naples approved the study. Written informed consent and assent were obtained from all parents before any procedures.

Patients underwent physical examination. Body weight was measured by a balance beam scale, the child being undressed, height was measured by a Harpenden stadiometer and body mass index (BMI) was calculated by dividing the weight by the height squares. *z* scores for BMI were calculated by using the lambda-mu-sigma method (18). Waist circumference was measured with a flexible tape measure while the subjects were standing, after normal expiration, at the midpoint between the lowest rib and the iliac crest.

Waist-to-height ratio (WHtR) was calculated to obtain an age-independent measure of body composition, particularly of abdominal fat deposits (19)

Pubertal stage according to Tanner criteria was assessed. Measurements of systolic and diastolic blood pressure (BP) were taken three times at the left arm, whereas subjects were seated and the mean of the last two measures was used for analysis. All measurements were taken by the same operator.

A blood sample was drawn from each patient at 0800 h after an overnight fast. Triglycerides and high-density lipoprotein cholesterol was measured. Triglyceride levels were determined by an enzymatic colorimetric test with lipid-clearing factor. Immunoreactive insulin was assayed by IMX (Abbott Diagnostics). The mean intra- and interassay coefficients of variation (CV) were 4.7 and 7.2%, respectively. Serum PTH levels were measured with a commercially available chemioluminescence immunoassay (interassay CV, 2.5%). ELISA commercially available kits were used for IL-6 (intra-assay CV, 3.4%; interassay CV, 5.2%) and for adiponectin (intra-assay CV, 4.4%; interassay CV, 5.5%) measurements. Analyses were performed in the same laboratory.

Children and adolescents were defined as obese if the BMI exceeded the 95th percentile for age and sex according to Italian charts (20).

Metabolic syndrome was defined using previously published definition (19). Metabolic syndrome was considered present when the child had three or more of the following criteria: central adiposity (waist circumference $\geq$ 90th percentile for age and sex) (21), triglycerides at least 150 mg/dL (1.7 mmol/l), high-density lipoprotein cholesterol $\leq$ 40 mg/dL (1.03 mmol/l), systolic BP or diastolic BP at least 90th percentile for age, sex, and height (22), fasting plasma glucose at least 100 mg/dL (5.6 mmol/l) or previously diagnosed type 2 diabetes.

Insulin resistance was assessed using the homeostasis model assessment (HOMA) and, according to a recent report (23) were considered as affected by increased insulin resistance children with HOMA greater than 3.

Levels of total 25-hydroxyvitamin D (D2 and D3) were measured by the method of ELISA according to the manufacturer's instructions (DLD Diagnostika, GMBH,) with the intra-assay and interassay coefficients of variation 5 and 7.8%, respectively. Vitamin D–binding protein was also measured by ELISA (R&D Systems,) with the intra-assay and interassay coefficients of variation 5.9 and 6.1%, respectively.

Genomic DNA was extracted from peripheral whole blood with a DNA extraction kit (Promega). Patients were genotyped for two common single-nucleotide polymorphisms in the coding region of the vitamin D–binding protein gene (rs4588 and rs7041) by Taqman allelic discrimination assay on ABI 7900HT Real Time PCR system (Applied Biosystems). Predesigned assay primers and probes were purchased from Applied Biosystems.

Concentrations of bioavailable 25-hydroxyvitamin were calculated in 33 homozygotes, for whom we could use a single genotype–specific binding affinity constant on the basis of the presence of a single vitamin D–binding protein variant according to the equation: $[\text{Bio D}] = [D_{\text{Free}}] + [D_{\text{Alb}}] = (K_{\text{alb}} \cdot [\text{Alb}] + 1) \cdot [D_{\text{Free}}]$ where $[D_{\text{Free}}] = [D_{\text{DBP}}] \div K_{\text{DBP}} \div ([\text{Total DBP}] - [D_{\text{DBP}}])$ (15).

Differences between means relative to total 25-Hydroxyvitamin D, bioavailable 25-Hydroxyvitamin D, and PTH serum levels among different categories of patients were evaluated with the Student *t* test.

Both simple regression and multiple regression analysis, including sex, pubertal stage, and age as covariates were used to correlate total 25-Hydroxyvitamin D, bioavailable 25-Hydrohyvitamin D, and vitamin D–binding protein with BMI *z* scores and HOMA and to correlate vitamin D–binding protein with adiponectin and IL-6. Data are expressed as means and SDs. We considered statistically significant a $P < .05$. Statgraphics Centurion XVII software for Windows (Adalta) was used for all the statistical analyses.

Results

The clinical and biochemical characteristics of the 84 children involved in the study are shown in Table 1. Sixty-three children have a BMI greater than the 95th percentile and are, therefore, obese. Twenty-seven children have the criteria for metabolic syndrome diagnosis.

Concentrations of bioavailable 25-hydroxyvitamin D were calculated in 33 children homozygotes for the two single-nucleotide polymorphisms in the coding region of the vitamin D–binding protein (rs4588 and rs7041) (Table 1) and for whom we were therefore able to use a single genotype–specific binding affinity constant on the basis of the presence of a single vitamin D–binding protein variant. This method has been validated in a previous paper (15), in which calculated bioavalable 25-hydroxyvitamin D concentrations seemed correlated with direct measurement of bioavailable 25-hydroxyvitamin D.

Obese children showed total 25-hydroxyvitamin D levels lower compared with nonobese children (21.3 ± 6.7 ng/mL vs 29.6 ± 11.7 ng/mL; $P = .0004$) (Figure 1). No differences concerning serum PTH levels were found between the two groups of children (14.5 ± 6.7 pg/mL vs 14.7 ± 6.3 pg/mL; $P > .05$). An inverse correlation between BMI *z* score and total 25-hydroxyvitamin D has been found in the whole population investigated (Figure 2A). The correlation remained significant including sex,

Table 1. Clinical, Biochemical, and Genetic Characteristics of the 84 Children Involved in the Study

Characteristics	Statistics
Age, y	10.5 ± 2.7 (4.5–15.8)
Male/Female	46/38
Pre-pubertal	43 (51%)
BMI	28.7 ± 7.4 (15–48.4)
BMI, *z* score	2.3 ± 1.5 (−1.5/+4.1)
WHtR	0.57 ± 0.07
Metabolic syndrome	27 (32%)
Adiponectin, micrg/mL	17.3 ± 5.3
PTH, pg/mL	14.6 ± 6.6
IL-6, pg/mL	9 ± 6.4
HOMA-IR	2.6 ± 1.7 (0.4–8.4)
Total 25(OH)D, ng/mL	22.36 ± 8.7 (7.6–55.3)
DBP, μg/mL	341.9 ± 148.3 (101.1–751.1)
Bioavailable 25(OHD, ng/mL[a]	2.7 ± 1.5 (0.5–6.2)
Patients homozygotes for different DBP genotypes	Gc1S/Gc1S 24 (28.5%) Gc2/Gc2 9 (10.7%)

Abbreviations: Gc1S, rs 7041-G and rs 4588-C; Gc2, rs 7041-T and rs 4588-A.

Values are expressed as mean ± SDs unless otherwise specified.

Ranges or percentages are in brackets.

[a] Bioavailable 25(OH)D has been evaluated in 33 children.

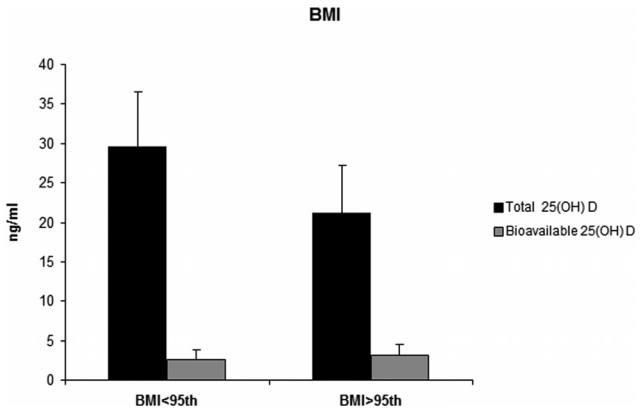

Figure 1. Levels of total and bioavailable 25-hydroxyvitamin D by obesity. Children who were obese (BMI > 95th percentile) had lower levels of total 25-hydroxyvitamin D compared with nonobese chidren, but bioavailable 25-hydroxyvitamin D does not differ among the two groups.

pubertal stage, and age as covariates ($r^2 = 0.17$; $P = .01$). A similar correlation was found between total 25-hydroxyvitamin D levels and abdominal fat expressed by WHtR ($r = -0.26$; $P = .02$). Bioavailable 25-hydroxyvitamin D levels, on the contrary, were not different among obese and not obese children (3.1 ± 1.6 ng/mL vs 2.6 ± 1.2 ng/mL; $P > .05$) (Figure 1) and no correlation was shown between BMI *z* score and bioavailable 25-hydroxyvitamin D (Figure 2B). Levels of both total 25-hydroxyvitamin D

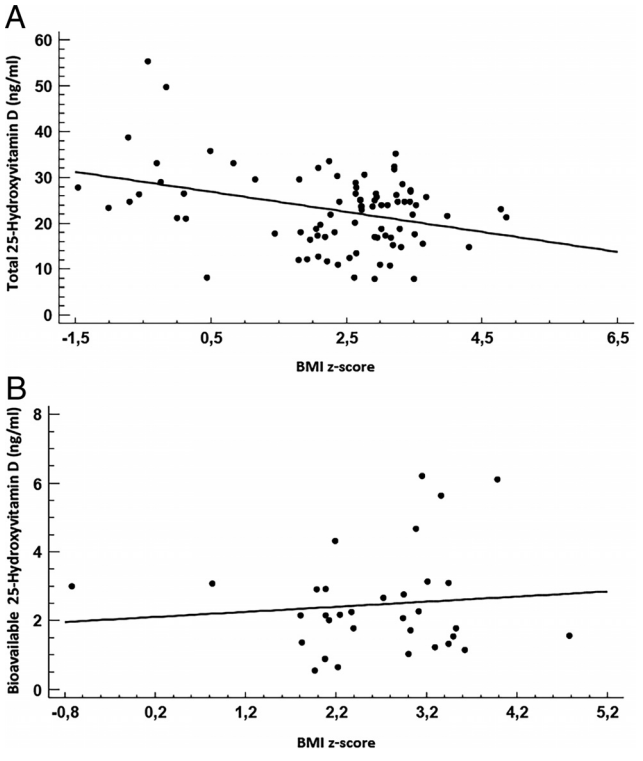

Figure 2. Simple regression correlating, in a group of 84 children, (A) BMI *z* score with total 25-hydroxyvitamin D ($r = -0.35$; $P = .0014$) and, in a subgroup of 33 children, (B) BMI *z* score with bioavailable 25-hydroxyvitamin D ($r = 0.01$; $P > .05$).

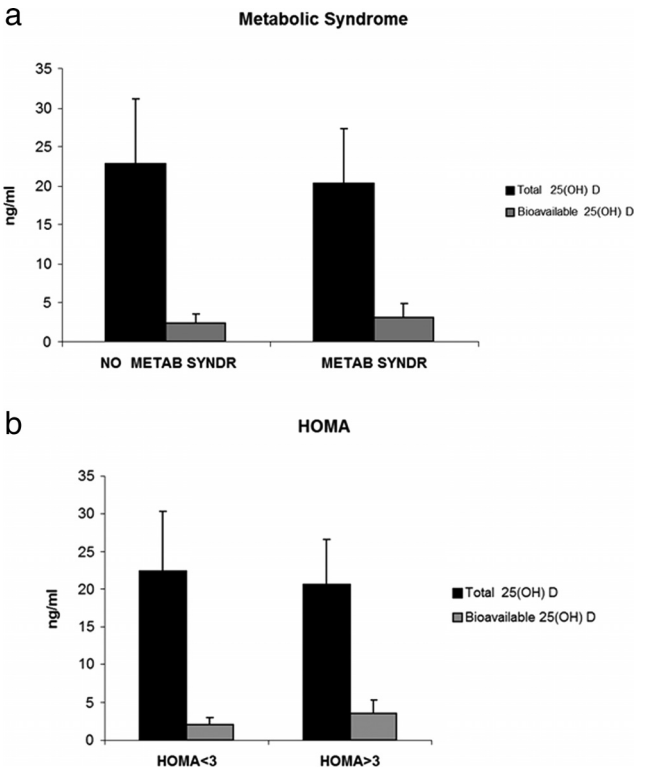

Figure 3. Levels of total and bioavailable 25-hydroxyvitamin D by metabolic syndrome (A). No statistically significant difference are present. Levels of total and bioavailable 25-hydroxyvitamin D by insulin resistance (B). Children with HOMA > 3 have higher levels of bioavailable 25-hydroxyvitamin D, but total 25-hydroxyvitamin D does not differ ($P > .05$).

and bioavailable 25-hydroxyvitamin D were not different in the children with metabolic syndrome compared with the children without metabolic syndrome (Figure 3A). Analyzing the concentrations of total 25-hydroxyvitamin D in the group of children with HOMA less than 3 compared with the children with higher insulin resistance (HOMA > 3) we did not find significant differences (22.2 ± 8 ng/mL vs 20.5 ± 6.4 ng/mL; $P > .05$), whereas insulin-resistant children showed higher bioavailable levels of 25-hydroxyvitamin D compared with noninsulin-resistant children (3.4 ± 1.4 ng/mL vs 2.0 ± 0.9 ng/mL; p: 0.013) (Figure 3B). Furthermore, we found a direct correlation between HOMA and bioavailable 25-hydroxyvitamin D (Figure 4A) and, interestingly, in the same 33 patients, an inverse correlation between insulin resistance and vitamin D–binding protein concentrations (Figure 4B). Both correlations remained significant including sex, pubertal stage, and age as covariates ($r^2 = 0.29$; $P = .02$; and $r^2 = 0.31$; $P = .04$, respectively). A direct correlation was present between vitamin D–binding protein and adiponectin ($r = 0.39$; $P = .02$), whereas no correlation has been found between vitamin D–binding protein and IL-6 ($r = -0.1$; $P > .05$).

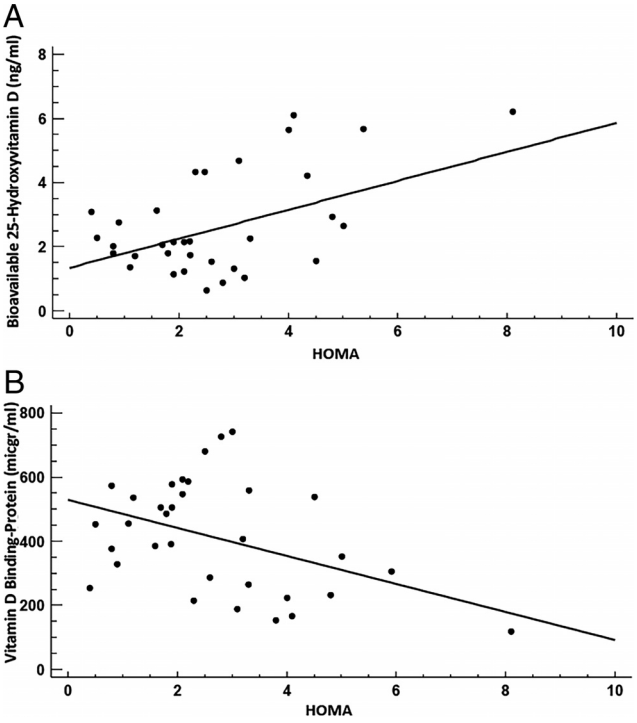

Figure 4. Simple regression showing, in a subgroup of 33 children, (A) a direct correlation between insulin resistance (HOMA) and bioavailable 25-hydroxyvitamin D ($r = 0.49$; $P = .006$) and (B) an inverse correlation between HOMA and vitamin D-binding protein ($r = -0.40$; $P = .024$).

Discussion

Differing from total 25-hydroxyvitamin D, which also in our study is low in obese children, we showed that the levels of bioavailable 25-hydroxyvitamin D in obese children seem to be similar to those in nonobese children.

However, in a study recently performed in humans with a range of clinical conditions (24), calculated that bioavailable 25-hydroxyvitamin D levels varied considerably from direct measurements of free 25-hydroxyvitamin D, these differences were attributed to the lack in the calculation of vitamin D–binding affinity, which, likely contributed to estimation errors. In our study, to obtain a reliable assessment of the portion of bioavailable 25-hydroxyvitamin D we also calculated the vitamin D–binding affinity for each child.

According to the free-hormone hypothesis (16, 17), only hormones liberated from binding proteins are able to enter cells to perform biological actions and, consistently with this hypothesis, some recent studies (13–15) have suggested that many of the biologic actions of vitamin D in humans are inhibited by the binding with the vitamin D–binding protein. This suggests that low total 25-hydroxyvitamin D levels do not uniformly suggest vitamin D deficiency. Like thyroid hormone-binding globulin and SHBG (25), vitamin D–binding protein may act as a serum carrier and reservoir, prolonging the circulating half-life of 25-hydroxyvitamin D while at the same time regulating its immediate bioavailability to target tissues (11). Thus, hormonal activity and sufficiency may be reflected by the amounts of bioavailable vitamin, not by total serum levels.

The finding of comparable levels of PTH despite different levels of total 25-hydroxyvitamin D in the two groups of obese and nonobese children may strengthen this concept.

The reduction in total 25-hydroxyvitamin D we have observed in obese children is counterbalanced by a parallel reduction of vitamin D–binding protein, driven by insulin resistance, which is usually increased in obese subjects. This leads to concentrations of bioavailable 25-hydroxyvitamin D in insulin-resistant children higher than those found in children with low levels of insulin resistance.

Therefore, low levels of vitamin D–binding protein in obese insulin resistant children may give protection against the manifestation of vitamin D deficiency despite low levels of total 25-hydroxyvitamin D.

In accord to our data, a recent report has shown that vitamin D–binding protein was lower in obese adolescents compared with those of normal weight and there was an inverse relationship between insulin levels and vitamin D–binding protein, even when corrected for adiposity (26). It was, therefore, suggested that insulin might suppress the production of vitamin D–binding protein (26).

Most studies found that low total 25-hydroxyvitamin D levels in obese subjects were associated with cardiometabolic risk factors, elevated insulin resistance, and the presence of metabolic syndrome (27–29), but several did not find such associations (30, 31). Many of these studies are observational and only document an association without causality, despite attempt to control for known confounders (5). Also, the results of the few studies reporting the effectiveness in improving metabolic parameters and insulin resistance of vitamin D supplementation in obese children and adolescents are contrasting (32–35). The notion of bioavailable 25-hydroxyvitamin D may help to look at these contrasting reports using a different perspective.

To better understand the crucial role of insulin resistance in modulating vitamin D–binding protein concentration and, therefore, vitamin D bioavailability, we have evaluated the association of IL-6, a biochemical marker of adipose tissue inflammation, with the vitamin D–binding protein levels. No association was found. A direct correlation, on the contrary, was found between vitamin D–binding protein and adiponectin. The combined evaluation of these data suggests that the more that inflammation may be hyperinsulinemia itself to affect vitamin D–binding protein concentrations. Additional studies

with different approaches will be useful for understanding the intriguing relationship between insulin resistance and vitamin D–binding protein.

Limitations

First, sample size, particularly considering that bioavailable 25-hydroxyvitamin D has been evaluated in 33 of 84 children, is limited and may adversely affect the power of our analysis. Therefore, the lack of finding a significant association between bioavailable 25-hydroxyvitamin D and obesity as well as between total 25-hydroxyvitamin D and metabolic syndrome might be due to the relatively small sample size. We must consider, however, that the sample size has been enough to put in evidence the association between total 25-hydroxyvitamin D and obesity.

Second, a longitudinal study design would have allowed for a better evaluation of the link between 25-hydroxyvitamin D bioavailable and insulin resistance.

Conclusion

In conclusion, our data 1) show that obese children, although having low concentrations of total 25-hydroxyvitamin D, present levels of bioavailable 25-hydroxyvitamin D similar to those of normal-weight children; 2) demonstrate that this finding is due to a reduced concentration of vitamin D–binding protein found in obese children; and 3) suggest that the increased insulin resistance usually present in obesity may be associated to this reduction. As a result, these data may provide a different point of view for understanding the vitamin D status in childhood obesity.

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References

1. **Lobstein T, Jackson-Leach R, Moodie ML, et al.** Child and adolescent obesity: Part of a bigger picture. *Lancet*. 2015;385(9986):2510–2520.
2. **Earthman CP, Beckman LM, Masodkar K, Sibley SD.** The link between obesity and low circulating 25-hydroxyvitamin D concentrations: Considerations and implications. *Int J Obes*. 2012;36:387–396.
3. **Vimaleswaran KS, Berry DJ, Lu C, et al.** Causal relationship between obesity and vitamin D status: Bi-directional Mendelian randomization analysis of multiple cohorts. *PLoS Med*. 2013;10:e1001383.
4. **Blum M, Dolnikowski G, Seyoum E, et al.** Vitamin D(3) in fat tissue. *Endocrine*. 2008;33:90–94.
5. **Rosen CJ, Adams JS, Bikle DD, et al.** The nonskeletal effects of vitamin D: An Endocrine Society scientific statement. *Endocr Rev*. 2012;33:456–492.
6. **Dong Y, Pollock N, Stallmann-Jorgensen IS, et al.** Low 25-hydroxyvitamin D levels in adolescents: Race, season, adiposity, physical activity, and fitness. *Pediatrics*. 2010;125:1104–1111.
7. **Kumar J, Muntner P, Kaskel FJ, Hailpern SM, Melamed ML.** Prevalence and associations of 25-hydroxyvitamin D deficiency in US children: NHANES 2001–2004. *Pediatrics*. 2009;124:362–370.
8. **Reis JP, von Mühlen D, Miller ER 3rd, Michos ED, Appel LJ.** Vitamin D status and cardiometabolic risk factors in the United States adolescent population. *Pediatrics*. 2009;124:371–379.
9. **Ross AC, Manson JE, Abrams SA, et al.** The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: What clinicians need to know. *J Clin Endocrinol Metab*. 2011;96:53–58.
10. **Holick MF, Binkley NC, Bischoff-Ferrari HA, et al.** Evaluation, treatment, and prevention of vitamin D deficiency: An Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2011;96:1911–1930.
11. **Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M.** Vitamin D and DBP: The free hormone hypothesis revisited. *J Steroid Biochem Mol Biol*. 2014;144A:132–137.
12. **Arnaud J, Constans J.** Affinity differences for vitamin D metabolites associated with the genetic isoforms of the human serum carrier protein (DBP). *Hum Genet*. 1993;92:183–188.
13. **Powe CE, Ricciardi C, Berg AH, et al.** Vitamin D-binding protein modifies the vitamin D-bone mineral density relationship. *J Bone Miner Res*. 2011;26:1609–1616.
14. **Bhan I, Powe CE, Berg AH, et al.** Bioavailable vitamin D is more tightly linked to mineral metabolism than total vitamin D in incident hemodialysis patients. *Kidney Int*. 2012;82:84–89.
15. **Powe CE, Evans MK, Wenger J, et al.** Vitamin D-binding protein and vitamin D status of black Americans and white Americans. *N Engl J Med*. 2013;369:1991–2000.
16. **Mendel CM.** The free hormone hypothesis. Distinction from the free hormone transport hypothesis. *J Androl*. 1992;13:107–116.
17. **Hammond GL.** Access of reproductive steroids to target tissues. *Obstet Gynecol Clin North Am*. 2002;29:411–423.
18. **Cole TJ.** The LMS method for constructing normalized growth standards. *Eur J Clin Nutr*. 1990;44:45–60.
19. **Santoro N, Amato A, Grandone A, et al.** Predicting metabolic syndrome in obese children and adolescents: Look, measure and ask. *Obes Facts*. 2013;6:48–56.
20. **Cacciari E, Milani S, Balsamo A, et al.** Italian cross-sectional growth charts for height, weight and BMI (2 to 20 yr). *J Endocrinol Invest*. 2006;29:581–593.
21. **Fernández JR, Redden DT, Pietrobelli A, Allison DB.** Waist circumference percentiles in nationally representative samples of African-American, European-American, and Mexican-American children and adolescents. *J Pediatr*. 2004;145:439–444.
22. **Rosner B, Prineas RJ, Loggie JM, Daniels SR.** Blood pressure nomograms for children and adolescents, by height, sex, and age, in the United States. *J Pediatr*. 1993;123:871–886.
23. **Shashaj B, Luciano R, Contoli B, et al.** Reference ranges of HOMA-IR in normal-weight and obese young Caucasians. *Acta Diabetol*. 2015;[Epub ahead of print].
24. **Schwartz JB, Lai J, Lizaola B, et al.** A comparison of measured and calculated free 25(OH) vitamin D levels in clinical populations. *J Clin Endocrinol Metab*. 2014;99:1631–1637.
25. **Vermeulen A, Verdonck L, Kaufman JM.** A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab*. 1999;84:3666–3672.
26. **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;99:178–183.
27. **Gagnon C, Lu ZX, Magliano DJ, et al.** Low serum 25-hydroxyvitamin D is associated with increased risk of the development of the metabolic syndrome at five years: Results from a national, population-based prospective study (The Australian Diabetes, Obesity and Lifestyle Study: AusDiab). *J Clin Endocrinol Metab*. 2012;97:1953–1961.
28. **Olson ML, Maalouf NM, Oden JD, White PC, Hutchison MR.** Vitamin D deficiency in obese children and its relationship to glucose homeostasis. *J Clin Endocrinol Metab*. 2012;97:279–285.
29. **Kayaniyil S, Harris SB, Retnakaran R, et al.** Prospective association of 25(OH)D with metabolic syndrome. *Clin Endocrinol*. 2014;80:502–507.
30. **de las Heras J, Rajakumar K, Lee S, Bacha F, Holick MF, Arslanian SA.** 25-Hydroxyvitamin D in obese youth across the spectrum of glucose tolerance from normal to prediabetes to type 2 diabetes. *Diabetes Care*. 2013;36:2048–2053.
31. **Boonchaya-anant P, Holick MF, Apovian CM.** Serum 25-hydroxyvitamin D levels and metabolic health status in extremely obese individuals. *Obesity*. 2014;22:2539–2543.
32. **Belenchia AM, Tosh AK, Hillman LS, Peterson CA.** Correcting vitamin D insufficiency improves insulin sensitivity in obese adolescents: A randomized controlled trial. *Am J Clin Nutr*. 2013;97:774–781.
33. **Wongwiwatthananukit S, Sansanayudh N, Phetkrajaysang N, Krittiyanunt S.** Effects of vitamin D(2) supplementation on insulin sensitivity and metabolic parameters in metabolic syndrome patients. *J Endocrinol Invest*. 2013;36:558–563.
34. **Kelishadi R, Salek S, Salek M, Hashemipour M, Movahedian M.** Effects of vitamin D supplementation on insulin resistance and cardiometabolic risk factors in children with metabolic syndrome: A triple-masked controlled trial. *J Pediatr (Rio J)*. 2014;90:28–34.
35. **Nader NS, Aguirre Castaneda R, Wallace J, Singh R, Weaver A, Kumar S.** Effect of vitamin D3 supplementation on serum 25(OH)D, lipids and markers of insulin resistance in obese adolescents: A prospective, randomized, placebo-controlled pilot trial. *Horm Res Paediatr*. 2014;82:107–112.