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Research paper

Decreased circulating 25-(OH) Vitamin D concentrations in obese female children and adolescents: Positive associations with Retinol Binding Protein-4 and Neutrophil Gelatinase-associated Lipocalin

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ABSTRACT

OBJECTIVE: Hypovitaminosis D has been associated with adult as well as childhood obesity. Retinol-binding-protein-4 (RBP-4) and Neutrophil Gelatinase-associated Lipocalin (NGAL) are altered in obese individuals. The aim of this study was to examine circulating 25-(OH) Vitamin D (25-(OH) D) concentrations according to BMI and its associations with RBP-4 and NGAL in female children and adolescents. DESIGN: Seventy-nine (79) children, aged 8-16 years, were studied and divided into four groups: 19 control (BMI z-score range -2.15 - 1.24), 20 overweight (1.34 – 2.49), 20 obese (2.50 – 2.87) and 20 ultra-obese (3 - 4.37). Patients were derived from a Pediatric Obesity Clinic. Plasma 25-(OH) D, RBP-4 and NGAL concentrations were measured with specific assays. RESULTS: Plasma 25-(OH) D concentrations were decreased significantly in the ultra-obese (p=0.005) and marginally in the obese group (p=0.05) compared to the control group. In the entire BMI range, Spearman correlations revealed strong positive associations between 25-(OH) D and RBP-4 (r=0.349, p=0.002) and between 25-(OH) D and NGAL (r=0.338, p=0.003). CONCLUSIONS: 25-(OH) D is deficient in a clinical population of obese female children and adolescents, whereas in the entire BMI range 25-(OH) D is associated with RBP4 and NGAL concentrations. Longitudinal studies are needed to reveal the role of these associations in metabolic alterations related to childhood and adolescent obesity and associated metabolic morbidities.

Key words: Children, 25-(OH) D, NGAL, Obesity, RBP-4, Vitamin-D

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INTRODUCTION

Childhood obesity, which has doubled over the last 20 years and is now an epidemic, is one of the greatest public health challenges of the 21st century.[1] Obese children and adolescents, especially those with central adiposity, are more likely to develop glucose intolerance, insulin resistance, dyslipidemia as well as high blood pressure, conditions that comprise the metabolic syndrome. The above comorbidities have been linked to an increased risk for diabetes type 2 and cardiovascular disease in later life.[2]

Obesity has further been associated with 25-hydroxy Vitamin D [25-(OH) D] deficiency. Serum 25-(OH) D, which is the reflection of the 25-(OH) D status in the body, is low in obese individuals,[3,4,5] occasionally with a consequent rise in serum PTH and secondary hyperparathyroidism.[6] It has been shown that there is an increased level of 25-(OH) D breakdown in subcutaneous tissues compared to the skin, suggesting decreased bioavailability of 25-(OH) D in such tissues and, therefore, in the circulation of obese individuals.[7] An inverse association between 25-(OH) D and body fat is evident in various studies of different populations of obese adults.[8,9]

25-(OH) D in obese individuals is thought to be stored in body fat compartments resulting in its decreased bioavailability in the serum and its circulatory deficiency.[10,11] 25-(OH) D stimulates the metabolism of fatty acids and suppresses lipogenesis. Body composition analysis in relation to all areas of adipose tissue – subcutaneous and visceral – demonstrates an independent, negative association between amount of adipose tissue and 25-(OH) D levels.[11] Similar results were observed in cohorts of obese children and adolescents in whom the prevalence of low 25-(OH) D increases with age.[12] It seems that the western lifestyle, in which outdoor activities have been replaced by sedentary indoor behaviors (such as television and computer games), results in reduced sunlight exposure, which can further decrease 25-(OH) D production, especially among obese adolescents. Seasonal variation, especially winter time, has recently been included as a risk factor for vitamin D deficiency, as highlighted by Tolppanen et al.[13]

Apart from 25-(OH) D, there is evidence that Retinol Binding Protein-4 (RBP-4)[14] and Neutrophil Gelatinase-associated Lipocalin (NGAL),[15] which belong to the lipocalin protein family, are associated with insulin resistance[16,17] and metabolic syndrome.[18] Impaired glucose tolerance states, such as obesity, have been associated with elevated RBP-4 concentrations. We have previously shown that these two proteins have an unexpectedly inverse relation to Body Mass Index (BMI) in young individuals as opposed to adults.[19]

The aim of the current study was to compare circulating 25-(OH) D concentrations between groups of overweight, obese and ultra-obese children in comparison to a control group of healthy, normal weight children. We have previously presented RBP-4 and NGAL concentrations in the same cohort of children and we used these data also here.[19] Furthermore, we aimed to investigate associations between 25-(OH) D with RBP-4 and NGAL concentrations. Since many adipokines (such as adiponectin and leptin) show sexual dimorphism and low concentrations of RBP4, NGAL and 25-(OH) D have been reported mainly in women,[20] we decided to examine only female children and adolescents.

PARTICIPANTS AND METHODS

Participants and methods as well as the allocation in groups have been previously described in detail.[19] The study was approved by the Ethics Committee of the "Aghia Sophia" Children's Hospital and was performed according to the Helsinki Declaration.[21] Written informed consent was obtained from the participants and their parents. Inclusion criteria were: children and adolescents 8-16 years old, assessed at the Pediatric Obesity Clinic. Exclusion criteria were: 1) underlying chronic illnesses (cardiac, hepatic, renal); 2) chronic use of medications; 3) syndromic obesity; 4) intellectual disabilities or psychiatric disorders; and 5) chromosomal disorders. Children with obesity-related clinical or metabolic alterations were not excluded from the study. The terms "overweight" and "obese" were defined according to the most recent Greek BMI charts[14] (BMI = weight in kilograms divided by height squared in meters), which is amended for child and adolescent obesity. BMI z-scores were calculated based on the Greek growth charts.[22] All girls underwent a complete physical examination to determine weight, height and BMI, blood pressure

and pubertal status (based on Breast Tanner Stage) by a certified pediatrician. Girls in Tanner stage 1 were classified as prepubertal, whereas girls in Tanner stages 2-5 were classified as pubertal.

In this study, a local population of 79 patients, Greek girls aged 8-16, was examined and was stratified in 4 subgroups according to their BMI z-score: a) control group (-2.15-1.24), b) overweight group (1.34 – 2.49), c) obese group (2.50 – 2.87) and d) ultra-obese (3-4.37).

The blood samples for all measurements were collected in the overnight fasting condition during a scheduled morning visit, at 8.00-9.00 a.m., over 1 calendar year. There was no seasonal variation in blood sampling. The blood was centrifuged and the serum obtained was stored at -85^{0}C. Fasting glucose and insulin levels were measured with Siemens Advia 1800 Clinical Chemistry System (Siemens Medical Solutions, Tarrytown, NY, USA) and the automated chemiluminescence Siemens ACS180 System (Siemens Medical Solutions, Tarrytown, NY, USA), respectively. The Homeostasis assessment model (HOMA) according to the formula HOMA=Fasting Insulin (mIU/ml) x Fasting glucose (mmol/L)/ 22.5 was used as a marker of insulin resistance. RBP4 was measured with a solid-phase ELISA technique (Immundiagnostik, AG Bensheim, Germany) and NGAL was determined by a solid phase ELISA technique (R&D Systems, Minneapolis, MN, USA). CVs for RBP-4 are 5% and 9.7%, whereas for NGAL 3.1-4.1% and 5.6 to 7.9%, respectively. 25-(OH) D3 was determined using Elecsys 25-OH-D(3) assay (Roche), a direct electrochemiluminescence immunoassay which employs a polyclonal antibody against 25-(OH) D3 using the Roche e411 Cobas immunoassay analyzer (Roche Diagnostics, Mannheim, Germany). CVs for VitD are 5.4-5.7 % and 6.9-9.9 %. Our trust provides internal quality audits. All of the 25-(OH) D samples with minimum value 4ng/ml are tested twice. In general, epidemiological studies in the Greek population have shown low 25-(OH) D concentrations in the entire BMI range.[10]

STATISTICAL ANALYSIS

Continuous variables are presented as mean ± standard deviation (SD), or median and interquartile range (25th and 75th percentile) in cases of skewed distributions, while categorical variables are presented as percentages. The level of significance was set to ≤0.05. Comparisons of means were performed by Mann-Whitney and Kruskall-Wallis tests. Spearman tests were used for correlations. Log transformation of selected variables was performed, but no additional information was revealed. The effect of age, NGAL, RBP-4 and BMI z-score in relation to 25-(OH) D was examined, using linear regression. The statistical tools used were SAS version 9.2 and SPSS version 18 for Windows 7.

RESULTS

As described previously, participants included female patients aged 8 to 16 years who were divided into 4 subtypes according to their BMI z-score: 20 overweight (BMI z-score 1.34-2.49), 20 obese (2.50-2.87), 20 ultra-obese (also referred to as extremely obese) (>3) and 20 healthy lean matched in age and gender as a control group (<1.24) [Table 1]. Population clinical characteristics and family history are presented in Table 1. Concentrations of 25-(OH) D did not differ between pubertal and prepubertal children. (Kruskal Wallis p value = 0.9).

Statistical analysis showed that the distribution as well as the median of 25-(OH) D is different amongst

Table 1. Population Characteristics

	Control	Overweight	Obese	Ultra-Obese
Number	(24.1%)	(25.3%)	(25.3%)	(25.3%)
Breast Tanner Stage (BTS) I/ II-V (BTS I %)	3/17 (15%)	3/17 (15%)	3/17 (15%)	2/18 (10%)
BMI z score range	<1.24	1.34 – 2.49	2.50-2.87	>3
Age (range) in years	9 – 14	9 – 14	9 – 16	8 – 14
1st degree relative with DM type 2	5%	5%	10%	40%
2nd degree relative with DM type 2	20%	25%	55%	65%

the four groups. Lower levels of 25-(OH) D were found in the most obese patients of our sample. Figure 1 displays the median 25-(OH) D by BMI category across the four groups. RBP4, NGAL, 25-OH-D and PTH across the four groups are calculated as median (25^{th} – 75^{th} percentile) and are presented in Table 2.

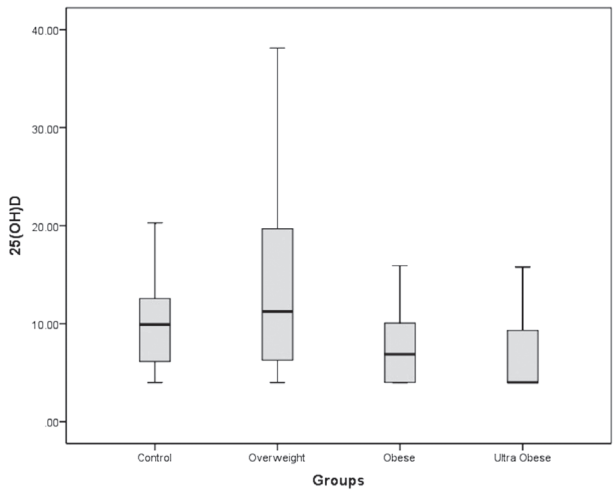

Figure 1. Boxplot of 25-(OH) D (ng/ml) by BMI category.

Table 2. Circulating concentrations of 25-(OH) D, PTH, RBP4, NGAL in Control, Overweight, Obese and Ultra-Obese groups. p value of Mann-Whitney test

	Control	Overweight	Obese	Ultra-Obese
25-(OH) D (ng/ml)	Q_1 =5.8 M = 9.9 Q_3=13.0	Q_1 = 5.9 M = 11.3 Q_3= 20.5 (p=0.643)	Q_1 = 4.0 M = 5.4 Q_3 = 10.6 (p=0.05)	Q_1= 4.0 M = 4.0 Q_3 = 9.7 (p=0.005)*
PTH (pg/ml)	Q_1=18.1 M=26.4 Q_3=34.6	Q_1 = 19.7 M = 23.6 Q_3 = 32.0 (p=0.518)	Q_1 = 19.7 M = 26.0 Q_3= 38.5 (p=0.693)	Q_1 = 24.0 M = 32.5 Q_3 = 38.7 (p=0.439)
RBP-4 (mg/L)	Q_1=18.7 M= 23.8 Q_3=28.7	Q_1= 22.6 M = 26.9 Q_3 = 30.9 (p=0.182)	Q_1 = 18.6 M = 26.3 Q_3 = 29.6 (p=0.955)	Q_1 = 16.3 M = 18.8 Q_3= 22.7 (p=0.01)*
NGAL (μg/L)	Q_1=14.0 M= 18.2 Q_3=32.2	Q_1 = 5.9 M = 13.7 Q_3 = 28.7 (p=0.369)	Q_1= 5.1 M = 11.1 Q_3 = 17.4 (p=0.025)*	Q_1 = 5.2 M = 8.8 Q_3 = 23.2 (p=0.116)

Q_1: 25^{th} percentile; M: median; Q_3: 75^{th} percentile; p: p value of Mann-Whitney test between control group and overweight, obese, ultra-obese groups

* statistically significant

As indicated in Table 3, fasting glucose concentrations were within the normal range in each group, and similar between groups. Fasting insulin concentrations were significantly higher in the ultra-obese group than in the control group. Similarly, insulin resistance (as reflected by HOMA index) was significantly increased in the obese and the ultra-obese groups compared to the control group.

Our data did not reveal significant correlation between BMI z-score and 25-(OH) D overall. PTH correlated with neither BMI z-score nor 25-(OH) D.

The Kruskal-Wallis test showed that the medians of 25-(OH) D by BMI range are different in the groups examined (p=0.006). The Mann-Whitney U-test for 25-(OH) D demonstrated significant differences between the control and ultra-obese groups (p=0.005). The same holds for the control and obese groups (p=0.05), however, a larger sample is required to provide more evidence, as the level of significance is close to 0.05.

Linear associations between RBP-4 and 25-(OH) D in the entire BMI range as well as between NGAL and 25-(OH) D are explored through Pearson's correlation coefficient (r=0.277, p=0.015), (r=0.4, p<0.001), respectively. Indeed, Spearman's correlation showed positive monotonic relations between 25-(OH) D and RBP4 (r=0.349, p=0.002), as well as between 25-(OH) D and NGAL (r=0.338, p=0.003).

The low level of detection for 25-(OH) D is 4 ng/ml. Epidemiological studies in the Greek population have shown low 25-(OH) D concentrations in the entire BMI range.[10]

Both RBP4 and NGAL are positively correlated with 25-(OH) D. Using linear regression, 25-(OH) D

Table 3. Fasting glucose, fasting insulin and HOMA index in control, overweight, obese and ultra-obese groups

	Control	Overweight	Obese	Ultra-Obese
Fasting glucose (mg/dL)	84±2	81±1	84±2	82±2
Fasting insulin (mIU/L)	11.6±1.7	14.4±1.4	15.4±1.5	18.6±2.1**
HOMA index	1.99±0.4	2.89±0.3	3.06±0.4*	3.41±0.4*

*p<0.01, **p<0.001

was shown to be positively affected by the two proteins RBP-4 and NGAL and negatively affected by age. NGAL and RBP4 had a strong positive relation with 25-(OH) D, whereas age had a negative one, as depicted in Table 4. The association of 25(OH) D with BMI z-score was not significant (p-value=0.682).

Table 4. Association between 25-(OH) D, age, RBP-4 and NGAL

		95% Confidence Interval		
25-(OH) D	**Unstandardized Coefficients**	**Lower Bound**	**Upper Bound**	**p**
(Constant)	16.461	**5.784**	**2.846**	0.006
RBP-4	0.311	**0.124**	**2.507**	0.014
NGAL	0.139	**0.046**	**3.023**	0.003
Age	-1.393	**0.454**	**-3.069**	0.003

DISCUSSION

We investigated the impact of BMI elevations on circulating 25-(OH) D and associations of this hormone with RBP-4 and NGAL (previously measured) in female obese children and adolescents. We found that 25-(OH) D was lower in ultra-obese young females than in controls, and that 25-(OH) D concentrations were significantly associated with RBP-4 and NGAL.

There are many adult studies on 25-(OH) D deficiency and its effects, whereas only a few studies have been published conducted in children. In adults, weight loss of about 10% may raise levels of 25-OH-D and this increment seems to correlate with improved insulin resistance.[23,24] 25-(OH) D deficiency has also been linked to insulin resistance and the metabolic syndrome. Previous studies suggested that the impact of 25-(OH) D deficiency on insulin resistance could not be attributed entirely to obesity.[25] Studies of Pacifico et al. demonstrated that adiposity and hypertension were inversely related to low levels of 25-(OH) D.[25] In a recent study, Chrousos et al. determined a positive correlation between adiponectin and 25-(OH) D, suggesting that the latter is a potential link between insulin resistance and diabetes mellitus type 2.[26] Computed tomography (CT) imaging, which allows reliable characterization of subcutaneous and visceral adipose tissue, suggests that levels of 25-(OH) D may be related to variation in regional adiposity.[27] There are many adult studies[28] to support the inverse relations between 25-(OH) D and BMI, proposing that 25-(OH) D is stored in large adipose tissue deposits and therefore its bioavailability is reduced.[29]

There is evidence to support the hypothesis that in obese individuals, an increased volume of distribution of 25-(OH) D is due to its sequestration in hypertrophic adipose tissue.[30] As suggested by Olson et al., an increased clearance of 25-(OH) D may be due to increased uptake by adipose tissue,[30] which may also promote the production of active metabolite vitamin D of 1,25-(OH) 2D and therefore have a negative effect on hepatic synthesis of 25-(OH) D.[31] Mansour et al.[32] showed that older children are more prone to develop 25-(OH) D deficiency than younger ones (P=0.000) and that the intake of 25-(OH) D decreases with increasing age, as found also in other studies.[33] This is latter view, however, disagrees with the report by McGillivray[34] showing higher levels of 25-(OH) D deficiency in the younger age group. Recent studies have shown that in obese children low levels 25-(OH) D were associated with increased markers of oxidative stress, inflammation and endothelial activation.[35] It has been observed that increased total central adiposity and decreased exercise frequency are directly related to insulin resistance.[36] Exercise reduces levels of fasting insulin and improves insulin sensitivity through structural and biochemical changes in skeletal muscle.

Studies of Alemzadeh et al[6] have shown that children with 25-(OH) D deficiency had reduced sensitivity to insulin compared to those with adequate levels. There was also a negative correlation between 25-(OH) D and body fat. Reduced levels of vitamin in obese subjects seem to occur because of enhanced binding of 25-(OH) D to peripheral fat.[37] 25-(OH) D is therefore positively correlated to insulin sensitivity, but negatively correlated to HbA1c, suggesting that obese children are deficient in 25-(OH) D and have a higher risk of developing impaired glucose tolerance and so are predisposed to type 2 diabetes.[38]

The main insulin-stimulated glucose transporter GLUT-4 is down-regulated preferably in adipocytes in insulin-resistant states.[39] In mouse studies, the genetic knockout of GLUT-4 has been associated

with high levels of RBP-4.[40] Yang et al. have studied subjects with obesity, type 2 diabetes and subjects without any disease but with a strong family history of the same comorbidities and found that an increase in RBP4 correlates with insulin resistance in all patients, demonstrating that RBP4 could be used as a tool to predict risk factors for cardiovascular disease.[41] There are very few and conflicting studies on obese children or adolescents suggesting any type of correlation between BMI and 25-(OH) D, RBP-4 and NGAL. In our study, the incidence of 1st degree relatives with diabetes type 2 was 5% for the control and overweight group, 10% for the obese and 40% for the ultra-obese group. We can therefore argue that ultra-obese patients are more likely to develop diabetes type 2, having lower 25-(OH) D concentrations and higher levels of RBP4 and NGAL.

A linear association between Lipocalin-2 and 25-(OH) D, as well as between Retinol-Binding-Protein-4 and 25-(OH) D, is evident from the results of this study. This means that the latter has an increasing trend as the NGAL and RBP4 levels increase in our female population. Studies of Gavi et al[42] show a positive correlation between RBP4, adiposity and an increased inflammatory state, whereas Wang et al[43] have reported similar results regarding Lipocalin-2. There are studies, though, that do not support the above,[44,45] while similarly, there are no studies showing a direct association of 25-(OH) D, RBP4 and Lipocalin 2 in obese female children and adolescents. We therefore underline the importance of further investigation in this area.

Our study had several limitations. The sample size is relatively small. Since all blood sampling was obtained throughout a calendar year, as a consequence, there was no seasonal variation of the values of vitamin D. Furthermore, this is a cross-sectional study and there are limited confounding factors as well as lack of differentiation between causality and result. Moreover, we have not included patients' nutrient intake nor their level of physical activity, which would have given a more holistic approach to the subject.

In conclusion, there is increasing prevalence of vitamin D deficiency amongst children and adolescents even in countries with adequate sunlight exposure. Our study aids in the comprehension of pathophysiological mechanisms related to hypovitaminosis D in obese children and adolescents revealing correlations with novel markers of obesity. Further investigation, taking into account broader parameters, is therefore warranted of larger groups of young obese individuals, which could potentially lead to new dietary measures.

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