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Pediatric RESEARCH

REVIEW ARTICLE

Vitamin D deficiency in children: a challenging diagnosis!

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The concern about the assessment of vitamin D status is growing. Numerous publications warn about the high prevalence of vitamin D deficiency, as well as the potential role of vitamin D in non-bone health outcomes. The status of vitamin D is usually assessed by measuring serum total 25-hydroxyvitamin D (25OHD) concentration. This is the major circulating form of vitamin D and keeps an inverse correlation with serum parathyroid hormone (PTH) concentration. A value of 25OHD of 20 ng/ml is generally assumed as threshold of vitamin D sufficiency in epidemiologic studies because serum PTH tends to increase when the 25OHD concentration stands below this value. In pediatric population, very few studies have analyzed this issue and the negative relationship between serum 25OHD and serum PTH is not clear, which is the suitable circulating concentration of 25OHD and the threshold of deficiency being matters of controversy. The majority of 25OHD circulates in serum tightly bound to a globulin (DBP). According to the free hormone hypothesis, protein-bound hormones are not biologically available and it is the free form that exerts or facilitates the physiologic actions. If this is true, factors that affect DBP may alter the interpretation of total serum 25OHD measurements.

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SCIENTIFIC AND ECONOMIC IMPACT OF VITAMIN D

The number of scientific publications related to vitamin D has remarkably increased in the last years. Thus, more scientific articles have been published in the 21st century on vitamin D than on any other vitamin.[1] Up to 4900 articles on vitamin D were added to MEDLINE database in 2017. The vast majority of current publications on vitamin D are focused on its extraskeletal role and less than 5% are related to rickets or its prevention,[2,3] although the major physiologic function of vitamin D is to maintain normal serum calcium and phosphorus levels and to ensure bone mineralization. Figure 1. The potential link between an adequate vitamin D status and large variety of diseases has given rise to a growing research on the concept of vitamin D deficiency and the relationship between serum vitamin D levels and the prevalence of certain chronic and severe diseases, such as cancer, mental disorders, autoimmune, infectious and cardiovascular diseases, asthma[4–17] and even between the vitamin D status and the mortality rates (premature death) of general population.[18,19] Therefore, many articles alert on the need of increasing universally the intake of vitamin D as a measure of preventing not only rickets but many other diseases.[20–23] Other authors insist on the need of obtaining scientific evidence before recommending specific prophylaxis or treatment.[1,2,24–26] Since 2010, the Cochrane Collaboration has published 10 reviews (Cochrane Database of Systematic Reviews) on the use of vitamin D in the prevention or treatment of problems not related to the mineral health[14,18,27–29] and the conclusions are always the same: more trials are needed and no firm evidence supports the beneficial effect of vitamin D supplementation.

High prevalences of deficiency in vitamin D throughout the world have been found and estimates of the prevalence of hypovitaminosis D range from 1% to 95 % according to the threshold set as deficiency.[22,30–35] In children, the optimal serum concentration of vitamin D has not been established[36] and may change in different stages of life.[37]

The concern originated in the scientific community on the new and promising functions of vitamin D and the warning on the potentially high prevalence of vitamin D deficiency have caused a marked increase in the demand for measurement of serum concentrations of 25-hydroxyvitamin D (25OHD).[26] The Ontario Health Technology Assessment reported that the total annual cost of vitamin D testing multiplied by 20 in Canada between 2004 and 2009.[38] Likewise, the Glasgow Royal Infirmary estimated a rise of 20,000 vitamin D tests in two years in Scotland.[26] This growing request of serum 25OHD concentration measurements accounts for an economic burden, likely unjustified, and the clinical benefit is rather questionable.[39,40]

VITAMIN D SYNTHESIS AND METABOLISM

The main source of vitamin D in humans (90%) is the cutaneous synthesis. The amount of vitamin D production in the skin depends on the incident angle of the sun and, thereby, on the latitude, season, and time of the day. The precursor molecule, previtamin D, is synthesized from 7-dehydrocholesterol in the epidermis and dermis, during exposure to ultraviolet rays in the range of 290 to 315 nm, by temperature-dependent, non enzymatically regulated reaction. It is subsequently transformed into vitamin D which then binds to the vitamin D-binding protein (DBP) and is transported to the liver and metabolized to 25OHD, through an enzymatic process involving 25-hydroxylase (CYP27A1). The 25OHD undergoes a second hydroxylation in the kidney, and less importantly in other tissues, by 1-alpha hydroxylase (CYP27B1) enzymatic activity, to become 1,

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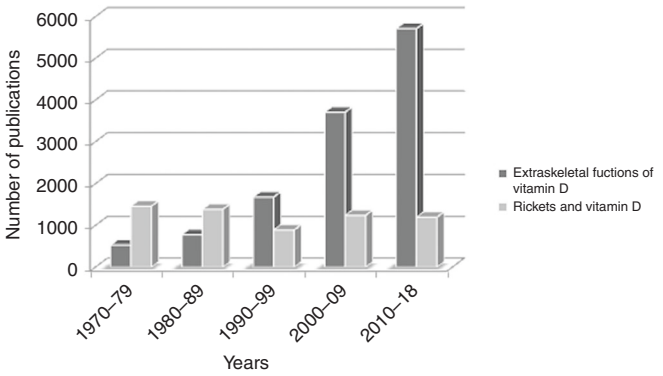


Fig. 1 Scientific impact of vitamin D. Data obtained from MEDLINE database show the growing number of publications during the last decades, the vast majority of current publications being on the extraskeletal functions of vitamin D rather than on vitamin D and rickets

25-dihydroxyvitamin D [1, 25$(OH)_2$ D]. The efficiency of vitamin D_3 synthesis in the skin is dependent on the number of ultraviolet B photons that penetrate into the epidermis. With prolonged solar exposure, pre-vitamin D_3 forms inert metabolites or is changed back to 7-dehydrocholesterol so that toxicity does not occur.[41–43] The vitamin D synthesized by humans is vitamin D_3 (cholecalciferol). Vitamin D_2 (ergocalciferol) is another form of vitamin D of vegetable origin. A low percentage of vitamin D, ~10%, enter the body, either as D_2 or D_3, through intestinal absorption from foods naturally containing vitamin D, foods fortified and vitamin D supplements.[3,44] Serum 25OHD is the major circulating form of vitamin D and it is clinically used to assess vitamin D status because its half-life is long and its synthesis is not directly regulated by hormones. Although the influence of inheritance on serum 25OHD levels is not well established, recent genome-wide association studies have shown association of serum 25OHD concentrations with six loci in European population, estimating the variability in single nucleotide polymoprhisms up to a 7.5% of serum 25OHD values.[45]

The 1, 25$(OH)_2$ D is the biologically active form of vitamin D, and it performs its function by interacting with its nuclear receptor, the vitamin D receptor (VDR).[42]

The synthesis of 1, 25$(OH)_2$ D is stimulated by parathyroid hormone (PTH) and by reduced levels of serum phosphate and suppressed by fibroblast growth factor 23 (FGF23). FGF23 is secreted by osteocytes in the bone matrix, and participates in the regulation of phosphate homeostasis with Klotho, a coactivator of FGF23. FGF23 secretion is correlated with serum 25OHD concentrations.

Circulating 25OHD and 1,25$(OH)_2$ D are tightly bound to DBP and albumin, with less than 1% circulating unbound. The free hormone hypothesis postulates that protein-bound hormones are not biologically available and that unbound hormones are biologically active, and therefore factors affecting DBP should be considered for the interpretation of 25OHD levels.[46]

The VDR is the specific receptor of vitamin D in the target cells. The 1,25$(OH)_2$ D-VDR compound complexes with retinoic acid X receptor (RXR) in the nucleus, forming 1,25$(OH)_2$D-VDR-RXR, which binds to the vitamin D-responsive element (VDRE) and regulates the transcription of various genes in the target cells.[42] At least 37 cell types express VDR.[41]

VITAMIN D FUNCTIONS

Vitamin D is essential for health. Its major physiologic function is related to the homeostasis of mineral metabolism throughout life, by maintaining serum calcium and phosphorus concentrations within the normal physiologic range to ensure bone mineralization, favoring the intestinal absorption of both nutrients and promoting their release from bone to the bloodstream.[47] Since the 1980s, it is known that there is extrarenal expression of 1-α hydroxylase as well as expression of VDR in cell types not directly involved in the classical endocrine functions of vitamin D.[2] Nowadays, up to 2000 genes have been described to be regulated by vitamin D.[21,48,49] Elevated levels of 1, 25$(OH)_2$ D found in individuals with granulomatous disorders prompted a potential role of vitamin D in cell proliferation. Its biologic role in proliferation and apoptosis along with its presence in most tissues and several epidemiological studies associating solar exposure, vitamin D, and many types of cancer,[50] aroused the interest in vitamin D as a cancer preventive agent.[4,5,8,51] Vitamin D supplementation in childhood may reduce the risk of developing type 1 mellitus diabetes.[10] However, several prospective studies in adults have led to non uniform results.[52,53] The presence of a VDRE in the promoter of the gene for cathelicidin, antimicrobial peptide with function in the killing of intracellular Mycobacterium tuberculosis, suggested that vitamin D intervenes in the innate immunity.[54] Many studies have evaluated the effect of supplementation with vitamin D in the prevention of infectious diseases with different results.[55,56] A recent Cochrane review by Yakoob et al[14] on vitamin D supplementation to prevent infections in children under five years of age concluded that no effect on death or respiratory infections can be demonstrated. Other associations between vitamin D and certain diseases, including cardiovascular risk (obesity, hypertension, hyperlipidemia, peripheral vascular disease, coronary artery disease, myocardial infarction, and heart failure…), still need to be confirmed.[57] The US Preventive Services Task Force and the Institute of Medicine (IOM) have recently stated that no sufficient data are available to recommend vitamin D status screening in routine clinical practice.[24,44] The Women's Health Initiative Calcium-Vitamin D Trial did not find decreased risk of cancer, cardiovascular disease or sudden death in postmenopausal women supplements with vitamin D and calcium.[8,19,58,59] Although the beneficial effects of vitamin D on bone metabolism have been well demonstrated, more trials on the extraskeletal effects of vitamin D are needed to clarify current knowledge and the lack of evidence is even greater in pediatric population.[2,60]

VITAMIN D STATUS ASSESSMENT

There is general consensus on considering the measurement of serum 25OHD as the most suitable marker of vitamin D status because many studies in adults have demonstrated their association with biochemical variables, such as PTH, and clinical findings, such as bone mineral density (BMD) and fracture risk.[61] However, these associations are not observed in all the studies and vary in different races, populations, and ages.[36,37,62]

Furthermore, technical issues in the determination of 25OHD need to be taken into account for the interpretation of values because 25OHD assays have different affinities for vitamin D_2 and D_3, leading to lower measured levels in regions where vitamin D_2 is predominantly used in supplementation or food fortification.[38,61]

In recent years, other potential markers of vitamin D status are being investigated including the vitamin D metabolite ratio 25OHD /24,25$(OH)_2$D,[62] and the free and bioavailable forms of 25OHD,[46,61] but there is no yet enough evidence to incorporate their use into daily practice.[61,63]

Recently, the European Food Safety Authority (EFSA) has published a Technical Report about the Dietary Reference Values for nutrients.[64] These values indicate the amount of a nutrient which must be consumed on a regular basis to maintain health in an otherwise healthy individual (or population). The Table 1 summarizes these reference values for the main nutrients related

to mineral metabolism (calcium, phosphorus, and vitamin D). This report also states that the available evidence on non-musculoskeletal health outcomes is insufficient to be used as criterion for setting Dietary Reference Values for vitamin D.

FREE HORMONE HYPOTHESIS

The free hormone hypothesis attributes the biologic activity of hormones to free or non-bound fractions to their carrier proteins rather than to the circulating total protein concentrations as it occurs with sex steroids or thyroid hormones.[65] According to this hypothesis, the bound fraction of the hormone could not freely cross the cell membrane to interact with nuclear binding proteins whereas unbound free small lipophilic ligands could cross cell membranes to exert effects.[66] The binding to DBP would impair the delivery of 25OHD to vitamin D-activating 1-alpha-hydroxylase in target cells, and therefore the free vitamin D fraction would better reflect the functional status of vitamin D.[67,68]

Until few years ago, free 25OHD serum concentrations were usually calculated by means of a complex mathematical formula including total 25OHD, DBP, and albumin serum values and their affinity constants.[69] The implementation of a new technique for the direct determination of free 25OHD has prompted studies on this topic in adults. Free 25OHD has been shown to be more strongly associated with mineral bone parameters than total 25OHD levels in healthy adults,[70,71] and in certain clinical situations.[72] In pediatric population, no reference values are available and very few studies have been carried out in children with chronic diseases. In premature infants and in children with cystic fibrosis, a strong correlation between total and free 25OHD concentrations has been reported. In children with ulcerative colitis, a recent study showed significant association between the free and bioavailable, but no total 25OHD levels and the degree of clinical activity of the disease.[73–75] We recently provided novel information on the values of free 25OHD serum concentrations in healthy children.[76] We found a high correlation between the levels of free and total 25OHD and lack of significant correlation between free 25OHD and PTH levels. By contrast, another recently published study analyzing this issue in a limited sample of children and adolescents concluded that the cut-off of free vitamin D equivalent to sufficient status of total vitamin D is 9.8 pmol/L and also found that directly measured free 25OHD showed more physiologically expected correlations with phosphocalcic metabolism biomarkers than calculated free 25OHD.[77] Table 2. Insufficient evidence is currently available to recommend the use of free 25OHD as a preferential marker of vitamin D status in pediatric population. Further studies are necessary to find out whether assessment of free 25OHD may be of clinical usefulness in specific groups of patients.

Table 1. Daily reference intakes for vitamin D (VD), calcium, and phosphorus in children older than 6 months

Child's age	VD[a] (μg)	Calcium (mg)	Phosphorus[a] (mg)
7–11 months	10	280[a]	160
1–3 years	15	450[b]	250
4–10 years	15	800[b]	440
11–14 years	15	1150[b]	640

[a]Means AIs = Adequate intake. That is the average observed daily level of intake by a population group (or groups) of apparently healthy people that is assumed to be adequate
[b]Means PRIs = Population reference intakes. That is the level of nutrient intake that is adequate for virtually all people in a population group

VITAMIN D-BINDING PROTEIN (DBP)

The vitamin D and its metabolites, circulate in blood mostly bound to a specific DBP, also known as GC-globulin. The active vitamin D metabolite, 1, 25$(OH)_2$ D, reaches the target cells through the transmembrane receptor, megalin, or low density lipoprotein-related protein 2 (LRP2). This mechanism is shared with other lipophilic molecules such as glucocorticoids, vitamin A, sex hormones, and thyroid hormones.[65] DBP has a molecular weight similar to albumin, is water-soluble and is primarily produced by the liver. The DBP binds 85–90% of the total circulating 25OHD and 85% of the total 1, 25$(OH)_2$ D, although its affinity for 25OHD is much higher. A small percentage of 25OHD circulates bound to albumin and chylomicrons that behave as non-specific transporters. The affinity of albumin for 25OHD is less than that of DBP, but the plasma concentration of albumin is higher.

There are three major polymorphisms for DBP (GC1F, GC1S, and GC2), which give rise to six common phenotypes for this protein: Gc1S/1S, Gc1S/2, Gc1F/1 F, Gc1S/1F, Gc1F/2, and Gc2/2,[78] with different affinities for vitamin D and its metabolites. These phenotypes have a different relative distribution according to the races, the Gc1F phenotype being the most frequent in Africans whereas the GC1S predominates in Europeans. Powe et al.[62] reported that African Americans had much lower DBP concentrations than US Caucasians and, despite their much lower total 25OHD concentration, their calculated free or bioavailable 25OHD was equal or even slightly higher than in Caucasians. Racial differences in the prevalence of common genetic polymorphisms provide a likely explanation for this observation. In contrast to the above stated, recent data using a variety of polyclonal antibodies indicate that the mean concentrations of DBP are, to a large

Table 2. Studies on free 25-hydroxyvitamin D (25OHD) in children

First author, year of publication	*N*	Population	Key findings
Hanson C, 2018	32	Infants < 32 weeks of gestational age	Supplementation with vitamin D3 increased the free fraction of 25OHD.
Lee MJ, 2015	25	Patients with cystic fibrosis, aged 4.5–32.5 years	High correlation between total and free 25OHD in cystic fibrosis. Unnecessary to use free 25OHD to more accurately assess vitamin D status
Mantecon, 2018	241	Healthy children, aged 0–14 years	The correlation between serum free and total 25OHD concentrations was high and significant. No significant correlation was found between PTH and free 25OHD. The correlation between total 25OHD and PTH was inverse and significant.
Sauer, 2018	388	Patients with ulcerative colitis, aged 4–17 years	Bioavailable but not total 25OHD was significantly associated with clinical symptoms.
López-Molina, 2018	66	Healthy children, aged 2–18 years	Directly measured free vitamin D correlated better with markers of phosphocalcic metabolism than total 25OHD and calculated free 25OHD in a population of healthy children.

extent, race and genotype independent.[79] Therefore, data from former studies should be reanalyzed and DBP measurements should be better standardized.

CONCEPT OF VITAMIN D DEFICIENCY

To set the threshold of serum 25OHD values indicative of vitamin D sufficiency would require to find a clinically relevant, easily measured variable dependent on the levels of 25OHD. Rickets might be this clinical variable in pediatrics, as proposed by IOM in 1997,[44] but studies on children from around the world have not supported an absolute threshold level of serum 25OHD concentration for the occurrence of rickets. The majority of pediatric patients with rickets have serum levels of 25OHD below 10 ng/ml, but patients with rickets caused by vitamin D deficit and serum 25OHD concentrations greater than 20 ng/ml have also been reported. Furthermore, it is possible that data on 25OHD concentrations and rickets are confounded by calcium intake.[60] A review of the scientific literature on this topic carried out by US Agency for Healthcare Research and Quality concludes that the available evidence is very limited for an association between 25OHD concentrations and bone health.[3]

In 2005, Hollis et al.[80] demonstrated an inverse correlation between total 25OHD and PTH levels in adult population, describing a clear plateau effect and a point from which on the decrease of 25OHD concentrations results in an abrupt rise of PTH levels. This serum 25OHD value of 20 ng/ml was subsequently defined as the deficiency threshold.[60] Studies carried out in infants and children[30,32,33,47,81,82] do not confirm this cut-off point and the value of 20 ng/ml cannot be assumed as the serum 25OHD concentration indicative of vitamin deficiency in pediatric population.[36] Atapattu et al. indicated that the deficiency in vitamin D based on the elevation of PTH was best defined by a serum 25OHD value of < 13.6 ng/ml in children and adolescents. These authors also argued that the threshold for the skeletal effects of vitamin D should not be based purely on 25OHD levels because deficient calcium supply often coexists with vitamin D deficiency and this should be taken into account.[83] It is of note that the relationship between serum PTH and 25OHD concentrations is, to some extent, age-dependent, higher concentrations of PTH being found for a given value of 25OHD in older individuals.[37] These observations underline the importance of undertaking pediatric studies without automatically extrapolating conclusions derived from studies in adults.

In relation to overall health, there is currently no consistent evidence to define new sufficiency thresholds related to the non mineral metabolism-related functions of vitamin D.[2] Based on this lack of evidence, the IOM has concluded that it is premature to recommend vitamin D administration for the prevention of non-musculoskeletal diseases,[44] regardless of the sufficiency threshold. The Committee on Nutrition of the European Society for Pediatric Gastroenterology Hepatology and Nutrition recommended a serum 25OHD concentration < 20 ng/ml to indicate vitamin D deficiency, but acknowledging at the same time that there is only scarce evidence on the correlation of 25OHD serum concentrations with health outcome.[60] This Committee also notes the implicit difficulty in assessing 25OHD serum concentrations because the substantial inter-assay differences of commercially available 25OHD tests limit the comparison among studies.[60,84] In the recently published summary of Consensus Recommendations of the "Global Consensus Recommendations on Prevention and Management of Nutritional Rickets", a serum 25OHD concentration < 12 ng/ml is recommended as indicative of vitamin D deficiency.[85] The value of the 25OHD cut-off is not uniform since several international societies have recommended different thresholds.[86]

It is a matter of current research the role of the genetic aspects in the metabolism of vitamin D the degree of heritability of concentrations of 25OHD. A larger genome-wide association study (GWAS) identified variants in four loci (GC, NADSYN1/DHCR7, CYP2R1, CYP24A1) involved in vitamin D transport, cholesterol synthesis, and hydroxylation, suggesting that these genetic variation could identify individuals with greater risk of vitamin D insufficiency.[87] Subsequent studies have confirmed the role for common genetic variants in the regulation of circulating 25OHD concentrations and estimate a 7.5% of heritability attributed to these genetic variations, and assuming the existence of other low frequency variants with larger effects that were not investigated. Also they find a specific genetic link between the vitamin D and autoimmune diseases. No genetic correlation was found with other traits studied.[45]

The knowledge about the genetic architecture of this trait could provide a better understanding of the regulation of vitamin D metabolism, but larger studies are required to identify additional common single nucleotide polymorphisms.

In conclusion, many factors may be involved in the definition of vitamin D deficiency and especially in children. Figure 2.

SUMMARY OF CONTROVERSIES AND CONCERNS

1. The measurement of serum total 25OHD is the most widely used parameter to clinically assess the vitamin D status, but the threshold concentration indicative of vitamin D deficiency remains to be established in children.

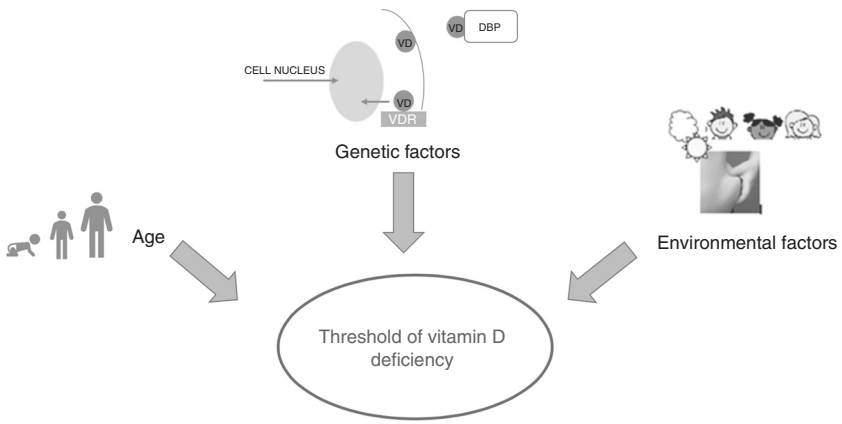


Fig. 2 The threshold of vitamin D (VD) deficiency in children may be influenced by age (needs for calcium and VD change with growth velocity), genetic factors [polymorphisms of VD binding protein (DBP) have different affinities for VD and its metabolites and the structure of VD receptor (VDR) influences on the interaction with DNA in the cell nucleus] and environmental conditions (season, sun exposure, pollution, skin pigmentation, latitude, diet, cultural factors etc.)

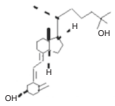
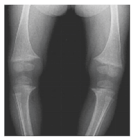
Vitamin D functions 	The main function of vitamin D is the maintenance of phosphocalcic metabolism, especially in periods of accelerated growth, but so far there are no clinical trials in children clarifying their role beyond the bone health.
Vitamin D status 	The serum total 25OHD concentration is generally used as indicator of vitamin D status at the moment but other potential markers of vitamin D status, such as the serum free 25OHD concentration, are currently being investigated.
Vitamin D deficiency 	A concentration of 25OHD >20 ng/ml seems to exclude vitamin D deficiency in healthy children but concentrations below this value do not necessarily associate with abnormal clinical or biochemical manifestations of vitamin D deficiency.

Fig. 3 Key points on vitamin D functions, status and deficiency in children

2. The threshold of deficiency may vary depending on age and individual, genetic, and environmental circumstances.
3. In pediatric population, a clinical marker having enough sensitivity to reflect the vitamin D status has not been described.
4. The determination of free 25OHD might be useful in certain clinical situations to assess vitamin D status.
5. As for the clinical assessment of vitamin D status, the extrapolation to pediatric population of data obtained in adults can give rise to inadequate conclusions and inappropriate recommendations.

In summary, the assessment of vitamin D status is of particular interest in childhood because growth in height and active bone formation may imply different needs from those of adult population. However, the best indicator of adequate vitamin D status in children remains to be defined and it is not known the clinical meaning of "low" serum 25OHD concentration in apparently healthy children not showing typical manifestations of vitamin D deficiency. Figure 3.

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AUTHOR CONTRIBUTIONS

Each author meets the *Pediatric Research* authorship requirements and contributed to study's conception and design. Mª.A.A. wrote the first draft of the manuscript and the three authors reviewed it several times and approved the current version.

ADDITIONAL INFORMATION

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