Original Article
Central Eur J Paed 2022;18(2):91-99
DOI 10.5457/p2005-114.321

Vitamin D Assessment in Patients with Multisystem Inflammatory Syndrome and SARS-CoV-2 Infection

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Received: May 31 2021; **Accepted:** August 26 2022

Abstract

Objective - To assess 25(OH)D status in children with Multisystem Inflammatory Syndrome (MIS-C) associated with SARS-CoV-2 infection, and the association between serum levels of 25(OH)D and inflammatory marker values. **Patients and methods** - This retrospective study was conducted at the Clinic for Children's Diseases, University Clinical Center, Tuzla in the period from November 2020 to November 2021. **Results** - The study included 23 children with MIS-C with a median age of 6.9 years, and 22 children with acute SARS-CoV-2 infection, with a median age of 1.1 years. Both groups of children had low serum levels of 25(OH)D in the range of deficiency; median levels of 25(OH)D in MIS-C children were lower (median 44.60 nmol/L) than in children with acute SARS-CoV-2 infection - median 52.45 nmol/L. There was no statistically significant difference in 25(OH)D levels between the two groups of children included in the study (P=0.33). The same number of children in both groups had adequate serum levels of 25(OH)D. The children in both groups had elevated markers of inflammation, but we did not find a significant correlation between the values of 25(OH)D and the inflammatory marker values. **Conclusion** - Deficiency of 25(OH)D could be one of the precipitating factors that lead to the development of both MIS-C - related SARS-CoV-2 and more severe forms of acute infection. 25(OH)D enriched food, as well as supplementation with 25(OH)D, should be considered a long-term strategy in all high-risk children and adolescents.

Key Words: Vitamin D ▪ 25(OH)D ▪ Multisystem Inflammatory Syndrome in Children ▪ SARS-CoV-2.

Introduction

The uncommon complication of SARS-CoV-2 infection, the Multisystem Inflammatory Syndrome in Children (MIS-C), is characterized by unremitting fever and prominent cardiovascular involvement, including shock, coronary-artery aneurysms, symptoms of multiple organ system dysfunction, and high values of inflammatory markers. The incidence of MIS-C is 5.1 per 1,000,000 persons, and 316 per 1,000,000 people younger than 21 years with SARS-CoV-2 infection (1, 2). MIS-C occurs 3 to 4 weeks after a SARS-CoV-2 infection and consists of a host-dependent reaction to past infections, meaning that it is compatible with an antibody- and/or immunocomplex-mediated disease. Vitamin D [25-hydroxy vitamin D-25(OH)D] has proven immunomodulatory properties, modulates both innate and adaptive immunity, reduces concentrations of pro-inflammatory cytokines, and increases concentrations of anti-inflammatory cytokines (3-6). Cells involved in innate and adaptive immune responses express the enzymes required to activate and respond to 25(OH)D. Studies have shown that 25(OH)D tends to suppress the

immune response, consequently low concentrations are associated with an increase in pro-inflammatory mediators and a more active disease (7-10).

Vitamin D (cholecalciferol) is synthetized in the skin, under ultraviolet-B (UV-B) radiation, but also derived from the intake of animal food and supplements. It is initially hydroxilated in the liver, resulting in the inactive circulating form, 25-hydroxyvitamin D, calcidiol. This form is then hydroxylated in the kidneys to yield inalcitriol, the active form of vitamin D. The immune cells are able to convert calcidiol locally into calcitriol, which acts as a circulating hormone, and is the most potent natural ligand of the vitamin D receptor of target cells, including monocytes, macrophages, as well as T and B lymphocytes. For that reason vitamin D has been characterized as a modulator of innate and adaptive immune responses (8, 9). Recent research has shown that vitamin D has an anti-inflammatory effect, and the stronger the inflammatory process in the disease, the greater the need for vitamin D (10, 11). The etiology of this association may be related to the immune modulatory role of vitamin D, and the accepted pathophysiology of MIS-C, which is an exaggerated immune response (12).

The aims of this study were to assess 25(OH)D status in children with MIS-C, and also to examine whether there was an association between serum levels of 25(OH)D and the values of inflammatory markers.

Patients and Methods

This retrospective study was conducted at the Department of Rheumatology, Immunology and Allergy and the COVID-19 department of the Clinic for Children's Diseases, University Clinical Center (UCC), Tuzla, from November 2020 to November 2021.The medical records of 23 children with MIS-C and 22 children with acute SARS-CoV-2 infection were analyzed. The diagnosis of MIS-C was established in accordance with the American College of Rheumatology Clinical Guidance for Multisystem Inflammatory Syndrome in Children Associated with SARS-CoV-2 and Hyperinflammation in Pediatric COVID-19 (7). SARS-CoV-2 infection diagnosis was based on the criteria given by the World Health Organization (16). Exclusion criteria included: failure to fulfill the diagnostic criteria for MIS-C and acute SARS-CoV-2 infections, children with chronic conditions (asthma, juvenile idiopathic arthritis, chronic renal insufficiency, children with hematooncological diseases, vasculitis), those on immunosuppressive therapy or chemo-therapy, and those whose parents refused to allow their child to take part in the study. The following were analyzed: age, gender, 25(OH) D levels, and inflammation markers (C-reactive protein (CRP), leucocyte and platelet count, procalcitonin, D–dimer, fibrinogen, ferritin, lactate dehydrogenase (LDH) and interleukin-6 (IL-6). The pathological cut-off values are summarized in Table 1. SARS-CoV-2 detection was performed by taking nasopharyngeal swabs, following the standard procedure for polymerase chain reaction (PCR) testing, and they were analyzed at the Clinic for Microbiology of UCC Tuzla. Serology tests were analyzed with a commercial enzyme linked immunosorbent ELISA assay [anti-SARS-CoV-2 immunoglobuline M (IgM) and immunoglobuline G (IgG)] (EuroImmun™, Bussy-Saint-Martin, France) with cut-off values of IgM >4 IU/mL and IgG >11 IU/mL. Assessment of 25(OH)D levels in the serum was conducted, where blood was taken by the standard procedure and analyzed at the Polyclinic for Laboratory Diagnostics of the UCC in Tuzla. The blood samples for 25(OH)D were centrifuged at 3,000 revolutions per minute for 10-minutes. After centrifuging, the separated serum was kept in flacons at a temperature of -70°C until analysis. Values of 25(OH)D were determined by ADVIA Centaur assay, and the cut off value was 75 nmol/L. The ADVIA Centaur Vitamin D total assay is an 18 minute automated direct competitive chemiluminescent immunoassay that detects 25(OH)D in serum or plasma. This assay uses a proprietary releasing reagent and a monoclonal antibody. The assay range is 9.3 nmol/L to 375 nmol/L. Values <25 nmol/L were considered to indicate insufficiency, 25-50 nmol/L deficiency, and

Table 1. The Pathological Cut-off Values of Laboratory Markers

Laboratory findings	Cut-off values
WBC* (×10⁹/L)	<5x10⁹/L
Neutrophils (×10⁹/L)	<1.4x10⁹/L
Lymphocytes (×10⁹/L)	<1.4x10⁹/L
RBC† (×10¹²/L)	<4x10¹²/L
Hemoglobin (g/ L)	<109 g/L
Platelets (×10⁹/L)	<150x10⁹/L
Fibrinogen (g/L)	>3.6 g/L
LDH‡ (U/L)	≥400 U/L
Ferritin (µg/L)	>700 ng/mL
CRP§ (mg/L)	>5mg/L
Prokalcitonin (ng/mL)	>2.0 ng/mL
D-dimer (µg/mL)	≥1.5 µg/mL
25(OH)D (nmol/L)	75 nmol/L
IL-6‖ (pg/mL)	≥15 pg/mL

*White blood cells; †Red blood cells; ‡Lactate dehidrogenase; §C-reactive protein; ‖Interleukin-6.

50-75 nmol/L insufficient values, since adequate 25(OH)D levels were ≥75 nmol/L.

Twenty seven healthy children, matched for gender and age to the children with MIS-C and children with acute SARS-CoV-2 infection, not using supplements containing vitamin D or calcium within the past three months, with no associated chronic diseases, were included as controls.

Ethics Statement

The study protocol was approved by the Ethics Committee of UCC, Tuzla, No.: 02-09/2-20/21. The inform consent has been signed by parents of all the children included in the study.

Statistical Analysis

Statistical data analysis was conducted using the biomedical software application "MedCalc for Windows, Version 15.11.4" (MedCalc Software, Ostend, Belgium). Distribution of variables determined by the Kolmogorov-Smirnov test. The variables with distorted distribution were shown with a median as a measure of the central value. The Mann-Whitney and Kruskal-Wallis tests were used to test statistically significant differences between the samples. Spearman's correlation coefficient was used to measure the correlations between the variables. Difference were considered significant when $P<0.05$.

Results

In the period from November 2020 to November 2021, 23 (12.30%) of 187 children hospitalized at the Department of Rheumatology, Immunology and Allergy of the Clinic for Children's Diseases, UCC Tuzla, had MIS-C. The study included 10 girls and 13 boys, with a median age at the time of diagnosis of 6.9 years (range: 3.5 - 11.2 years). Initially, there were 26 children with suspected MIS-C. Three were excluded due to alternative diagnoses (septic arthritis, encephalitis, and urinary tract infection). Five MIS-C children were initially treated in the intensive care unit (two of them developed cardiogenic shock, one had severe respiratory distress). The clinical signs and symptoms of the children with MIS-C and SARS-CoV-2 infections are summarized in Table 2.

Roughly the same number of children, 10 girls and 12 boys, with a median age 1.1 years (range: 0.5-3.3 years) at diagnosis were treated in the COVID-19 department due to acute SARS-CoV-2 infection. The control group consisted of 27 healthy children (13 boys and 14 girls) with a median age of 4.2 years (IQ range: 0.3-9.3years). There was a statistically significant difference in age but not in gender distribution ($P<0.0001$ and $P=0.56$) between the children with MIS-C and the children with acute SARS-CoV-2 infections. The children in control group were matched with examined groups of children, there was no difference in age ($P=0.22$ and $P=0.29$) or gender ($P=0.58$ and $P=0.72$) compared to the children with MIS-C or with children with acute SARS CoV-2 infections.

All children in examined groups had fever, analyzing the frequency of common symptoms we do not find statistically significant differences between

Table 2. The Clinical Signs and Symptoms of 23 MIS-C Children and 22 Children with COVID-19 Infections Included in the Study

Clinical characteristics	Children with MIS-C* N (%)	Children with COVID-19† N (%)
Gender		
Male	13 (56)	12 (54.53)
Female	10 (43)	10 (45.45)
Signs and symptoms		
Fever	23 (100)	22 (100)
Sore throat	17 (73)	9 (39.13)
Pneumonia	17 (73)	17 (77.27)
Conjunctivitis	17 (73)	2 (9.09)
Rash	17 (73)	-
Abdominal pain	16 (69.5)	5 (22.73)
Diarrhea	15 (65.2)	5 (22.73)
Myalgia	14 (60.86)	-
Cervical lymphadenopathia	13 (56)	1 (4.54)
CNS‡manifestation	10 (43)	4 (18.18)
Vomiting	10 (43)	3 (13.04)
Cough	9 (39.13)	17 (77.27)
Oral mucose changes	7 (30.4)	-
Acute kidney injury	3 (13.04)	-
Rhinorrhea	3 (13.04)	9 (39.13)
Shock	2 (8.69)	-
Dyspnea	1 (4.34)	6 (27.27)

*Multisystem inflammatory syndrome in children; †Corona virus disease 2019; ‡Central nervous system.

the examined groups in the frequency of cough (P=0.79), CNS manifestations (P=0.12) or pneumonia (P=0.79). However, for the dominant symptoms of children with MIS-C, a statistically significant difference was observed in the frequency of occurrence compared to children with acute SARS-CoV-2 in whom these symptoms were not frequent (Table 2), sore throat (P=0.025), dyspnea 0.02, abdominal pain (P=0.001), diarrhea (P=0.004), conjunctivitis (P=0.00001) and cervical lymphadenopathy P=0.0001, vomiting P=0.04. There was a statistically significant difference in age but not in gender distribution (P<0.0001 and P=0.56) between the children with MIS-C and the children with acute SARS CoV-2 infections.

Both, examined groups of children had low (range of deficiency) serum levels of 25(OH)D. The median levels of 25(OH)D in the MIS-C children were lower, at 44.60 nmol/L (IQ range: 38.40-77.20), than in the children with acute SARS-CoV-2 infection, with a median of 52.45 nmol/L (IQ range: 17.60-178.0), where as those levels were 71.31 nmol/L (IQ range: 41.4-89.62) in the control group. However, there was no statistically significant difference in 25(OH)D levels between the two examined groups of children included in the study (P=0.33), nor did we observe significant differences for the serum levels of 25(OH)D of the control group of children compared to the levels of MIS-C children (P=0.22) or in children with acute SARS-CoV-2 infection (P=0.17). The same number of children in the two examined groups had adequate serum levels of 25(OH)D (Table 3).

Table 3. Serum 25(OH)D Levels in Children with MIS-C, children with COVID-19 and children of control group

Serum 25(OH)D levels	Children with MISC* N (%)	Children with COVID-19† N (%)	Children of control group N (%)
Insufficiency <25nmol/L	1 (4.30)	3 (13.60)	3 (11.1)
Deficiency 25-50 nmol/L	15 (65.20)	6 (27.30)	5 (18.6)
Inadequate levels 50-70 nmol/L	2 (8.69)	8 (36.40)	7 (25.9)
Adequate levels ≥75 nmol/L	5 (21.70)	5 (22.70)	12 (44.4)

*Multisystem Inflammatory Syndrome in Children: †Corona virus disease 2019.

Similar serum levels of 25(OH)D were found in girls [median 45.70 nmol/L (IQ range: 36.80-61.57] and boys [median 44.2 nmol/L (IQ range: 35.20 - 108.00)] with MIS-C, also in girls with SARS-CoV-2 infection, with a median of 43.50 nmol/L (IQ range: 35.05-60.72). The SARS-CoV-2 boys and control group boys had slightly higher serum levels of 25(OH)D, with a median of 62.40 nmol/L (IQ range: 23.20-102.0) and 69.50 nmol/L (IQ range: 83.38- 121.0), while girls in the control group had significantly higher levels of 25(OH)D with median 80.0 nmol/L (IQ range: 33.2-111.45). No statistically significant differences in 25(OH)D concentrations between the girls and boys of the examined groups nor in the control group were noticed, P=0.37, P=0.07 and P=0.87 respectively. However, a moderate but not significant correlation (r=0.43, P=0.07; 95%CI: -0.18-0.80) was noticed between serum levels of 25(OH) D and the gender of MIS-C children; while there was no statistically significant correlation between gender and levels of 25(OH)D in children with SARS-CoV-2 infection, in the control group of children either. Analyzing 25(OH)D status with regard to the age of children, no statistically significant correlation was found between the levels of 25(OH)D and the ages of children with MIS-C (r=0.11, P=0.26; 95%CI: 0.38-0-58), or between the levels of 25 (OH)D and the age of children with SARS-CoV-2 infection (r=0.16, P=0.11; 95%CI: -0.49-0.32), however negative weak correlation found in children of control group (r=-0.30, P=0.13; 95%CI: -0.62-0.10).

All MIS-C children had elevated values of CRP and ferritin, and increased procalcitonin values were found in 20/23 (86.9%) children. However, a statistically significant difference in the values of the analyzed inflammatory parameters among the children of the examined groups was for: neutrophils, lymphocytes, thrombocytes, fibrinogen, ferritin, CRP and D dimer (Table 4). The values of IL-6 and SARS-CoV-2 serology tests was estimated in only the MIS-C children.

Nineteen of the 23 (82.6%) MIS-C children had increased levels of IL-6 [median 130.30 (IQ range: 24.20-789.0 μg/mL)]; all MISC-C children had positive IgM [median 8.25 (IQ range: 0.76-18.30 IU/mL)], but 22/23 (95.7%) had positive IgG [median 16.46 (IQ range: 4.90-30.78 IU/mL)] antibodies for SARS-CoV-2. The laboratory findings of the children included in the study are summarized in Table 4.

After assessing the serum levels of 25(OH) D and the values of inflammatory markers in the MIS-C children, no significant correlation was found: CRP (r=0.20, P=0.28; 95%CI: -0.19-0.58), ferritin (r=0.10, P=0.64; 95%CI: -0.49-0.32), D dimer (r=0.04, P=0.83; 95%CI: -0.38-0.25) and prokalcitonin (r=0.09, P=0.68; 95%CI: -0.49-0.33) but also no significant correlation was found for serum levels of 25(OH) D with other common parameters: leukocytes (r=0.02, P=0.80; 95%CI: -1.53-1.95), neutrophils (r=0.094, P=0.15; 95%CI: -3.27-0.55), lymphocytes (r=0.27, P=0.22; 95%CI: -0.15-0.61), platelets (r=0.10, P=0.31; 95%CI: -0.31-0.49) or for fibrinogen (r=0.01, P=0.85; 95%CI: -8.03-6.75). In the group of children with SARS-CoV-2 infection, a significant correlation was found only between the levels of 25(OH)D and procalcitonin (r=0.44,

Table 4. Summary of Laboratory Findings in 23 Children with MIS-C and 22 Children with COVID-19 Included in the Study

Laboratory findings	Children with MIS-C* Median (range); IQR‡	Children with COVID-19† Median (range); IQR	P value††
WBC§ ($\times 10^9$/L)	10.36 (7.20-17.65)	7.75 (1.72-19.80)	P=0.27
Neutrophils ($\times 10^9$/L)	6.32 (0.32-19.24)	2.57 (0.61-8.59)	P=0.025
Lymphocytes ($\times 10^9$/L)	2.29 (0.34-17.9);	42.50 (3.15-5.86)	P<0.0001
RBC‖ ($\times 10^{12}$/L)	4.32 (3.49-4.92)	3.91 (3.50-4.54)	P=0.27
Hemoglobin (g/ L)	129.0 (98-141)	101.0 (84-180)	P=0.21
Platelets ($\times 10^9$/L)	154.0 (53-546)	331.0 (62-615)	P<0.0001
Fibrinogen (g/L)	4.08 (3.60-5.30)	2.30 (4.20-18)	P=0.03
LDH¶ (U/L)	298.0 (214-348)	284.0 (210-373)	P=0.24
Ferritin (µg/L)	557.0 (258-3070.40)	130.60 (23.70-749)	P<0.0001
CRP** (mg/L)	190.0 (15.30-424)	23.90 (0.10-312)	P<0.0001
Prokalcitonin (ng/mL)	3.20 (1.93-220)	0.11 (0.01-0.80)	P<0.0001
D-dimer (µg/mL)	3.20 (0.27-4.60)	1.30 (0.60-4.30)	P=0.002
25(OH)D (nmol/L)	44.60 (38.40-77.20)	52.45 (17.60-178)	P=0.33

*Multisystem inflammatory syndrome in children; †Corona virus disease 2019; ‡Interquartile range; §White blood cells; ‖Red blood cells; ¶Lactate dehydrogenase; **C-reactive protein; ††Kolmogorov-Smirnov test.

P=0.04; 95%CI: 0.01-0.78), while for other markers of inflammation we found no association**.**

Discussion

At the moment, there have been a limited number of studies regarding the relationship between 25(OH)D and SARS CoV-2 infections or MIS-C in children. Current evidence suggests that MIS-C is a post-infectious, immunologically mediated disorder, which occurs as a result of a host immune response to SARS-CoV-2 characterized by a cytokine storm (17-19). The median age of the MIS-C children in our study was 6.9 years (range: 3.5- 11.2). Similarly, in the studies conducted by Tristan et al. (20) and Rivera et al. (21) the median age was 8.8 years (range: 3.5- 11.2 years) and 8 years (range: 1-13 years), respectively. Also, many other studies reported that the children most affected with MIS-C were between the ages of 6 and 11 (7, 22, 23, 24). Such observations are expected given the fact that research has shown that older children and adolescents are more likely to develop severe SARS-CoV-2 infections and MIS-C, and that they are at higher risk of vitamin D deficiency (25-27).

However, in our study we did not find any statistically significant correlation between the levels of 25(OH)D and the age of the children with MIS-C (r=0.11, P=0.26), or with acute SARS-CoV-2 infection (r=0.16, P=0.11), whose median age was significantly lower, 1.1 years; for the control group of healthy children, the correlation of 25(OH)D values was negative, weak and statistically insignificant (r=-0.30, P=0.13).

Consistent evidence of MIS-C being mediated by amplified inflammatory responses to SARS-CoV-2 and the regulatory actions of 25(OH)D on pro-inflammatory cytokine signaling, further substantiates the possible role of 25(OH)D in MIS-C (29). The largest number of MIS-C children in our study, 17/23 (73.9%), had low serum levels of 25(OH)D, and only 5/23 (21.7%) MIS-C children had adequate values of 25(OH)D. Similar results were reported by Darren et al. (25), where 78% of children were 25(OH)D deficient, while sufficient 25(OH)D was present in only 11% of MIS-C children in their study. The same researchers pointed out that children with severe MIS-C, who required treatment in a pediatric intensive care unit, had lower mean 25(OH)D concentrations compared

with children who had a milder form of MIS-C, but they found no statistically significant differences in values of 25(OH)D between these two groups of children (19.5 v. 31.9 nmol/L; P=0.11). Rivera et al. (21) found adequate values of 25(OH) D in only 5.8% of children with MIS-C in their study, indicating an association between severe MIS-C and severe vitamin D deficiency. Children with severe vitamin D deficiency were at increased risk of severe MIS-C disease (OR: 28.8; 95%CI; 2.9–286.4; P<0.01). However, Darren et al. (25) did not observe any association between 25(OH) D concentrations and disease severity in children with MIS-C in their study, but concluded that their study was not adequately powered to evaluate that relationship. Although Rivera et al. (21) found an association between low 25(OH)D values and the severity of MIS-C, they did not find any significant association between 25(OH)D levels in MIS-C children and inflammatory markers. Similar results were reported by Daneshkhah et al. (29), and these researchers did not find any significant correlation between inflammation markers and levels of 25(OH)D in children with MIS-C or SARS-CoV-2 infection.

Our results are consistent with the above results, since we did not notice any significant association between 25(OH)D levels in MIS-C children and inflammatory markers. The immunomodulatory activity of vitamin D may play a key role in the host immune response to SARS-CoV-2 infection, given that the increased risk of acute viral respiratory infections with vitamin D deficiency and the potential protective effects of supplementation have been widely reported (21, 31). In the study by Yilmaz and Şen (31), children with acute SARS-CoV-2 infection had significantly low values of 25(OH)D, and 72.5% of them had values of 25(OH)D at the level of deficient or insufficient. Similarly, Alpcan et al. (32) reported low values of 25(OH)D levels in children with acute SARS-CoV-2 infection, with a median value of 21.5 nmol/L. We also noticed low serum levels of 25(OH)D in children with acute SARS-CoV-2 infection in our study, with median values of 52.45 nmol/L, and only 5/22 (22.7%) of children had adequate serum levels of 25(OH)D. Since these were children with more severe clinical manifestations of acute SARS-CoV-2 infection, they required hospital treatment, and our results are in line with the results of other researchers. Thus, Rivera et al. (21), Merzon et al. (33) and D'Avolio et al. (34) reported that low vitamin D levels were a risk factor for acute SARS-CoV-2 infection, with increased morbidity and hospitalization.

Severe infections, such as SARS-CoV-2 infections, can lead to intracellular consumption of vitamin D during the immune process (11). This is supported by earlier reports on the properties of vitamin D and their implications for acute viral respiratory syndrome caused by SARS-CoV-2 infections (17,18,19). The rational of vitamin D supplementation in SARS-CoV-2 infection in children is based on the results of studies which investigated the impact of vitamin D status on infections due to influenza viruses. Inflammatory reactions involved in the process of infectious diseases reduce vitamin D levels, which could explain why low vitamin D levels are reported (35).

The Limitations of the Study

Our study had several limitations. Firstly, we had a small sample size. Secondly, there was a lack of data on 25(OH)D status in children before acute infection with SARS-CoV-2 and MIS-C. Thirdly, there was a lack of dynamic monitoring of 25(OH) D levels, since the assessment was made in one single time point. The level of IL-6 was not evaluated in children with SARS-CoV-2 infection because it is not done routinely in University Clinical Center Tuzla and the method is in a high price range for a developing country like Bosnia and Herzegovina.

Conclusion

25(OH)D immunomodulatory activity plays an important role in the host immune response to infections, so 25(OH)D deficiency could be one of the precipitating factors for both the more severe form of acute infection, and the development of

MIS-C-related to SARS-CoV-2. Supplementation with 25(OH)D has been shown to be useful in infections with other viruses although it is not yet clear whether a similar benefit can be achieved in MIS-C. 25(OH)D - enriched food and 25(OH)D supplementation for all high-risk children and adolescents should be considered a long-term strategy.

Author's Contributions: Conception and design: AHK, AĆ, AP, AS, DS, MK and DM. Acquisition, analysis and interpretation of data: AĆ, AHK, AP. Drafting the article AHK, AĆ, AP, AS. Revising the article critically for intellectual content: AHK, AĆ, AP, AS, MK. Approved final version of the manuscript: AHK, AĆ, AP, AS, DS, MK and DM.

Conflict of Interest: The authors declare that they have no conflict of interest.

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