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Abstract

Context Vitamin D status has previously been associated with cardiometabolic risk markers in children and adolescents. In particular, it has been suggested that children with obesity are more prone to vitamin D deficiency and unfavorable metabolic outcomes compared to healthy-weight children.

Objective To conduct a longitudinal study assessing this association in children and stratify by body mass index (BMI) category.

Methods Children from the pan-European IDEFICS/I.Family cohort with at least one measurement of serum 25-hydroxyvitamin D (25(OH)D) at cohort entry or follow-up (n=2,171) were included in this study. Linear mixed-effect models were used to assess the association between serum 25(OH)D as an independent variable and z-scores of cardiometabolic risk markers [waist circumference (WC), systolic (SBP) and diastolic blood pressure (DBP), high (HDL) and low density lipoprotein, non-HDL, triglycerides (TRG), apolipoprotein A1 and B (ApoB), fasting glucose (FG), homeostatic model assessment for insulin resistance (HOMA-IR), metabolic syndrome score] as dependent variables.

Results After adjustment for age, sex, study region, smoking and alcohol status, sports club membership, screen time, BMI, parental education, and month of blood collection, 25(OH)D levels were inversely associated with SBP, DBP, FG, HOMA-IR and TRG. The HOMA-IR z-score decreased by 0.07 units per 5 ng/ml increase in 25(OH)D. 25(OH)D was consistently associated with HOMA-IR irrespective of sex or BMI category.

Conclusion Low serum 25(OH)D concentrations are associated with unfavorable levels of cardiometabolic markers in children and adolescents. Interventions to improve vitamin D levels in children with a poor status early in life may help to reduce cardiometabolic risk.

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity worldwide among all non-communicable diseases. CVD and diabetes mellitus account for 19.4 million global deaths per year (1, 2). Unfavorable cardiometabolic risk markers such as high systolic (SBP) and diastolic blood pressure (DBP), dyslipidemia and dysglycemia markers as well as increased body mass index (BMI), waist circumference (WC) and metabolic syndrome (MetS) score in childhood have been shown to track into adulthood (3-5) and to promote the development of CVD and diabetes mellitus (6-8). Therefore, preventing these diseases early in life is a key public health goal.

A poor vitamin D status which is usually and reliably determined as low serum concentration of 25-hydroxyvitamin D [25(OH)D] (9) has been linked to unfavorable levels of cardiometabolic risk markers such as high blood pressure and insulin resistance in adults (10) and children (11). Vitamin D deficiency in children and adolescents is widespread in European countries (12, 13).

Results from cross-sectional studies in children and adolescents revealed an inverse association between vitamin D status and cardiometabolic risk markers, in particular insulin resistance, fasting glucose (FG), triglycerides (TRG), non-high density lipoprotein (Non-HDL), low density lipoprotein (LDL), apolipoprotein B (ApoB), and hypertension as well as a positive association with HDL and apolipoprotein A1 (ApoA1) (14-26) although not all studies reported associations (27, 28).

Longitudinal cohort studies with a large number of children and adolescents are rare and show inconsistent results (25, 29, 30). Due to the potential protective effects on cardiometabolic health and the widespread poor vitamin D status, several randomized controlled trials (RCTs) have been conducted. Their results were combined in two recent systematic reviews and meta-analyses which investigated the effects of vitamin D supplementation on cardiometabolic risk markers in children and adolescents in 9 RCTs including 954 participants (31) and 14 RCTs including 1800 participants (32), respectively. In both studies, no overall beneficial effect of supplemental vitamin D was observed, which may be attributed to the short follow-up duration in the RCTs. Interestingly, a clinically relevant reduction of homeostatic model assessment for insulin resistance (HOMA-IR) was observed per 10 nmol/l increase of serum 25(OH)D level among participants with overweight/obesity in a meta-regression analysis in one of the reviews (32). This may indicate that at least in this group higher serum 25(OH)D levels can help to lower HOMA-IR levels. In a subgroup analysis of the other meta-analysis (31), a decrease of FG and TRG was observed only in the high vitamin D supplementation group indicating that higher dosages might be required to show any beneficial effect. On the other hand, in populations with sufficiently high 25(OH)D levels, no associations between 25(OH)D and cardiometabolic risk markers or lower odds ratios for unfavorable

cardiometabolic risk markers were reported (16, 33). Therefore, the baseline or achieved vitamin D status may explain divergent results between studies and needs to be considered particularly in longitudinal and interventional studies (31). Another important factor in the association of vitamin D status and cardiometabolic risk markers seems to be adiposity (31, 34, 35). However, the effect of adiposity is difficult to elucidate as it can act as a confounder as well as a potential effect modifier (36). The confounding effects are based on its lowering effect of serum 25(OH)D levels and deteriorating most cardiometabolic risk markers (10). Additionally, the evidence that vitamin D is absorbed by adipose tissue, leading to lower bioavailability of vitamin D (21, 37, 38) may explain the role of obesity as an effect modifier. Thus, adjusting for BMI and stratification of study participants by weight status is required.

We hypothesize that children who have low 25(OH)D levels have unfavorable cardiometabolic risk markers and that this association is even stronger in children with overweight or obesity. Our study aims to address the following hypotheses: i) Serum 25(OH)D levels are associated with cardiometabolic risk markers (WC, SBP, DBP, HDL, LDL, non-HDL, TRG, ApoA1, ApoB, FG, HOMA-IR, MetS score) after adjustment for relevant confounders. ii) Changes in serum 25(OH)D levels over time are associated with trajectories of cardiometabolic risk markers. iii) The associations differ between children with normal weight and children with overweight/obesity?

Methods

Study population

Children from eight European countries (Belgium, Cyprus, Estonia, Germany, Hungary, Italy, Spain, and Sweden) participated in the IDEFICS/I.Family cohort. The baseline examination included 16,229 children aged 2 to 9 in 2007/2008 (Wave 1=T_0), and follow-up examinations were carried out after 2 (Wave 2=T_1) and 6 years (Wave 3= T_3). Parental questionnaires were collected at T_2 but the data were not used in the present analysis. Additional children were recruited and entered the cohort at T_1 and T_3. All survey waves included physical examinations of the children as well as the collection of blood samples. Questionnaires concerning health-related behaviors (39) and dietary intakes (40) were completed by a parent for children aged up to 12 years and self-reported by children aged 12 years and older. Fasting blood samples were drawn in the morning after an overnight fast of at least 8 hours. The same standardized assessments and procedures were applied in each survey wave. Further details on the study design are described elsewhere (41, 42). Parents provided written

informed consent before their child entered the study. Additionally, children aged 12 years and older gave written consent, while younger children gave oral consent. All eight study centers obtained ethics approval from their local institutional review boards prior to the start of the study. The IDEFICS/I.Family cohort has been registered under ISRCTN62310987. In the present paper, we performed a longitudinal analysis in children with serum 25(OH)D measurements available in at least one survey wave.

Anthropometric measurements and blood pressure

For the examination of body weight and height, children wore light clothes and no shoes. Weight was measured to the nearest 0.1 kg using a TANITA digital scale, (TANITA Europe GmbH, Sindelfingen, Germany) and height to the nearest 0.1 cm using a stadiometer (Seca GmbH & Co. KG., Hamburg, Germany). Age and sex-specific BMI z-scores calculated based on Cole and Lobstein (43) were used in the analysis. WC was measured midway between the lowest rib margin and the iliac crest to the nearest 0.1 cm in an upright position with relaxed abdomen and feet together (non-elastic tape: Seca 200; seca, Birmingham, UK) and age-, sex- and height-specific reference values were used to derive WC z-scores (44).

Systolic (SBP) and diastolic blood pressure (DBP) were measured in a seated position on the right arm with an automated oscillometric device (Welch Allyn, Inc., 4200B-E2, Skaneateles Falls, NY, USA) after at least 5 minutes of rest and an additional recording after a 2-min break. A third measurement was taken in case of a > 5% difference between the first two readings and the mean value of the two measurements with the smallest difference was used. The cuff length was chosen depending on the child's arm circumference. According to previously described methods, age-, sex- and height-specific reference values were applied to calculate z-scores for SBP and DBP based on the data collected in the IDEFICS/I.Family cohort (45).

Markers of glycemic disorders

Measurement of FG at T_0 was conducted either with capillary blood from a finger prick or with venous blood from venipuncture using a point-of-care analyzer (Cholestech LDX, Cholestech Corp., Hayward, CA, USA). At T_3, FG was assessed from NaF plasma by an enzymatic UV test (Cobas c701, Roche Diagnostics GmbH, Mannheim, Germany) in a central laboratory. Serum insulin concentrations were measured in a central laboratory at both time points. A luminescence immunoassay (AUTO-GA Immulite 2000, Siemens, Eschborn, Germany, RRID:AB_2756390) was used at T_0 and assessment at T_3 was conducted by multiplex analysis with electrochemiluminescence technology from Meso Scale Discovery (MSD) using a MULTI-SPOT® Assay System (Human Leptin, Insulin Assay Kit, RRID:AB_2819057). HOMA-IR was calculated as fasting insulin

(μIU/ml) × FG (mg/dl)/405. Based on the IDEFICS/I.Family cohort data, age- and sex-specific reference values were used to calculate z-scores for FG, insulin and HOMA-IR according to previously described methods (46) considering separate reference curves for T_0 and T_3 for FG as the laboratory methods for these parameters differed between the two time points.

Markers of dyslipidemia

At T_0 total cholesterol, HDL and TRG were assessed using a point-of-care analyzer (Cholestech LDX, Cholestech Corp., Hayward, CA, USA). LDL was calculated based on the Cholestech results applying the Friedewald formula: LDL = Total cholesterol – HDL – TRG/5.0 (mg/dl). At T_3, a homogeneous enzymatic colorimetric test was used for HDL and LDL and an enzymatic colorimetric test for total cholesterol and TRG assessment (in all cases: Cobas c701, Roche Diagnostics GmbH, Mannheim, Germany). Non-HDL was calculated as the difference between total cholesterol and HDL. ApoA1 and ApoB were measured using N antisera against human ApoA1 (RRID:AB_2935776) and ApoB (RRID:AB_2935777) for BN ProSpec System with immuno-nephelometry (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) at T_0 and T_3. Age- and sex-specific reference values were used to calculate HDL z-scores according to previously described methods (47). As no age trend was found for TRG, sex-specific reference percentiles were used to calculate the z-scores. Stata module STNDZXAGE was used for calculating z-scores of LD, non-HDL, ApoA1 and ApoB; with standardization on age, sex and survey.

Metabolic syndrome score (MetS score)

The MetS score was previously defined for children (48). To derive the score, the age- and sex- (for blood pressure also height-) specific reference values were used for waist circumference, SBP, DBP, HDL, TRG, and FG in children and adolescents according to previously described methods (44-47) based on the data collected in the IDEFICS/I.Family cohort. Separate reference curves were estimated for T_0 and T_3 for FG, HDL and TRG and used for the analysis as the laboratory methods for these parameters changed at T_3. Based on these reference values, z-scores were calculated and summed up (HDL multiplied by -1) to derive the MetS z-score, which was lower in children with a more favorable metabolic profile (48).

25-hydroxyvitamin D

Serum 25(OH)D concentrations were measured at T_0 and T_3 by the chemiluminescence assay, IDS-iSYS 25-Hydroxy Vitamin D^s (Immunodiagnostic Systems Ltd, Boldon, United Kingdom) in a central laboratory. This

assay covers a measurement range of 10 to 275 nmol/l (4 to 110 ng/ml) and is aligned to the National Institute of Standards and Technology Standard Reference Material (NIST SRM) 2972 (NIST, Gaithersburg, Maryland, USA) according to the manufacturer information.

Pubertal status, health-related behaviors, and ultraviolet radiation (UV) exposure

Girls were classified as pre-menarcheal or post-menarcheal according to their age at menarche, and boys were classified as pre-pubertal or pubertal according to whether the voice change had set in. The corresponding information was self-reported confidentially on a paper by children aged 8 years and above at T_3 and then put into a sealed box without the study staff being able to see it. A previous analysis in this cohort confirmed that this definition provided similar results compared to Tanner stages (49). Health-related behaviors including alcohol consumption and smoking habits were self-reported by children aged 12 years and older at T_3. All questionnaires for children aged 12 years and older were completed online on a tablet in a confidential environment. Behaviors of younger children were reported by parents. These proxy questionnaires did not include questions on smoking or alcohol consumption as younger children were assumed to be non-smokers and non-drinkers. Binary indicator variables were created for alcohol intake and smoking of ever smokers/drinkers vs. non-smokers/non-drinkers based on the number of occasions reported for alcohol intake/cigarette smoking in a lifetime. A variable indicating whether the child was a member of a sports club (yes/no) was used as a proxy for physical activity. The total screen time including TV, DVD, video, computer and games-console use, in units of hours for the whole week was used as a proxy for sedentary behavior. Month of blood sample collection and study region were used to consider UV radiation exposure.

Parental education and early life factors

The highest educational attainment of parents according to the International Standard Classification of Education (ISCED) was used and grouped into three categories: low (ISCED 0,1,2); medium (ISCED 3,4) and high level (ISCED 5,6) (50). Mothers reported the gestational week of birth which was used as a binary variable (delivered at term vs. born preterm, i.e. $\leq 37^{th}$ week of gestation), the birth weight (g), and total breastfeeding duration (in months, including combinations of breastfeeding with infant formula and food). Weight and height of the mother were self-reported and maternal BMI at cohort entry of the child was calculated as weight (kg) divided by height (m) squared.

Analysis dataset

The present analysis used T_0 and T_3 measurements as 25(OH)D concentrations were not measured at T_1. Our analysis dataset included participants with measurements of 25(OH)D from T_0 and/or T_3 (n=2171), thus resulting in 1,505 children with 25(OH)D measurements at both T_0 and T_3, 133 children with measurements only available at T_0 and 533 children with measurements only at T_3 (Supplementary Figure 1) (51).

Statistical analysis

Data were shown as mean ± SD or median with an interquartile range as appropriate. To assess differences in levels of serum 25(OH)D between cohort entry and follow-up examination for different categories of cardiometabolic risk markers across different examinations, all risk markers were categorized into "always normal" (levels of cardiometabolic risk markers < 90th percentile at all examinations), "high to normal" (levels decreased from ≥ 90th percentile to < 90th percentile from T_0 to T_3), "normal to high" (levels increased from < 90th percentile to ≥ 90th percentile from T_0 to T_3), and "always high" (levels were consistently ≥ 90th percentile at all examinations; except for HDL and ApoA1: ≥ 10^{th} percentile was defined as normal and < 10^{th} percentiles as low). We determined mean differences and standard deviations of 25(OH)D between T_0 and T_3 at each of the above-mentioned categories of cardiometabolic risk markers using a paired t-tests.

We assessed longitudinal associations between serum 25(OH)D and cardiometabolic risk markers using linear mixed-effect regression models with age as random slope. In this model, one level accounts for differences between individuals, whereas the other level for changes with age within individuals (52). The serum concentration of 25(OH)D was the exposure variable and cardiometabolic risk markers were the outcome variables. Exposure and outcomes were considered as continuous variables. The between-subject effect estimate referred to the association between 25(OH)D and a cardiometabolic risk marker. We also assessed fixed-effect interaction on a multiplicative scale between age and 25(OH)D to represent the rate of change of the association between 25(OH)D and cardiometabolic risk markers with age.

The description of the crude model is as follows: let y_{ij} be j-th measurement of the i-th child (e.g., z-scores of cardiometabolic risk markers), M_{ij} is 25(OH)D, age_{ij} is the age of the child and ϵ_{ij} is the error term for child *i* at measurement time *j*, then the crude model without adjustment was specified as follows:

$$y_{ij} = \gamma_{0i} + \gamma_{1i}\mathrm{age}_{ij} + \epsilon_{ij} \text{ with}$$

$$\gamma_{0i} = \beta_{00} + \beta_{01}\mathrm{M}_{ij} + u_{0i}$$

$$\gamma_{1i} = \beta_{10} + \beta_{11}\mathrm{M}_{ij} + u_{1i}$$

where β_{00} is the overall mean intercept, β_{10} is the overall mean slope and u_{0i} and u_{1i} express how much the intercept and slope, respectively, of child i deviates from the average intercept and slope with respect to the child's age.

All models were adjusted for age in years and sex with age as a random slope. The so-called 'adjusted models' also included study region, lifetime smoking and alcohol status, membership in a sports club, screen time per week, BMI (except for models assessing associations with MetS score and WC), parental education and month of blood sample collection. Model diagnostics were evaluated by examination of homoscedasticity, normality of residuals and random intercepts and were evaluated and observed to be satisfactory. The marginal effect of 25(OH)D on cardiometabolic risk markers at different ages was calculated using effect estimates from the adjusted model as part of posthoc analysis. To assess any effect modification, analyses were stratified by BMI (dichotomized based on the categories by Cole & Lobstein (43): normal weight vs overweight or obese) and sex. Several sensitivity analyses were performed: (i) To compare our findings with the previously used cut-offs for vitamin D status (53, 54), we categorized 25(OH)D levels into deficient (< 20 ng/ml), insufficient (20-29 ng/ml) and normal (30-50 ng/ml). (ii) We also evaluated a flexible dose-response relationship between 25(OH)D and cardiometabolic markers using linear spline with knots at 10, 20, 30 and 40 ng/ml. (iii) We additionally adjusted for metabolic and early life factors on our adjusted models to evaluate if factors such as pubertal status, birth weight, duration of breastfeeding, preterm birth and maternal obesity attenuate the association.

To account for changes in health-related behaviors and BMI over time, all covariates were treated as time-varying. Results were reported as regression coefficients with 95% confidence intervals. All statistical analyses were performed using Stata 17 (RRID:SCR_012763).

Results

Characteristics of the study population

A total of 2,171 children were included in the analysis (Supplementary Figure 1) (51). The characteristics of the study population are shown in Table 1. At T_0 the mean age of the children was 6.17 years (n=2,149), and after 6 years of follow-up (T_3) the mean age was 11.95 years (n=2,168). The median serum 25(OH)D concentrations at T_0 and T_3 were 18.0 ng/ml and 17.9 ng/ml, respectively, which is below the threshold for normal levels of 30 ng/ml. Hence, 47.4% of the children were classified as having a deficient status at T_0 while 59.5% were below the limit at T_3. The percentage of children classified as overweight or obese increased with age and was 16.6% at T_0 and 22.6% at T_3. Accordingly, the z-scores for the MetS score and some of its components (WC, blood

pressure and HOMA-IR) increased over time. Both the proportion of children who were members of a sports club and the mean daily screen time increased from T_0 to T_3 (Table 1). The included and excluded populations of children had similar characteristics at cohort entry (Supplementary Table 1) (51) except for smoking or alcohol consumption which is due to the fact that a larger proportion of the excluded participants entered the cohort at an older age only at T_3, while these behaviors were not assessed at T_0 because of the young age of the children. We observed that the levels of 25(OH)D attenuated from T_0 to T_3 in children with a persistently high HOMA-IR, whereas the 25(OH)D levels increased slightly from T_0 to T_3 when WC, FG, HOMA-IR, TRG, LDL, Non-HDL, MetS score and DBP were persistently normal. Additionally, 25(OH)D slightly increased from T_0 to T_3 in children with a normal to low HDL and with a high to normal SBP (Figure 1).

Longitudinal associations between serum 25(OH)D and cardiometabolic risk markers

We observed associations between 25(OH)D concentrations and SBP, DBP, FG, HOMA-IR and TRG. HOMA-IR z-score decreased by 0.07 units per 5 ng/ml increase in 25(OH)D (Table 2). The associations of 25(OH)D with WC, FG and HOMA-IR varied with a per year increase in age (Table 2). Figure 2 displays the association between 25(OH)D and the single risk markers at different ages. While the inverse association between 25(OH)D and WC and between 25(OH)D and HOMA-IR strengthened with age, the inverse association between 25(OH)D and FG was only observed in children $\leq$ 8 years of age.

Inverse associations persisted for FG and HOMA-IR after additional adjustments for preterm birth, birth weight, duration of breastfeeding, pubertal status, and maternal obesity (Supplementary Table 2) (51). The stratified analysis on sex showed similar protective associations in both sexes (Supplementary Table 3) (51). Figure 3 and Supplementary Table 4 (51) show the associations between serum 25(OH)D and cardiometabolic risk markers stratified by weight status. In children classified as overweight or obese, stronger inverse associations between 25(OH)D level and cardiometabolic risk markers were shown compared to normal weight children, with the exception of FG. The strongest associations were detected for MetS score, HOMA-IR and TRG.

Sensitivity analysis

When 25(OH)D levels were categorized into deficient, insufficient and normal vitamin D status, we observed that deficiency was associated with an increase of 0.33 units of HOMA-IR ($\beta = 0.33$; 95% CI = 0.07 to 0.59) compared to normal status (Table 3). On further testing for non-linear associations between 25(OH)D and cardiometabolic risk markers, we observed an inverse association with markers of dyslipidemia such as LDL and

non-HDL when levels of vitamin D were lower than 10 ng/ml. We also observed a modest inverse association with HOMA-IR at 25(OH)D levels between 30-39 ng/ml (Supplementary Figure 2) (51).

Discussion

Confirming our hypothesis, this population-based study in a large cohort of European children and adolescents shows that children with low 25(OH)D levels have unfavorable cardiometabolic risk markers after adjustment for relevant confounders including BMI and factors like physical activity and sedentary behavior. Additionally, children with overweight/obesity compared with children of normal weight and children with vitamin D deficiency compared with those with a sufficient vitamin D status are both at higher risk for unfavorable cardiometabolic risk markers. These associations are partially reflected in the trajectories of cardiometabolic risk markers by changes in 25(OH)D levels between T_0 and T_3 examination. In particular, the decrease of 25(OH)D levels over six years may have contributed to the observed persistent insulin resistance. Our results revealed age dependency in the associations of 25(OH)D levels with FG, WC and HOMA-IR which indicated that the inverse association with FG is strong in young children while the associations with WC and HOMA-IR became stronger with increasing age. This highlights the importance of early prevention of increased glucose levels as high HOMA-IR with increased age may indicate that glucose metabolism worsened and resulted in insulin resistance at an older age.

Notably, the association between low 25(OH)D and high HOMA-IR was consistently observed; it was mostly the strongest association and persisted independent of sex and weight status as well as after further adjustment for early life factors.

In the stratified analysis by weight status, associations between 25(OH)D and cardiometabolic risk markers pointed mostly to the same directions in both groups but were stronger in children with overweight/obesity, particularly for SBP, HOMA-IR, MetS score and blood lipids. This indicates a potentially higher benefit of an improved vitamin D status in this group.

Inverse associations with both FG and HOMA-IR, were also reported in previous cross-sectional studies (16, 19-21). In two further large cross-sectional analyses, a US study showed an inverse association for HOMA-IR in children and adolescents (55), whereas no such cross-sectional association was observed in children and adolescents from China where even an upward trend in FG in those with normal FG values with increasing 25(OH)D was detected (28).

No association with FG was identified, however an association with fasting insulin was observed in children and adolescents of a large longitudinal study in France who had a slightly better baseline 25(OH)D status in the comparable age group than our population (25). The study does not report HOMA-IR but the inverse association with fasting insulin in the adjusted model might also indicate an inverse association with HOMA-IR.
A higher HOMA-IR in children with obesity as observed in our study was also reported by others (23, 24). In a large Chinese cohort of children and adolescents, the interaction of insufficient vitamin D status and obesity explained 32% of the increased odds of hyperglycemia (16). Our results show a consistent inverse association with HOMA-IR which strengthened with age and which was independent of weight status and other confounding factors. This finding is also supported by a meta-regression analysis where HOMA-IR decreased by 0.51 units per 4 ng/ml increase of 25(OH)D among children and adolescents with overweight or obesity (32).

The role of vitamin D in insulin resistance may be explained by different mechanisms (56): (i) vitamin D affects the calcium flux through calcium channels of the pancreatic cell membranes and the Ca^{2+} homeostasis via an increase of cytoplasmic Ca^{2+} levels which activates exocytosis of insulin in the pancreatic β cells; (ii) in muscle and adipose tissue, vitamin D regulates Ca^{2+} levels and counteracts the parathormone-induced increase of Ca^{2+} and decrease of GLUT-1 and GLUT-4 glucose transporters on the cell membranes; (iii) vitamin D seems to indirectly inhibit the renin-angiotensin-aldosteron-system which influences cellular Ca^{2+} levels in skeletal muscle cells and impedes insulin action in peripheral tissues; (iv) vitamin D improves insulin sensitivity and signaling by induction of insulin receptor expression and activation of peroxisome proliferator-activated receptor delta (PPAR-δ); the latter reduces free fatty acid-mediated insulin resistance in the skeletal muscle; (v) vitamin D has indirect antioxidant properties by protecting cells from reactive oxygen species overproduction and increasing the production of antioxidants; thus it reduces oxidative stress which is involved in the development of hyperinsulinemia; (vi) vitamin D inhibits the release and activity of pro-inflammatory cytokines and suppresses pathways involved in their transcription and thus counteracts the overproduction of pro-inflammatory cytokines in hypertrophic adipose tissue which dysregulates signaling pathways and results in insulin resistance.
We assume that the association between 25(OH)D levels and HOMA-IR is bidirectional and the inverse association in our study can indicate both poor health-related behaviors, including obesity, which are associated with low 25(OH)D and high HOMA-IR, as well as a protective effect of higher 25(OH)D levels on insulin resistance.

A poor vitamin D status is associated with unfavorable cardiometabolic risk markers. In our population, most children and adolescents had an insufficient or deficient 25(OH)D status. Compared to a sufficient status, 25(OH)D deficiency was associated with higher HOMA-IR independent of the child's BMI. In the non-linear associations, we observed null associations between 25(OH)D and most cardiometabolic risk markers while a modest reduction in HOMA-IR levels was observed with vitamin D when 25(OH) levels were in the range of 30-39 ng/ml. A recent meta-analysis of 9 RCTs with vitamin D supplementation in children highlighted the role of the vitamin D status in reducing FG levels only when restricted to studies including vitamin D deficient children. Additionally, a decrease in FG and TRG became only apparent in studies with a total vitamin D supplementation of at least 5,000 µg (31) which indicates that lower dosages are insufficient to improve these risk markers. Accordingly, for successful treatment of vitamin D deficiency, a sufficient status may not be achieved if a low dosage is administered over a long period (57). Deficient children with obesity are even at risk of not benefiting from vitamin D supplementation at usual doses regardless of the duration of supplementation as shown in a recent meta-analysis of clinical trials, which indicated that higher doses may be needed in this vulnerable group (58).

Other large studies in children and adolescents found cross-sectionally inverse associations of vitamin D status with blood pressure (15) and a positive one with HDL cross-sectionally (15) and longitudinally (25). While our data do not reveal a consistent association of 25(OH)D with HDL, we observed an inverse association with SBP and DBP. Our results showed an inverse association with TRG which was also reported by a meta-analysis of cross-sectional studies (59) and in the subgroup of boys in the longitudinal analysis of another cohort study (30).

Limitations and strengths

Our study presents associations of serum 25(OH)D level with cardiometabolic risk markers controlling for the most relevant confounders. For some influencing factors we needed to consider self-reported proxy markers, e.g., screen time per week was used as a marker for sedentary behavior as it has been shown to be an appropriate proxy which is associated with accelerometer-derived objectively measured sedentary time (60); further, it is a marker for exposure to marketing of unhealthy food and diets (61). Additionally, sports club membership was used as a proxy for physical activity because previous results have revealed that it is associated with objectively measured time spent in moderate-to-vigorous (MVPA) (62) and vigorous physical activities (VPA) (63). Additionally, sports club members are more likely to reach MVPA recommendations (63). As comorbidities like metabolic syndrome components or diabetes mellitus type 1 (T1DM) were either not assessed or were observed in very few children (T1DM; n=5), we did not adjust for these conditions. Further, social desirability and other

factors may have led to information bias, particularly regarding sensitive information, e. g., on alcohol consumption or smoking. However, previous studies have shown that young adolescents report their alcohol consumption rather reliably (64). Though there were differences in blood collection techniques and assays for FG and lipid measurements between the two examinations, we believe these differences may have only resulted in non-differential misclassification thus attenuating the association towards the null. We used month of blood draw and region of residence as a proxy for UV radiation exposure which may not have sufficiently reflected the true exposure to UV radiation. However, our usage of serum 25(OH)D may have partly accounted for other UV-dependent factors such as time spent outdoors, dietary intake of vitamin D, area of skin exposed to the sun and skin pigmentation.

On the other hand, our study has several strengths as it provides longitudinal associations of serum 25(OH)D with cardiometabolic risk markers based on a large population of children and adolescents from Northern, Eastern, Western and Southern regions across Europe who were deeply phenotyped in terms of lifestyle behaviors, anthropometric measurements and biomarker data. Another strength is that we also tested for non-linear associations. All measurements and examinations were conducted following highly standardized protocols and adherence to them was ensured by standard operating procedures, central training for field personnel, and site visits.

The design of our study allowed association analysis considering serum 25(OH)D at two time points as well as several cardiometabolic risk markers and influencing variables at three time points. This longitudinal observation of a large European cohort of children provides stronger evidence as compared to cross-sectional studies. The external validity of our findings may also be considered higher than that of experimental intervention studies with mostly short-term vitamin D supplementation.

Conclusion

Low 25(OH)D levels are associated with unfavorable cardiometabolic markers in children and adolescents. Interventions to improve vitamin D levels in children with a poor status early in life may help to reduce the cardiometabolic risk. The consistent inverse association of 25(OH)D with HOMA-IR independent of sex and weight status indicates that a higher vitamin D status may be beneficial to most children, particularly to those with a deficient vitamin D status. Children being overweight or obese could benefit even more from a better status as many cardiometabolic risk markers seem to improve with increasing serum vitamin D levels in this group.

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Author contributions

MM and RN were responsible for the conceptualization. MW and MM wrote the original draft. The formal analysis was conducted by RN. RF gave advice on statistical methods. HC, SDH, GE, FL, II, LAM, DM, TV, and WA were responsible for the acquisition of data. Writing-review and editing was carried out by RN, RF, HC, SDH, GE, FL, II, LAM, DM, TV, and WA. All authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but can be provided on reasonable request to the IDEFICS/I.Family steering committee.

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Figure Legends

Figure 1: Change in levels of serum 25-hydroxyvitamin D with trajectory of cardiometabolic risk markers

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; DBP, diastolic blood pressure; FG, fasting glucose; HDL, high density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; LDL, low density lipoprotein; MetS, metabolic syndrome; TRG, triglyceride; SBP, systolic blood pressure. Apolipoprotein A1 and B were not measured at T_1.
always normal: levels of cardiometabolic risk markers < 90^{th} percentile at T_0, T_1 and T_3.
high to normal: levels of cardiometabolic risk markers decrease from ≥ 90th percentile at T0 or T0 +T1 to < 90th percentile at T1 +T3 or T3 respectively.
normal to high: levels of cardiometabolic risk markers increase from < 90th percentile at T0 or T0 +T1 to ≥ 90th percentile at T1 +T3 or T3 respectively.
persistently high: levels of cardiometabolic risk markers ≥ 90^{th} percentile at T_0, T_1 and T_3
p-values represent mean differences of 25(OH)D between T_0 and T_3 at each category of cardiometabolic risk markers using a paired t-test.
Dashed line indicates cut-off for vitamin D deficiency.

Figure 2: Posthoc analyses to evaluate the marginal effect of 25(OH)D on cardiometabolic risk markers at different ages

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index: DBP, diastolic blood pressure, FG, fasting glucose; HDL, high density lipoprotein, HOMA-IR, homeostatic model assessment for insulin resistance; LDL, low density lipoprotein; MetS, metabolic syndrome; SBP, systolic blood pressure; TRG, triglyceride.
Marginal effects are presented as regression coefficient that represent the ß unit change in the z-score of cardiometabolic risk markers per 5 ng/ml increase in 25(OH)D at different ages.
Associations are adjusted for age, sex, study region, lifetime smoking and alcohol status, membership in sport club, screen time/week, BMI, parental education status and month of blood sample collection with interaction term between 25(OH)D and age with age as a random slope. Waist circumference and MetS score were not adjusted for BMI.

Figure 3: Association between serum 25-hydroxyvitamin D and cardiometabolic risk markers stratified by weight status

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; CI, confidence interval; HOMA-IR, homeostatic model assessment for insulin resistance.
Normal weight children: 18.5 ≤ BMI < 25; overweight/obese children: BMI ≥ 25 (Cole & Lobstein 2012 (46)).
The ß coefficient represents the ß unit change in the z-score of cardiometabolic risk markers per 5 ng/ml increase in 25(OH)D.
Associations are adjusted for age, sex, study region, lifetime smoking and alcohol status, membership in sport club, screen time/week, parental education status and month of blood sample collection with age as a random slope.

Table 1: Characteristics of the analysis group at T_0 and T_3

Parameters		IDEFICS/I.Family analysis sample (n=2,171)	
		T_0 (n=2,149)	T_3 (n=2,168)
Sex, female: n (%)		1,039 (48.4)	1,046 (48.3)
Age: years		6.17 (±1.78)	11.95 (±1.82)
Study region	Belgium: n (%)	112 (5.2)	112 (5.2)
	Cyprus: n (%)	43 (2.0)	44 (2.0)
	Estonia: n (%)	374 (17.4)	379 (17.5)
	Germany: n (%)	355 (16.5)	357 (16.5)
	Hungary: n (%)	464 (21.6)	465 (21.5)
	Italy: n (%)	260 (12.1)	262 (12.1)
	Sweden: n (%)	253 (11.8)	252 (11.6)
	Spain: n (%)	288 (13.4)	297 (13.7)
BMI*	Thinness grade 1-3: n (%)	236 (11.0)	175 (8.1)
	Normal weight: n (%)	1556 (72.4)	1503 (69.3)
	Overweight/obese: n (%)	357 (16.6)	490 (22.6)
25-hydroxyvitamin D	Normal 30–50 ng/ml: n (%)	44 (2.1)	73 (3.4)
	Insufficient 20–29 ng/ml: n (%)	576 (26.8)	665 (30.7)
	Deficient < 20 ng/ml: n (%)	1,018 (47.4)	1,290 (59.5)
	missing	511 (23.8)	140 (6.5)
25-hydroxyvitamin D (ng/ml), $n(T_0)$ = 1,638; $n(T_3)$ = 2,038		18.00 (13.50; 22.00)	17.90 (13.80; 22.00)
Pubertal/post-menarcheal status[§]: n (%)		not observed	913 (42.1)
Ever smoking[#]: n (%)		not observed	136 (6.3)
Ever consumed alcohol[#]: n (%)		not observed	388 (17.9)
Membership in sports club: n (%)		1086 (50.5)	1449 (66.8)
Screen time per week (hours), $n(T_0)$ = 2,073; $n(T_3)$ = 2,014		11.53 (±7.17)	16.87 (±10.75)
WC z-score, $n(T_0)$ = 2,121; $n(T_3)$ = 2,149		0.11 (±1.34)	0.38 (±1.43)
SBP z-score, $n(T_0)$ = 2,095; $n(T_3)$ = 2,119		0.09 (±0.98)	0.13 (±0.99)
DBP z-score, $n(T_0)$ = 2,096; $n(T_3)$ = 2,119		0.04 (±0.97)	0.16 (±0.95)
TRG z-score, $n(T_0)$ = 1,987; $n(T_3)$ = 2,140		-0.02 (±0.80)	0.06 (±1.02)
LDL z-score, $n(T_0)$ = 1,977; $n(T_3)$ = 2,139		0.05 (±0.98)	0.00 (±0.97)
HDL z-score, $n(T_0)$ = 1,981; $n(T_3)$ = 2,140		0.02 (±1.00)	0.02 (±1.01)
Non-HDL z-score, $n(T_0)$ = 1,981; $n(T_3)$ = 2,139		0.02 (±0.99)	-0.02 (±0.96)
HOMA-IR z-score, $n(T_0)$ = 1,761; $n(T_3)$ = 1,543		0.04 (±1.08)	0.18 (±1.15)
FG z-scores, $n(T_0)$ = 1,947; $n(T_3)$ = 1,749		0.01 (±1.01)	0.09 (±1.00)
Apolipoprotein A1 z-score, $n(T_0)$ = 1,403; $n(T_3)$ = 2,092		0.00 (±1.01)	0.01 (±1.00)
Apolipoprotein B z-score, $n(T_0)$ = 1,401; $n(T_3)$ = 2,085		0.00 (±1.00)	0.00 (±1.00)
MetS z-score, $n(T_0)$ = 1,693; $n(T_3)$ = 1,793		0.17 (±2.63)	0.94 (±3.02)
BMI z-score, $n(T_0)$ = 2,149; $n(T_3)$ = 2,168		0.22 (±2.72)	0.78 (±2.75)
BMI (kg/m^2), $n(T_0)$ = 2,149; $n(T_3)$ = 2,168		15.70 (14.80; 17.00)	18.60 (16.75; 21.20)
WC (cm), $n(T_0)$ = 2,121; $n(T_3)$ = 2,149		52.70 (50.00; 56.80)	65.00 (60.00; 71.70)
SBP (mmHg), $n(T_0)$ = 2,095; $n(T_3)$ = 2,119		100.00 (94.50; 106.50)	107.5 (101.50; 113.50)
DBP (mmHg), $n(T_0)$ = 2,096; $n(T_3)$ = 2,119		62.50 (58.50; 67.00)	65.00 (61.00; 69.50)
TRG (mg/dl), (T_0) = 1,987; $n(T_3)$ = 2,140		45.00 (45.00; 59.00)	59.00 (46.00; 80.50)
LDL (mg/dl), (T_0) = 1,977; $n(T_3)$ = 2,139		93.60 (77.00; 111.80)	88.00 (74.00; 104.00)
HDL (mg/dl), $n(T_0)$ = 1,981; $n(T_3)$ = 2,140		52.00 (43.00; 61.00)	59.00 (50.00; 68.00)
Non-HDL (mg/dl), $n(T_0)$ = 1,981; $n(T_3)$ = 2,139		105.0 (88.00; 123.00)	97.00 (82.00; 114.00)
HOMA-IR, $n(T_0)$ = 1,796; $n(T_3)$ = 1,557		0.74 (0.43; 1.16)	1.27 (0.84; 1.89)
FG (mg/dl), $n(T_0)$ = 1,988; $n(T_3)$ = 1,768		84.00 (78.00; 90.00)	94.00 (90.00; 99.00)
Apolipoprotein A1(mg/dl), $n(T_0)$ = 1,403; $n(T_3)$ = 2,092		150.00 (135.00; 165.00)	138.00 (125.00; 152.00)
Apolipoprotein B (mg/dl), $n(T_0)$ = 1,401; $n(T_3)$ = 2,085		74.20 (64.90; 85.00)	62.20 (53.30; 72.40)

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; FG, fasting glucose; HDL, high density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; LDL, low density lipoprotein; MetS; metabolic syndrome score; SBP, systolic blood pressure; TRG, triglyceride; WC, waist circumference.

[§] Menarche in girls and voice change in boys have occurred, as self-reported by children aged ≥ 8 years at T_3

* Category for BMI was calculated using Cole and Lobstein, 2012 (46); # Children below 12 were assumed to be non-smokers and non-drinkers.

[a] Characteristics of the study participants are presented as number (percentages) for categorical variables and median (25^{th} and 75^{th} percentiles) or mean (±SD) for continuous variables. n stated in case of missingness.

Table 2: Longitudinal association between serum 25-hydroxyvitamin D and cardiometabolic risk markers

Cardiometabolic risk markers (z-score)	n	Crude[a]	n	Adjusted[b]	Interaction with age[c]
		β (95% CI)		*β* (95% CI)	*β* (95% CI)
Waist circumference[d]	2,160	-0.03 (-0.06; 0.00)	2,061	-0.03 (-0.06; 0.01)	**-0.01 (-0.02; -0.003)**
Systolic blood pressure	2,154	**-0.03 (-0.06; -0.01)**	2,053	**-0.03 (-0.06; -0.0002)**	0.00 (0.00; 0.01)
Diastolic blood pressure	2,154	-0.02 (-0.04; 0.01)	2,053	**-0.03 (-0.06; -0.004)**	0.00 (0.00; 0.01)
Fasting glucose	2,040	**-0.08 (-0.11; -0.05)**	1,916	**-0.06 (-0.09; -0.02)**	**0.01 (0.003; 0.02)**
HOMA-IR	1,956	**-0.12 (-0.15; -0.08)**	1,833	**-0.07 (-0.11; -0.03)**	**-0.01 (-0.02; -0.0002)**
High density lipoprotein	2,157	0.02 (-0.01; 0.04)	2,039	0.00 (-0.03; 0.03)	0.00 (0.00; 0.01)
Triglycerides	2,157	**-0.03 (-0.06; -0.01)**	2,039	**-0.03 (-0.06; -0.01)**	0.00 (-0.01; 0.01)
Low density lipoprotein	2,157	-0.02 (-0.04; 0.00)	2,038	-0.02 (-0.04; 0.01)	0.01 (0.00; 0.01)
Non-high density lipoprotein	2,157	**-0.02 (-0.05; -0.001)**	2,039	-0.02 (-0.05; 0.00)	0.01 (0.00; 0.01)
Apolipoprotein A1	2,152	0.02 (-0.01; 0.04)	2,030	0.01 (-0.02; 0.03)	0.00 (-0.01; 0.01)
Apolipoprotein B	2,148	**-0.03 (-0.05; -0.004)**	2,028	-0.02 (-0.05; 0.004)	0.00 (-0.01; 0.00)
Metabolic syndrome score[d]	2,030	**-0.12 (-0.20; -0.05)**	1,908	-0.09 (-0.17; 0.002)	-0.01 (-0.03; 0.01)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; HOMA-IR, homeostatic model assessment for insulin resistance.

The *ß* coefficient represents the *ß* unit change in the z-score of cardiometabolic risk markers per 5 ng/ml increase in 25(OH)D. The interaction *ß* coefficient represents the rate of change in the association between 25(OH)D and cardiometabolic risk markers (z-scores) per 1year increase in age on a multiplicative scale.

Associations at $p < 0.05$ are shown in bold.

[a] Crude model is adjusted for age and sex with age as a random slope;

[b] Adjusted model is additionally adjusted for study region, lifetime smoking and alcohol status, membership in sports club, screen time/week, BMI, parental education status and month of blood sample collection.

[c] Adjusted model including an interaction term between the 25(OH)D and age.

[d] Not adjusted for BMI.

Table 3: Association between insufficient and deficient 25(OH)D compared to normal levels of 25(OH)D with cardiometabolic risk markers

Cardiometabolic risk markers (z-score)	n	Insufficient[a]	Deficient[a]
		β (95% CI)	β (95% CI)
Waist circumference[b]	2,058	0.00 (-0.21; 0.21)	0.07 (-0.15; 0.29)
Systolic blood pressure	2,050	0.03 (-0.15; 0.22)	0.10 (-0.09; 0.28)
Diastolic blood pressure	2,050	0.11 (-0.07; 0.30)	0.18 (-0.01; 0.36)
Fasting glucose	1,916	0.10 (-0.12; 0.33)	0.20 (-0.03; 0.43)
HOMA-IR	1,833	0.23 (-0.03; 0.49)	**0.33 (0.07; 0.59)**
High density lipoprotein	2,036	-0.05 (-0.22; 0.12)	-0.06 (-0.23; 0.12)
Triglycerides	2,036	-0.09 (-0.26; 0.09)	0.00 (-0.18; 0.18)
Low density lipoprotein	2,035	-0.05 (-0.21; 0.10)	-0.05 (-0.21; 0.11)
Non-high density lipoprotein	2,036	-0.05 (-0.20; 0.11)	-0.03 (-0.19; 0.13)
Apolipoprotein A1	2,027	-0.02 (-0.20; 0.17)	-0.05 (-0.24; 0.13)
Apolipoprotein B	2,025	-0.04 (-0.21; 0.13)	-0.04 (-0.21; 0.13)
Metabolic syndrome score[b]	1,905	0.06 (-0.51; 0.63)	0.29 (-0.30; 0.87)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; HOMA-IR, homeostatic model assessment for insulin resistance.

The ß coefficient represents the ß unit change in the z-score of cardiometabolic risk markers in children with insufficient and deficient 25(OH)D compared to normal 25(OH)D levels.

Associations at $p < 0.05$ are shown in bold.

Associations are adjusted for age, sex, study region, lifetime smoking and alcohol status, membership in sport club, screen time/week, BMI, parental education status and month of blood sample collection with age as a random slope.

[a] deficient (< 20 ng/ml), insufficient (20-29 ng/ml), normal (30-50 ng/ml)

[b] Not adjusted for BMI