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ORIGINAL CONTRIBUTION

High maternal vitamin D levels in early pregnancy may protect against behavioral difficulties at preschool age: the Rhea mother–child cohort, Crete, Greece

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Abstract Animal studies suggest that prenatal vitamin D status may affect fetal brain growth. However, human studies are scarce with conflicting results. We aimed to investigate the association of maternal 25-hydroxyvitamin D [25(OH) D] levels with multiple neurodevelopmental outcomes at 4 years of age. We included 487 mother–child pairs from the prospective pregnancy cohort, "Rhea" in Crete, Greece. Maternal serum 25(OH) D concentrations were measured at the first prenatal visit (13 ± 2.4 weeks). Cognitive functions at 4 years were assessed by means of the McCarthy Scales of Children's Abilities. Behavioral difficulties were assessed by means of Strengths and Difficulties Questionnaire and Attention Deficit Hyperactivity Disorder Test. Children of women in the high 25(OH) D tertile (>50.7 nmol/l) had 37% decreased number of hyperactivity–impulsivity symptoms (IRR 0.63, 95% CI 0.39, 0.99, p_{trend} = 0.05) and 40% decreased number of total ADHD-like symptoms (IRR 0.60, 95% CI 0.37, 0.95, p_{trend} = 0.03) at 4 years of age, compared to children of women in the low 25(OH) D tertile (<38.4 nmol/l), after adjustment for several confounders. Similar associations were found with the hyperactivity/inattention score of the SDQ questionnaire. Children of mothers with high 25(OH) D levels had also fewer total behavioral difficulties (beta-coeff: −1.25, 95% CI −2.32, −0.19) and externalizing symptoms (beta-coeff: −0.87, 95% CI −1.58, −0.15) at preschool age. The observed associations were stronger in girls than in boys ($p_{for\ interaction}$ < 0.1). No association was observed between maternal 25(OH) D concentrations and cognitive function in preschoolers. Our results suggest that high maternal vitamin D levels in early pregnancy may protect against behavioral difficulties, especially ADHD-like symptoms at preschool age.

Keywords 25-hydroxyvitamin D · Pregnancy · ADHD · Behavior problems · Cognition · Preschool children

Abbreviations

ADHD	Attention deficit hyperactivity disorder
BMI	Body mass index
CI	Confidence interval
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
GAMs	Generalized additive models
IQ	Intelligence quotient
IRR	Incidence rate ratio

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MSCA	McCarthy Scales of Children's Abilities
SDQ	Strengths and Difficulties Questionnaire
SD	Standard deviation
TSH	Thyroid stimulating hormone

Introduction

Early pregnancy is a critical developmental time window for offspring growth and neurodevelopment [1]. Vitamin D has traditionally been viewed as a hormone essential for skeletal growth and calcium metabolism [2], but it has also multiple extraskeletal actions. As vitamin D amounts of the developing fetus are dependent on maternal stores, maternal vitamin D deficiency is of great concern for its consequences in the offspring. Recent data have shown a high prevalence of pregnant populations with vitamin D deficiency [3], even in countries with abundant sunshine [4]. In Europe, the prevalence of maternal vitamin D deficiency during pregnancy is similar or even higher in southern European countries, compared to central or northern countries, a phenomenon known as the vitamin D paradox in the Mediterranean region [5].

Low vitamin D concentrations during pregnancy have been related with fetal growth restriction [6], rickets [7], hypocalcaemia [8], respiratory tract infections [9], and allergic diseases [10]. Animal studies suggest that maternal vitamin D deficiency may impair fetus brain development [11]. Few epidemiological studies have examined so far the association between vitamin D status during pregnancy with offspring cognition [12–19] or behavioral difficulties [13–16, 20–22] with inconclusive results. Studies in the first half of pregnancy support an association of high maternal 25-hydroxyvitamin D [25(OH) D] levels, with improved mental and psychomotor development in infants [12], better receptive language development at 2 years of age [17], less language difficulties at 5 and 10 years of age [13], and a lower risk of developing ADHD-like symptoms in preschoolers [21]. Birth cohorts examining the impact of maternal 25(OH) D status in late pregnancy or cord blood levels on offspring neurodevelopment found very little [15, 18], or no association with offspring IQ [14] and no association with behavioral difficulties [14, 15] or ADHD diagnosis in mid childhood and adolescence [16, 22]. Differences in the sample size, timing of blood collection, and variation in outcome measures may partly explain the heterogeneity between studies. Additionally, most of them examined small sample sizes and had weak statistical power to detect a small to medium effect size.

We aimed to add to the above research more detailed data and investigate the associations of maternal 25(OH) D levels in early pregnancy with multiple neurodevelopmental outcomes, including neurocognitive function and behavioral difficulties at 4 years of age, in a prospective pregnancy cohort in Crete, Greece, after controlling for a wide range of confounders and effect modifiers.

Methods

Study design and population

The present study is part of the "Rhea" study, a prospective pregnancy cohort, at the prefecture of Heraklion, Crete, Greece. Detailed characteristics of the study population have been described elsewhere [23]. In brief, female residents who had become pregnant during the 12-month period starting in February 2007 participated in the study. Maternal inclusion criteria were the following: residents in the study area; pregnant women aged >16 years; no communication handicap. The study was approved by the Ethical Committee of the University Hospital of Heraklion (Crete, Greece) and all participants provided written informed consent after complete description of the study.

In total, 879 singletons participated at the 4-year follow-up, from October 2011 to January 2013, during which neurodevelopmental assessment was performed in 875 children (99.5%). We excluded 26 children with diagnosed neurodevelopmental disorders (i.e., pervasive developmental disorder), other severe medical disorders (i.e. plagiocephalus, microcephaly, hydrocephalus, brain tumor) and/or incomplete examination. Thus, 849 mother–child pairs were available for our analysis. From those, sufficient maternal serum from early pregnancy for 25(OH) D measurement was available for 497 mothers. We further excluded ten mother–child pairs with missing data for possible confounders. Thus, a cohort of 487 mother–child pairs (98% of the children with maternal 25(OH) D data and neurodevelopmental assessment) was available for the present analysis (Fig. 1). We observed no difference between the children included in the analysis and those that were excluded, except breastfeeding duration which was shorter in participants (Table S1).

Measures

Maternal 25(OH) D concentrations in early pregnancy

Maternal non-fasting serum samples during early pregnancy (13 ± 2.4 weeks) were collected in serum gel separator (BD 367958) tubes, centrifuged and stored at −80 °C until assayed. We used chemiluminescent immunoassay (CLIA) test (DiaSorin, Cat. No. 310600) to measure the total amount of 25(OH) D (both serum 25(OH) D_2 and 25(OH) D_3) [24]. The analytical range for the 25(OH) D assay was 10–375 nmol/L. Inter- and intra-assay precision

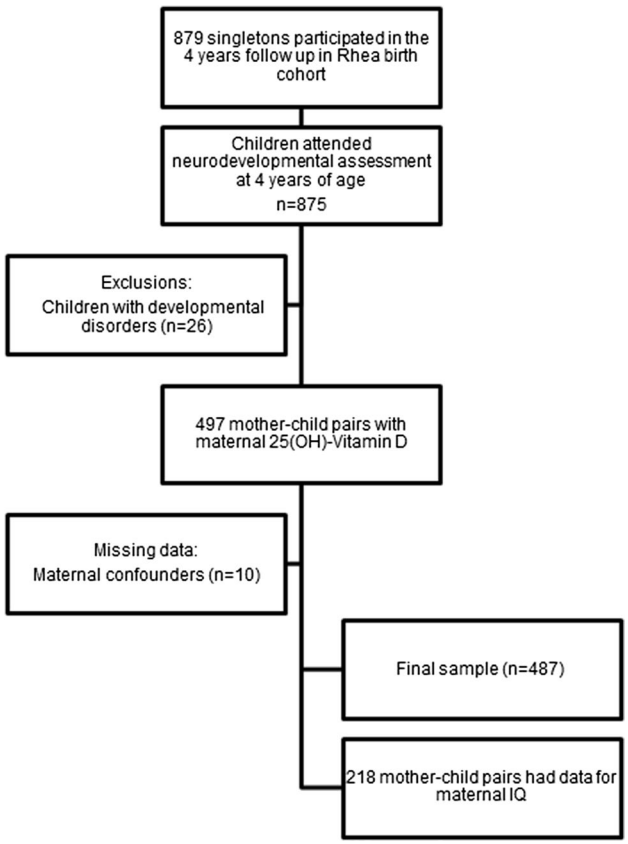

Fig. 1 Flowchart of participants

were <10 and <5%, respectively. We found a weak, but significant correlation between maternal vitamin D serum concentration and dietary intake as measured from a Food Frequency Questionnaire, which included 250 food items and was completed at the end of the first trimester of pregnancy (Spearman's $\rho = 0.12$; $p = 0.006$). FFQ did not take into account vitamin D taken from supplements. We asked about intake of vitamin D supplements in a separate questionnaire; however, none of the study participants were using such supplements. Maternal vitamin D concentration was treated as categorical divided into tertiles: tertile 1: <38.4 nmol/l; tertile 2: 38.4–50.7 nmol/l; tertile 3: >50.7 nmol/l.

Behavioral difficulties at 4 years of age

Information on children's behavior at 4 years of age was obtained via maternal report on two standardized child behavior scales. The parent version of the Strengths and Difficulties Questionnaire (SDQ) [25] is a 25-item behavioral screening instrument designed for children aged 3–16 years. It consists of five subscales generating scores for emotional symptoms, conduct problems, hyperactivity/inattention, peer relations problems, and prosocial behavior; all but the last one are summed to generate a total difficulties score, with a high score being less favorable (range 0–40). Two additional scores were calculated: the internalizing problems score by adding up the emotional and peer relationships subscales (range 0–20) and the externalizing problems score by adding up the conduct and hyperactivity subscales (range 0–20). The prosocial behavior scale provides information on protective factors of the child; a low score is less favorable. The SDQ has been translated and adapted for the Greek population [26]. Internal consistency (Cronbach's alpha) varied between 0.38 and 0.70.

The Attention Deficit Hyperactivity Disorder Test (ADHDT) [27] is designed to identify and evaluate ADHD symptoms in ages 3–23 years. It is composed of 36 items in three subscales; (1) hyperactivity, (2) inattention, and (3) impulsivity. All items were rated on a three-point scale (0 = never or rarely, 1 = mild, or 2 = severe).The ADHDT has been translated and adapted for the Greek population [28]. We used the ADHD Criteria of Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) to categorize ADHD-like symptoms. Three quantitative traits were generated for use in our analyses: (1) a count of the number of hyperactive–impulsive symptoms, (2) a count of the number of inattentive symptoms, and (3) a count of the total number of ADHD-like symptoms. In all three cases, a binary measure indicating the presence (severe) or absence (never/rarely or mild) of each symptom was measured and the totals were generated by summing over all symptoms, making the maximum number of symptoms 9, 9, and 18, respectively.

Neurodevelopmental assessment at 4 years of age

Children's cognitive and motor development was assessed by means of the McCarthy Scales of Children's Abilities (MSCA) at 4 years of age [29]. The MSCA is developed for children of ages 2½–8½ years, and includes five conventional subscales (verbal, perceptual performance, quantitative, memory, and motor). A general cognitive score was calculated by combining the verbal, perceptual performance, and quantitative scores [29]. Raw scores were standardized for child's age at test administration using a method for the estimation of age-specific reference intervals based on fractional polynomials. Standardized residuals were then typified having a mean of 100 points with a 15 SD to homogenize the scales. Higher scores represent better general cognition, language, or psychomotor development. The inter-rater reliability was very high for all scales (intra-class correlation coefficient ≥0.973). MSCA translation and cross-cultural adaptation were conducted according to the internationally recommended methodology. Internal consistency (Cronbach's alpha) varied between 0.76 and 0.89. Confirmatory factor analysis

supported good fit of the model ($\chi 2/df = 2$, CFI = 0.83, GFI = 0.97, RMSEA = 0.034) [30].

Procedure

Women were invited to provide blood and urine samples and to participate in a face-to-face interview at the first prenatal visit (mean (SD): 12.4 (1.6) weeks). Maternal intelligence quotient (IQ) was measured using the Raven's Standard Progressive Matrices test at the 4-year follow-up [31]. Children's cognitive and motor function at 4 years of age was evaluated by two trained psychologists through the McCarthy Scales of Children Abilities. The administration time ranged from 40 to 60 min. The examiners, also, noted critical comments about the difficulties or special conditions of the neurodevelopmental assessment, so as to evaluate the "quality of assessment" such as: no difficulties, difficulties due to physical problems (e.g., physical illness, tiredness, asleep), difficulties due to behavior problems (e.g., nervousness, shyness). Inter-observer variability was tested in a subsample of 12 children and was <1%. Additional information on children's behavior and ADHD-like symptoms was obtained via maternal report on the SDQ and ADHDT questionnaires. All testing was done at the University Hospital of Heraklion, and Medical Health Centres in the prefecture of Heraklion, Crete, Greece.

Potential covariates

Potential covariates included characteristics that have an established or potential association with the exposure and/or outcomes of interest including: maternal age at delivery (years), maternal education (low: ≤6 years of school; medium: ≤12 years of school; high: university or technical college degree), maternal origin (Greek; non-Greek), smoking during pregnancy (yes; no), parity (multiparous; primiparous), maternal pre-pregnancy BMI (kgr/m^2), maternal IQ measured using the Raven's Standard Progressive Matrices (Raven and Court 1996), first-trimester serum TSH levels, child's sex (male; female), birth weight (kgr), prematurity (preterm; non-preterm), and any breastfeeding duration (months, information on breastfeeding duration was collected during the 9th and the 18th months follow-up).

Statistical analysis

The primary outcomes of interest were SDQ scores, ADHD-like symptoms, and MSCA scores, at 4 years of age. SDQ and MSCA scores were treated as continuous variables, whereas ADHD-like symptoms were treated as quantitative traits. The primary exposure of interest was maternal 25(OH) D in early pregnancy. The distribution of mean 25(OH) D concentration was plotted by calendar month and showed a seasonal variation (Fig S1). As 25(OH) D concentrations followed a sinusoidal pattern, we fitted a cosinor model to the data to predict "deseasonalized" annual mean 25(OH) D concentration for each participant adjusted for month at blood collection.

Descriptive statistics were used to summarize the baseline characteristics of our study population. Bivariate comparisons of normally distributed variables were tested with ANOVA and non-normally distributed variables were tested with non-parametric Kruskal–Wallis test, whereas categorical variables were tested with Pearson's Chi-square test. Generalized additive models (GAMs) were applied to explore the shape of the relationships between 25(OH) D concentration in maternal serum and outcomes under study. These models did not indicate clear linear relationships (p gain defined as the difference in normalized deviance between the GAM model and the linear model for the same predictor < 0.10); thus, maternal 25(OH) D concentration was treated as categorical divided into tertiles. To test the dose–response relationship of 25(OH) D concentrations and outcomes of interest, p for trend was assessed ($p < 0.10$).

We used multivariate linear regression models to assess the association (beta-coefficient, 95% CI) of 25(OH) D levels in early pregnancy on SDQ and standardized MCSA scores at 4 years of age. We also examined the risk [incidence rate ratio (IRR), 95%] of the number of ADHD-like symptoms in association with 25(OH) D levels in early pregnancy using multivariate negative binomial regression models. Covariates were selected if they showed at least marginally significant association ($p < 0.1$) with exposures and outcomes of interest or if they modified the coefficient of maternal 25(OH) D concentration by at least 10% when included in the crude model. Information about child sex and age, the examiner who conducted the developmental testing, and quality of neurodevelopmental assessment were included as a priori confounders. Based on the previous criteria, we created two models: (1) the crude model, minimally adjusted for child sex and age for SDQ scores and ADHD-like symptoms as outcomes, and child sex, examiner, and quality of assessment for MCSA scores; (2) the adjusted model additionally adjusted for maternal age, education, parity, smoking during pregnancy, and pre-pregnancy BMI.

Because relations of 25(OH) D concentration with offspring neurodevelopmental outcomes could be confounded by maternal IQ, we repeated the analysis after adjusting for maternal IQ in a subsample ($n = 218$) for which information was available. To check for residual confounding, we also adjusted our final models for physical activity, maternal alcohol intake, and total energy intake during pregnancy as well as children's BMI at 4 years of age. We

further examined potential heterogeneity in associations related to maternal pre-pregnancy BMI, maternal TSH levels in early pregnancy, child's sex, birth weight z-score, and breastfeeding duration, by including a multiplicative interaction term in the models (statistically significant effect modification if p value < 0.10) and by stratifying the sample accordingly.

All hypotheses testing were conducted assuming a 0.05 significance level and a two-sided alternative hypothesis. Due to multiple hypotheses testing, Benjamini–Hochberg correction was performed post hoc to control for false discovery rate (FDR = 0.25). We used Stata S.E. version 13 for the statistical analyses (Stata Corp, Texas, USA).

Missing data

In the initial 842 SDQ and ADHDT questionnaires completed by parents at 4 years of age, there were missing values in 1–17 items of the ADHDT questionnaire for 114 subjects and in 1–9 items of the SDQ questionnaire for 57 subjects, respectively. Missing items were imputed to minimize the impact of lack of data and the identification of the number of ADHD-like symptoms based on imputed data. We applied ordinal logistic chained equations to multiply imputed missing values (mi impute procedure in STATA 13.0), and 20 imputed data sets were generated. We have repeated the analysis using cases with complete data and interpretation of results remained unchanged, even though some associations lost statistical significance due to sample size reduction (data not shown).

Results

Sample characteristics

The socio-demographic characteristics of our study population are described in Table 1. Most mothers had a medium education level (52.4%), 46% were primiparous, and 35% were smokers during pregnancy. The mean

Table 1 Maternal and child characteristics by maternal 25(OH) D levels in early pregnancy ($n = 487$)

	Maternal 25(OH) D levels				
	Overall	Tertile 1 < 38.4 nmol/l	Tertile 2 38.4–50.7 nmol/l	Tertile 3 > 50.7 nmol/l	p value[a]
Maternal 25(OH) D (nmol/l), *mean (SD)*	46.3 (15.4)	30.4 (6.3)	45.1 (3.5)	63.4 (10.4)	**<0.01**
Season of maternal blood collection, *n* (%)					0.05
Winter	90 (18.5)	33 (20.4)	29 (17.8)	28 (17.2)	
Spring	142 (29.2)	43 (26.5)	62 (38.1)	37 (22.9)	
Summer	149 (30.6)	50 (30.9)	46 (28.3)	53 (32.8)	
Autumn	106 (21.7)	36 (22.2)	26 (15.8)	44 (27.1)	
Maternal characteristics					
Age at delivery (yr), mean (SD)	29.7 (5.0)	30.1 (5.1)	29.4 (5.2)	29.6 (4.8)	0.66
Education, *n* (%)					0.93
Low	77 (15.8)	23 (14.2)	28 (17.2)	26 (16.1)	
Medium	255 (52.4)	88 (54.3)	85 (52.1)	82 (50.6)	
High	155 (31.8)	51 (31.5)	50 (30.7)	54 (33.3)	
Primiparous, *n* (%)	224 (46.0)	70 (43.2)	85 (52.2)	69 (42.6)	0.15
Smoking during pregnancy, *n* (%)	171 (35.1)	60 (37.1)	62 (38.1)	49 (30.3)	0.27
Pre-pregnancy BMI (kg/m^2), mean (SD)	25 (5.0)	26 (5.8)	25 (4.9)	24 (4.0)	**0.01**
Gestational weight gain (kg), mean (SD)	13.7 (5.6)	13.5 (6.5)	13.8 (5.4)	13.9 (4.8)	0.81
TSH levels(μIU/mL), mean (SD)	1.3 (1.1)	1.4 (1.1)	1.3 (1.0)	1.2 (1.2)	0.27
Child characteristics					
Sex, girl, *n* (%)	230 (47.3)	79 (48.8)	73 (44.8)	78 (48.2)	0.74
Birth weight (kg), mean (SD)	3.2 (0.5)	3.2 (0.4)	3.2 (0.5)	3.2 (0.4)	0.48
Gestational age (weeks), mean (SD)	38.3 (1.6)	38.2 (1.7)	38.2 (1.7)	38.4 (1.3)	0.52
Breastfeeding duration (months), mean (SD)	3.8 (4.1)	3.2 (3.4)	4.0 (4.7)	4.3 (4.0)	**0.03**
BMI (Kg/m^2) at 4 years, mean (SD)	16.5 (1.9)	16.7 (2.0)	16.4 (2.0)	16.4 (1.7)	0.16

BMI body mass index, *TSH* thyroid stimulating hormone

[a] Obtained by Kruskal–Wallis test for more than two independent variables, and $\chi 2$ test or Fisher exact test for categorical variables. Bolds indicate statistically significant differences at $p < 0.05$

(SD) concentration of maternal circulating 25(OH) D was 46.3 (15.4) nmol/l. The second tertile higher threshold (50.7 nmol/l) corresponded well with the recently used definition of vitamin D deficiency as a 25(OH) D concentration <50 nmol/l [31], indicating that two-thirds of pregnant women suffered from vitamin D deficiency. Women in the low 25(OH) D tertile (<38.4 nmol/l) had a higher mean BMI pre-pregnancy and were more likely to breastfeed their children for a shorter interval. We included 257 (52.7%) boys and 230 (47.3%) girls in the present analysis; the mean (SD) age was 4.2 (0.2) years and the mean (SD) birth weight was 3.2(0.5) kg.

Behavioral difficulties

Compared with children of mothers in the low 25(OH) D tertile (<38.4 nmol/l) in early pregnancy children of mothers in the high 25(OH) D tertile (>50.7 nmol/l) had 37% fewer hyperactivity–impulsivity symptoms (IRR 0.63, 95% CI 0.39, 0.99, $p_{trend} = 0.05$) and 40% fewer total ADHD-like symptoms (IRR 0.60, 95% CI 0.37, 0.95, $p_{trend} = 0.03$) at preschool age, after adjustment for several confounders (Fig. 2).

Similar associations were observed between 25(OH) D tertiles in early pregnancy and hyperactivity/inattention subscale score of SDQ questionnaire at preschool age (Table 2). Additionally, children of mothers in the high 25(OH) D tertile in early pregnancy had a significant score reduction in total behavioral difficulties (beta-coeff: −1.25, 95% CI −2.32, −0.19) and more specifically in the scale of externalizing symptoms (beta-coeff: −0.87, 95% CI −1.58, −0.15) at 4 years of age (Table 2). Effect estimates of the crude models for the associations between maternal vitamin D concentrations and behavioral outcomes under study did not differ substantially from the final models adjusted for maternal and child characteristics (Table 2, Table S2).

Neuropsychological outcomes

We did not find a significant association between maternal 25(OH) D tertiles in early pregnancy and offspring cognitive and motor function at preschool age in crude models, although we observed a trend of higher scores in almost all MSCA subscales among children of women in the high 25(OH) D tertile (>50.7 nmoll/l) (Table S2). Effect estimates remained to a large extent the same after adjustment for maternal and child characteristics (Table S3).

Effect modification–sensitivity analyses

Further analyses showed that the observed associations were more pronounced in girls than in boys (p for interaction <0.10, Table S4). We saw no evidence for a multiplicative interaction of maternal vitamin D tertiles in early pregnancy with maternal pre-pregnancy BMI, TSH levels, child's birth weight z score and breastfeeding duration.

Further adjustment for maternal intelligence in the subsample ($n = 218$) for which maternal IQ data were available did not change the direction of associations, though confidence intervals were wider, probably due to small sample size (Table S5). The observed associations remained substantially the same, after adjustment for physical activity, alcohol intake during pregnancy, total energy intake during pregnancy, and children's BMI at 4 years of age, although some of our main findings lost statistical significance, probably due to sample size reduction (Table S6). Because the effect of season is close to significant (Table 1) for our analysis, we repeated the analysis by including season in regression models and the associations remained the same (Table S7). To elucidate whether gestational diabetes, gestational hypertension, or prematurity modified the observed results, we performed a sensitivity analysis in which we excluded (a) women diagnosed with gestational diabetes ($n = 41$), (b) women diagnosed with gestational hypertension ($n = 21$), and (c) children born preterm (<37 gestational weeks, $n = 58$). We found no substantial differences in observed estimates (data not shown).

Discussion

In this prospective pregnancy cohort, we examined different domains of child neuropsychological and behavioral development at preschool age affected by vitamin D levels in early pregnancy. To our knowledge, this is the first study to examine the impact of maternal vitamin D status in early pregnancy on both cognitive function and behavioral difficulties in preschoolers. We showed that exposure to high 25(OH) D levels (>50.7 nmol/l) was associated with reduced number of hyperactivity–impulsivity and total ADHD-like symptoms, as well as total behavioral difficulties at preschool age. Our findings suggest that a vitamin D cutoff value of 50 nmol/l may be essential not only for bone health [32], but also for prevention of behavioral difficulties in the offspring. The observed associations persisted after adjustment for several maternal confounders and pre-pregnancy BMI. We also showed for the first time that child sex may modify the impact of vitamin D status in early pregnancy on offspring behavior at 4 years of age. We did not find a strong association between maternal vitamin D levels in early pregnancy and child cognitive and motor function at 4 years of age.

Although maternal vitamin D status has an important role in early brain development, data on the association between child neurodevelopment and vitamin D status in

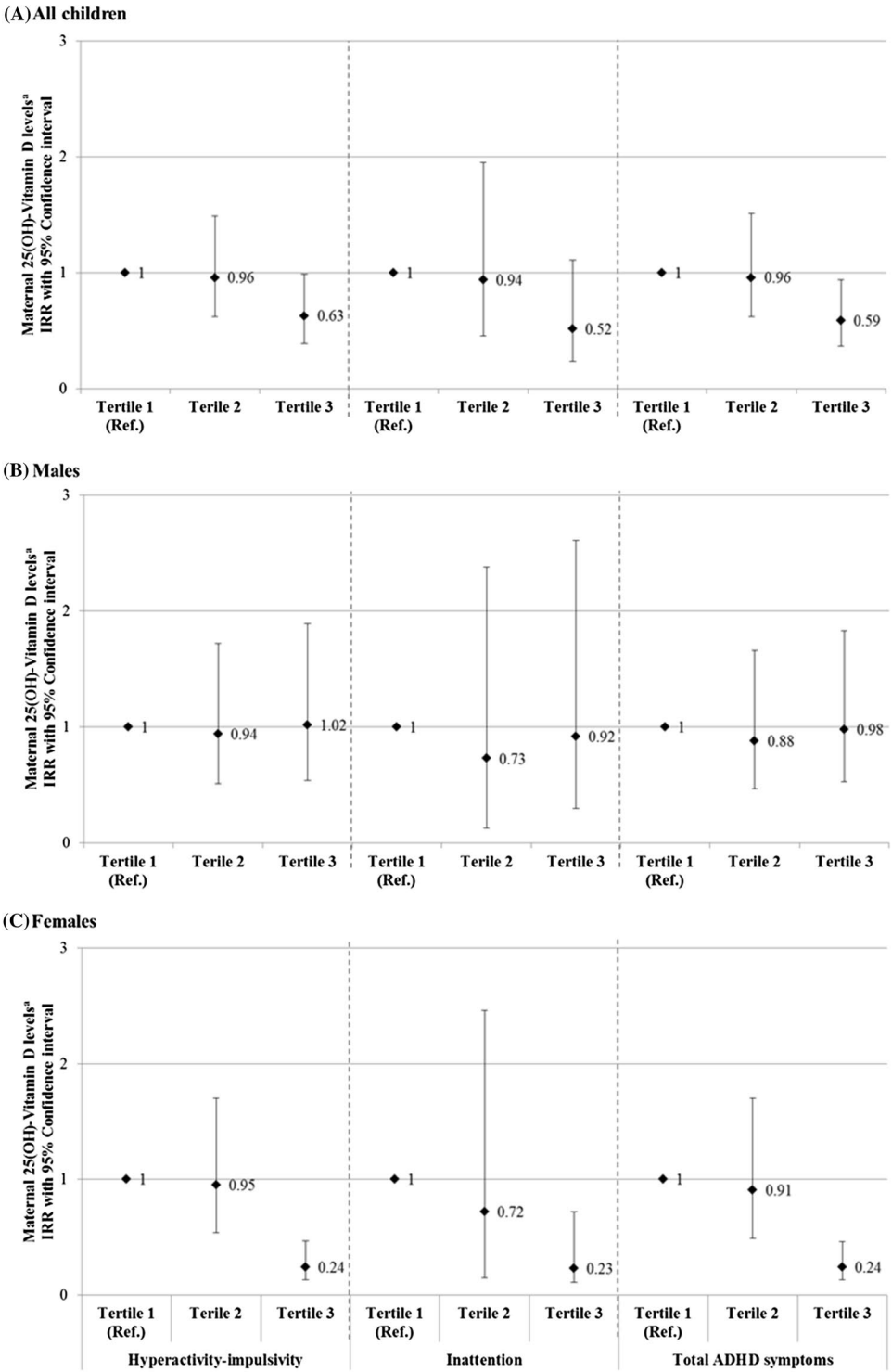

Fig. 2 Association [incidence rate ratio (IRR) with 95% confidence interval] of maternal 25(OH) D concentrations with ADHD-like symptoms in **a** all children, **b** males, and **c** females at 4 years of age. All models were adjusted for child age at assessment, maternal age, maternal education, parity, smoking during pregnancy, and maternal body mass index pre-pregnancy. The models including all children were additionally adjusted for child sex

early pregnancy are limited. Previous studies support that high maternal 25(OH) D levels in the first half of pregnancy were associated with improved mental and psychomotor development in infants [12], better receptive language development at 2 years of age [17], and less language difficulties at 5 and 10 years of age [13]. We examined a wide broad of cognitive abilities at 4 years of age and also found a trend of higher scores among children of women in the high 25(OH) D tertile. However, our results did not reach statistical significance probably due to the small sample size. We also found an inverse relationship between maternal vitamin D status in early pregnancy and behavioral difficulties, including ADHD-like symptoms at 4 years of age. Our results are consistent with the findings of Morales et al.

Table 2 Association of maternal 25(OH) D[a] levels in early pregnancy and offspring behavioral difficulties at 4 years of age ($n = 445$)

SDQ	Maternal 25(OH) D levels					
	Tertile 1 < 38.4 nmol/l		Tertile 2 38.4–50.7 nmol/l		Tertile 3 > 50.7 nmol/l	
	Ref.	β-coeff	95% CI	β-coeff	95% CI	$P_{\text{for trend}}$
Crude model						
Emotional symptoms	0	−0.04	−0.42, 0.33	−0.20	−0.58, 0.17	0.289
Conduct problems	0	0.15	−0.21, o.50	−0.14	−0.49, 0.21	0.426
Hyperactivity/inattention	0	−0.24	−0.73, 0.24	**−0.73**	**−1.21, −0.24**	**0.003**
Peer relationship problems	0	0.25	−0.06, 0.57	−0.26	−0.58, 0.05	0.093
Prosocial behavior	0	0.04	−0.37, 0.45	−0.05	−0.46, 0.35	0.793
Internalizing symptoms	0	0.21	−0.35, 0.77	−0.46	−1.02, 0.09	0.096
Externalizing symptoms	0	−0.09	−0.82, 0.64	**−0.87**	**−1.59, −0.14**	**0.018**
Total difficulties score	0	0.12	−0.98, 1.21	**−1.33**	**−2.42, −0.24**	**0.016**
Adjusted model						
Emotional symptoms	0	−0.07	−0.44, 0.29	−0.15	−0.52, 0.22	0.421
Conduct problems	0	0.11	−0.25, 0.46	−0.15	−0.50, 0.21	0.410
Hyperactivity/inattention	0	−0.35	−0.82, 0.13	**−0.72**	**−1.19, −0.25**	**0.003**
Peer relationship problems	0	0.23	−0.07, 0.54	−0.23	−0.54, 0.07	0.129
Prosocial behavior	0	0.07	−0.34, 0.48	−0.04	−0.44, 0.37	0.859
Internalizing symptoms	0	0.16	−0.38, 0.70	−0.38	−0.93, 0.16	0.157
Externalizing symptoms	0	−0.24	−0.95, 0.47	**−0.87**	**−1.58, −0.15**	**0.017**
Total difficulties score	0	−0.08	−1.14, 0.98	**−1.25**	**−2.32, −0.19**	**0.019**

Beta-coefficients (β-coeff) and 95% confidence intervals retained from linear regression models

Crude model: minimally adjusted for child sex and child age of assessment

Adjusted model: crude model further adjusted for maternal age, maternal education, parity, smoking during pregnancy, and maternal body mass index pre-pregnancy

SDQ Strengths and Difficulties Questionnaire

[a]Deseasonalized maternal 25(OH) D concentrations based on month at blood collection for each subject derived from the sinusoidal model. Bolds indicate statistically significant differences at $p < 0.05$, after Benjamini–Hochberg procedure for multiple testing correction

[21], who found a significant association between high 25(OH) D levels in early pregnancy and reduced number of ADHD-like symptoms in preschool children. However, we investigated more aspects of child behavior in our study and additionally found that high vitamin D levels in early pregnancy were associated with a reduced number of total behavioral problems, including externalizing symptoms at 4 years of age. Further adjustment for maternal IQ did not change the direction of the associations, suggesting a limited role of maternal genetic confounding.

It is well known that early pregnancy is a time window of particular vulnerability, as cortical structures critical to cognitive function and behavioral regulation are first formed. Maternal vitamin D performs a number of biological functions that are fundamental to early brain development [11], including proliferation and differentiation of brain cells [33], regulation of axonal growth [34], calcium signaling within the brain, and neurotrophic and neuroprotective actions [34]. In animal models, prenatal vitamin D deficiency has been associated with morphological changes [33] that may persist despite a postnatal return to normal vitamin D levels, resulting in abnormal behaviors in adulthood [35]. However, studies in humans are limited and plausible biological mechanisms are not clear yet.

In our study, we showed for the first time that child sex may modify the impact of maternal vitamin D status on offspring neurodevelopment. Higher levels of 25(OH) D in early pregnancy had a stronger protective effect on behavioral difficulties in females compared to males, especially on hyperactivity/inattention, externalizing symptoms and total ADHD-like symptoms. Limited data in adults have shown that immunomodulatory effects of vitamin D are significantly stronger in females than in males in multiple sclerosis patients supporting estrogen-promoted differences on vitamin D metabolism and action [36]. Whether there is also a functional synergy between estradiol and vitamin D action on prenatal brain development remains to be investigated.

With the vitamin D deficiency epidemic among pregnant women, the present results have important public health implications, which may be more profound in countries with higher prevalence of vitamin D deficiency.

Despite a hypothetical excess of sunshine hours in the Mediterranean region, maternal hypovitaminosis D remains common in pregnant populations of these countries [5]. In our study, we found that almost two-thirds of pregnant women (n = 313) had vitamin D deficiency [25(OH) D levels < 50 nmol/l] in early pregnancy [31]. Possible reasons for this paradox could be maternal darker skin pigmentation, poor dietary vitamin D intake, veiled clothing reducing sunshine exposure, and increased prevalence of obesity [5]. In addition, preventive strategies for maternal vitamin D deficiency in the Mediterranean region are lacking so far, as hypovitaminosis D is largely unrecognized and underrated in several South European countries [5].

The strengths of our study include its prospective population-based study design, well-established neurodevelopmental outcome measures, and control for several maternal and child characteristics. The inclusion of maternal intelligence, although available for a subsample of the total population, should be considered as an additional strength. We estimated maternal vitamin D status in early pregnancy by measuring circulating 25(OH) D concentration, a reliable indicator of vitamin D synthesis and intake. We minimized the potential effect of season in our results by using the deseasonalized variable of 25(OH) D in our analysis. Neurodevelopment assessment at preschool age was performed with the use of MCSA [29], a valid, standardized psychometric test which provides both a general level of child's intellectual functioning and an assessment of separate neurodevelopmental domains. In the present analysis, we used standardized neurodevelopmental scales (mean of 100 points with a 15 SD). There is extensive literature on the public health impact of a 1-point loss of a neuropsychological scale, but most are based on the effects of lead exposure on IQ [37]. Although a seemingly small change of a 1-point decrease in IQ score might not be relevant at the individual level, at the population level it is possible to shift the distribution of IQ to the left and increase the number of persons below the normal range [38].

A limitation of our study is the assessment of children's ADHD symptoms and behavioral difficulties by parent-reported measures, which could be different from assessments made by a health-care professional. However, both ADHDT and SDQ questionnaire are well established and widely used screening tools with high specificity and sensitivity. Although we incorporated extensive information on potential social and environmental factors that are associated with child neurodevelopment, we acknowledge that residual confounding because of other unmeasured confounders such as social class and child's vitamin D status may still occur.

In conclusion, our findings support a possible inverse relationship between vitamin D levels in early pregnancy and behavioral problems, especially hyperactivity/inattention, externalizing symptoms, and total ADHD-like symptoms in early childhood. These associations are more pronounced in females and may have important implications from a public health perspective. Whether these findings translate into long-term increased risk of abnormal behavior for the offspring of vitamin D-deficient women is unknown. However, unlike other causes of neurodevelopmental disorders, maternal vitamin D deficiency may be prevented. We speculate that appropriate supplementation during pregnancy may reduce the incidence of behavioral difficulties and ADHD-like symptoms later in life. Further investigation is needed to assess the long-term effects of vitamin D supplements in pregnancy on neuropsychological and behavioral development in offspring.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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