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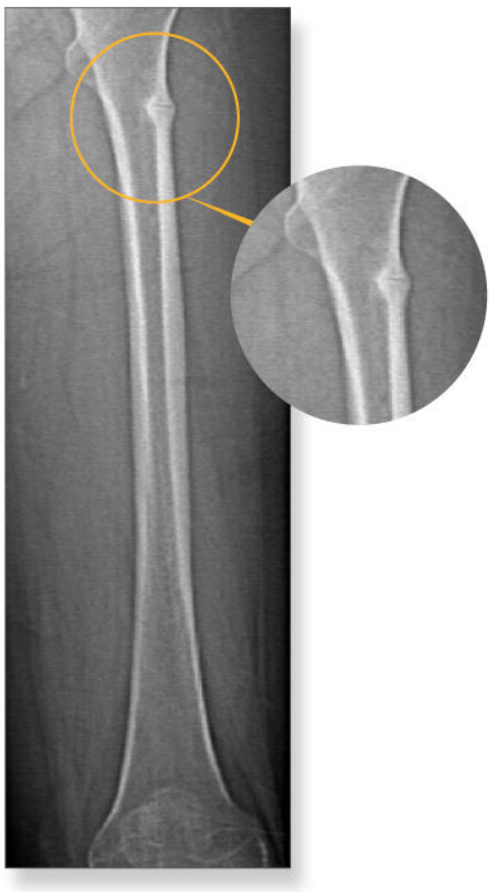

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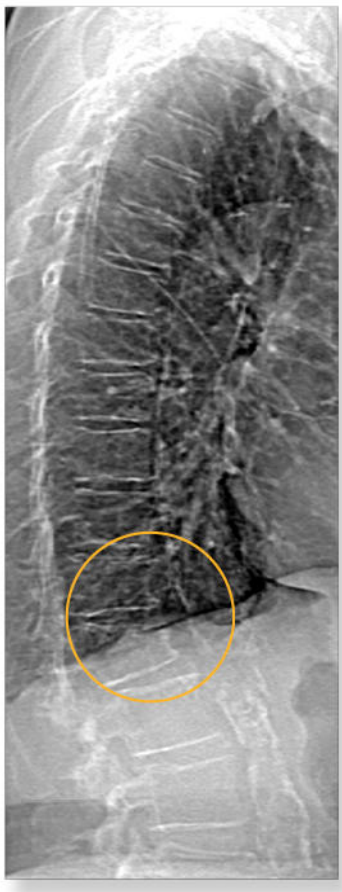

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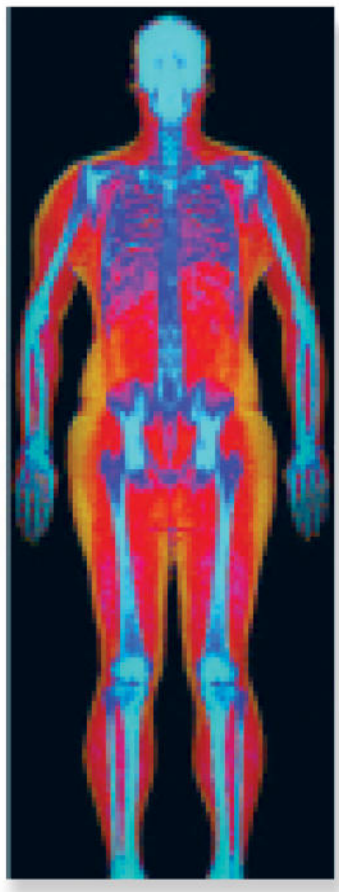

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ORIGINAL ARTICLE

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Relationship Between Neonatal Vitamin D at Birth and Risk of Autism Spectrum Disorders: the NBSIB Study

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ABSTRACT

Previous studies suggested that lower vitamin D might be a risk factor for autism spectrum disorders (ASDs). The aim of this study was to estimate the prevalence of ASDs in 3-year-old Chinese children and to examine the association between neonatal vitamin D status and risk of ASDs. We conducted a study of live births who had taken part in expanded newborn screening (NBS), with outpatient follow-up when the children 3-year old. The children were confirmed for ASDs in outpatient by the Autism Diagnostic Interview-Revised and Diagnostic and Statistical Manual of Mental Disorders (DSM)-5 criteria. Intellectual disability (ID) status was defined by the intelligence quotient (IQ < 80) for all the participants. The study design included a 1:4 case to control design. The concentration of 25-hydroxyvitamin D3 [25(OH)D3] in children with ASD and controls were assessed from neonatal dried blood samples. A total of 310 children were diagnosed as having ASDs; thus, the prevalence was 1.11% (95% CI, 0.99% to 1.23%). The concentration of 25(OH)D3 in 310 ASD and 1240 controls were assessed. The median 25(OH)D3 level was significantly lower in children with ASD as compared to controls ($p < 0.0001$). Compared with the fourth quartiles, the relative risk (RR) of ASDs was significantly increased for neonates in each of the three lower quartiles of the distribution of 25(OH)D3, and increased risk of ASDs by 260% (RR for lowest quartile: 3.6; 95% CI, 1.8 to 7.2; $p < 0.001$), 150% (RR for second quartile: 2.5; 95% CI, 1.4 to 3.5; $p = 0.024$), and 90% (RR for third quartile: 1.9; 95% CI, 1.1 to 3.3; $p = 0.08$), respectively. Furthermore, the nonlinear nature of the ID-risk relationship was more prominent when the data were assessed in deciles. This model predicted the lowest relative risk of ID in the 72rd percentile (corresponding to 48.1 nmol/L of 25(OH)D3). Neonatal vitamin D status was significantly associated with the risk of ASDs and intellectual disability. The nature of those relationships was nonlinear. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: AUTISM SPECTRUM DISORDERS; NEONATAL; VITAMIN D; 25 HYDROXYVITAMIN D3

Introduction

The prevalence of autism spectrum disorders (ASDs) has increased over recent years in much of the Western world.[1] Relatively little is known about the prevalence of ASDs in China. From 1986 to 2005, the estimated incidence of autism spectrum disorder was 5.49 per 10,000 in Hong Kong.[2] Furthermore, a meta-analysis shown that the long-term outcome of almost one-half of all individuals with autistic disorders was poor.[3] Thus, the need to understand the causes of ASDs and the underlying pathophysiology has become more meaningful.

Gene-environment interactions have recently been suggested play a role in the development of ASDs. However, the underlying mechanism or a specific metabolic target relevant to ASDs has not yet been identified. One study suggested that vitamin D deficiency—either during pregnancy or early childhood—may be an environmental trigger for ASDs in individuals genetically predisposed for the broad phenotype of autism.[4] Vitamin D deficiency was hypothesized to contribute to the increase in the incidence of ASDs.[5]

Population-based longitudinal studies on associations of vitamin D and ASDs has been reported.[6] In a meta-analysis, Wang and colleagues[7] found that levels of serum 25-hydroxyvitamin D [25(OH)D] in participants with ASDs were significantly lower than controls, suggesting that lower vitamin D level might be a risk factor for ASDs. Similarly, Mostafa and Al-Ayadhi[8] reported that autistic children had significantly lower serum levels of 25(OH)D than healthy children ($p < 0.001$),

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with 40% and 48% being vitamin D–deficient and vitamin D–insufficient, respectively. Furthermore, the vitamin D status might be affected by race, location and diet, sun, and lifestyle.[9–11] Therefore, it is necessary to study the relationship between vitamin D status and ASDs in the Chinese population. Using a population-based sample, the authors sought to estimate the prevalence ASDs in 3-year-old Chinese children and to directly examine the association between neonatal vitamin D status and risk of ASDs.

Materials and Methods

Study population

We conducted a cohort study of all native and singleton live births who had taken part in expanded newborn screening (NBS) in Beijing (NBSIB), China, from 2008 through 2010 ($n = 32{,}912$ births), with outpatient follow-up when the children were 3 years old. In 2006, new technology (liquid chromatography–tandem mass spectrometry [LC-MS/MS]) was introduced, which could look for a number of different chemicals at once using a tiny sample of blood. In Beijing, about 35 conditions (Expanded Newborn Screening Program, ENSP) were screened for as part of the NBS program.[12] Since 2006, the screening services had taken place in Center for Clinical Laboratory Development, Chinese Academy of Medical Science. Newborns who were participated in the ENSP need to meet the following two conditions: (i) more than 24 hours and less than 7 days after birth (the best time were between 48 and 36 hours after born); (ii) protein-containing feedings (formulas or breast milk) have started. The samples were collected by pricking the heels of the patients to get enough blood to fill a few circles on S&S Grade 903 filter paper.

For each child with a diagnosis of ASDs, we selected a 1-to-4 control sample to maximize statistical power, matching for possible variables, including gender, maternal age, paternal age, gestational age, parity, birth weight (±100 g), and time (±1 month) and hospital of birth (cohort effect). Children with four of those variables matched could be selected. Those controls were randomly selected from potential matches. The Jiangsu Normal University Institutional Review Board for the Protection of Human Subjects approved this study. Neither data nor specimens were collected until written informed consents were obtained from the parents.

Follow-up

In the NBS program, all children at the age of 3 years would obtain an opportunity for outpatient visits. This was a routine examination and free of charge. One month ahead of study endpoints (3 years old) the staff from screening center would contact the parents to determine the time of outpatient visits. In the outpatient follow-up, the Autism Diagnostic Interview-Revised (ADI-R)[13] and Diagnostic and Statistical Manual of Mental Disorders (DSM)-5 criteria[14] were used to confirm a diagnosis of ASDs. Those protocols were translated into Chinese Mandarin. Chinese-translated materials were provided in advance. Those works were finished by authors M-QL and Y-LZ, who had undergone a standard pre-job training according to a Mandarin Chinese version.[15] Reliability analysis of the diagnosis (internal consistency) yielded Cronbach's α of 0.90 in the pilot study ($n = 100$). In addition, 200 screen-negative cases were tested with the ADI-R, which suggested sensitivity with 94% and specificity with 88%.

Clinical variables

Maternal and paternal demographic characteristics, infant characteristics (gender, maternal age, paternal age, gestational age, birth weight, parity, time and hospital of birth, prolonged labor, parental depression, chronic complication before pregnancy, pregnancy-induced complication, mode of delivery, transfer to neonatal intensive care unit (NICU) after delivery, breech presentation, premature rupture of membranes, pre-eclampsia, fetal distress, meconium, birth injury or trauma, maternal hemorrhage, 5-min Apgar score, congenital infection, respiratory distress syndrome, neonatal encephalopathy, assisted ventilation, and congenital anomaly [defined by birth records]) were derived from birth certificates and hospital records of pregnant women. Maternal delivery payer source was derived from the infant's hospital discharge record. NBS results, family structure, family income, education background, whether ASDs diagnosis had been confirmed or expected before follow-up, and maternal, paternal, and sibling history of mental illness subdivided hierarchically as ASDs were obtained from outpatient follow-up. Family socio-professional category (low, medium, and high) was defined according to academic qualifications, occupation, and income. Intellectual disability (ID) status was defined by the intelligence quotient ($IQ < 80$) for all the participants.[16] IQ was assessed using the Combined Raven's Test and then converted to a standard IQ score according to Chinese children's norm.[17]

25(OH)D3 tested by LC-MS/MS

The concentration of 25(OH)D3 in children with ASDs and controls was assessed from neonatal dried blood samples. For each individual, 25(OH)D3 was extracted from 3.2 mm dried blood sample (DBS) punches, derivatized with 4-phenyl-1, 2, 4-triazoline-3, 5-dione (PTAD) prior to analysis. Samples were reconstituted in 50 μL of 40% acetonitrile/water and 25 μL was injected onto an API 3000 QTRAP Triple Quadrupole, Linear Ion-Trap LC-MS-MS mass spectrometer (Applied Biosystems, Foster City, CA, USA) connected to a Dionex Ultimate 3000 LC system. The assay method is highly sensitive and uses minimal sample cleanup to reduce sample loss during extraction, chemical derivatization to enhance 25(OH)D3 ionization, and liquid chromatography tandem mass spectrometry coupled with multiple reactant monitoring. From a previous small sample study ($n = 120$), we showed that the assay can reliably detect seasonal (within year) fluctuations and is strongly correlated with neonatal cord blood ($r = 0.885$; Supporting Fig. 1). Moreover, appropriate 25(OH)D3 concentrations can still be detected after prolonged storage time (greater than 5 years in our study). The correlation of 25(OH)D3 concentrations between samples collected and 5 years later can reach 0.943 ($n = 100$; $p < 0.001$).

We obtained external accuracy calibrators for 25(OH)D3 in frozen serum (standard reference materials 968e and 1950) from the National Institute of Standards and Technology (Gaithersburg, MD, USA). We prepared calibrators for DBS as described by Eyles and colleagues.[18] Briefly, 25(OH)D3 stock solutions were prepared in ethanol and spectrometrically verified. Ethanolic solutions of 25(OH)D were gently mixed with fresh whole blood from a healthy male adult donor for [25(OH)D3 44.6 (2.0) nmol/L, uncorrected for hematocrit] (0.25% vol/vol total ethanol [10 μL ethanol standard solution plus 3.99 mL blood]) 30 min. DBS (50 μL) was then spotted at a uniform flow rate with an automatic dispenser and dried at room

temperature overnight. Final calibrator concentrations were 5, 10, 25, 50, 80, and 125 nmol/L above the baseline blood 25 (OH) D concentrations.[19] The baseline concentration was subtracted to produce a 0 point in the calibration curve. We prepared sera calibrators in a similar manner. The concentration of 25(OH)D3 is reported in nanomoles per liter (nmol/L) and, as the protein-bound molecule is excluded from erythrocytes, results are reported as sera concentrations adjusted to account for the increased hematocrit in capillary blood. At the three concentrations examined (12.5, 25, 125 nmol/L), intraday recoveries from DBS were 84% to 116%. At these same concentrations, inter-run recoveries from DBS were 85–106%. Intra-run imprecision at these same concentrations was 10% to 15%. Inter-run imprecision from DBS ranged from 5% to 10%. With respect to the lower limit of quantification (LLOQ), our assay was able to detect 7.5 nmol/L of 25(OH)D3.

In this study, the concentration of 25(OH)D3 in ASD and controls were assessed from neonatal dried blood samples by LC-MS/MS. The validation of 25(OH)D3 measurement from dried blood had been illustrated in previous studies.[18,20,21] Eyles and colleagues[18] reported that both 25(OH)D3 and 25(OH)D2 can be reliably quantified in archived 3.2-mm dried blood spots, and another study found that 25(OH)D3 concentrations in neonatal cord serum and DBS were highly correlated ($r = 0.85$, $p < 0.0001$), and archived DBS samples provided a valid measure of perinatal vitamin D status.[20] Furthermore, Newman and colleagues[21] described that the blood spot assay for 25(OH)D3 and 25(OH)D2 provides a convenient and cost-effective alternative to serum assays and can be automated.

Statistical methods

Results are expressed as percentages for categorical variables and as means ± standard deviation (SD) and medians (interquartile ranges [IQRs]) for the continuous variables, depending on the normal or non-normal distribution of data. Shapiro-Wilk tests were used for normal distribution test. Proportions were compared using the chi-square test. Two-group comparison of not normally distributed data was performed using Mann–Whitney *U* test, and a two-tailed Student's unpaired *t* test was used for normally distributed continuous variables.

The main analyses were based on quartiles for 25(OH)D3 in the control sample, using conditional logistic regression. Owing to the matching scheme, this means that all relative risks were controlled for gender, maternal age, paternal age, gestational age, parity, time of day of birth, and hospital of birth. To investigate whether 25(OH)D3 allows predicting of both ASDs and ID, different statistical methods were used. In this study, we included a range of variables as potential confounds in the analyses. These include parental depression, chronic complication before pregnancy, pregnancy-induced complication, mode of delivery, breech presentation, premature rupture of membranes, fetal distress, meconium, birth injury or trauma, maternal hemorrhage, transfer to NICU after delivery, 5-min Apgar score, congenital infection, respiratory distress syndrome, neonatal encephalopathy, assisted ventilation, congenital anomaly, NBS results, family structure, family income, education background, and maternal, paternal, and sibling history of mental illness subdivided hierarchically as ASDs. For multivariate analysis, we included 25(OH)D3 and other theoretical confounders.

To examine 25(OH)D3 as a continuous variable, we also used second-degree fractional polynomials to explore the relationship between the variables of interest.[22] If the exposure-risk relationship was nonlinear, we planned further analyses to explore the nature of the relationship using more fine-grain quantiles and individually assessing the list of variables previously included as covariates for interaction effects with 25(OH)D3.

Last, vitamin D deficiency was defined as serum 25(OH)D concentrations <30 nmol/L.[23] The relationship between 25 (OH)D3 and ASD was also assessed in multivariate analysis. All statistical analysis was performed with SPSS for Windows, version 20.0 (IBM Corp., Armonk, NY, USA) and STATA 9.2 (Stata Corp, College Station, TX, USA), R version 2.8.1 (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org/). Statistical significance was defined as $p < 0.05$.

Results

In our study, 27,940 newborns were included and finished the 3-year follow-up (see Fig. 1). A total of 310 were diagnosed as having ASDs, thus the prevalence of ASDs was estimated to be 1.11% (95% CI, 0.99% to 1.23%). The prevalence in males was estimated to be 1.65% (95% CI, 1.44% to 1.86%), whereas the prevalence in females was estimated to be 0.52% (95% CI, 0.40% to 0.64%). The male-to-female ratio was 3.43:1, and the ratios of autistic disorders to other ASD subtypes was 2.44:1. The rate of children who had ID was significantly lower in controls as compared with ASDs groups (2.26% versus 32.58%, $p < 0.001$). Table 1 represents the baseline characteristics of the enrolled children with ASDs and controls.

In addition, the concentration of 25(OH)D3 in 310 children with ASDs and 1240 controls were assessed. Concentrations of 25(OH)D3 were compared based on four seasons of blood sampling. Significant seasonal differences in 25(OH)D3 concentrations were observed (analysis of variance [ANOVA]: $p = 0.004$; Supporting Fig. 2). The children with ASD in the summer have the highest 25(OH)D3 concentrations (median: 21.9 mmol/L; IQR, 17.2 to 38.2 mmol/L). Similarly, results were obtained from the control children (data not shown). Furthermore, we examined the relationship between several variables and vitamin D state including age in collection of the sample, sex, race, and diagnosis of neonatal hyperbilirubinemia. None of these analyses could account for vitamin D state ($p > 0.05$). Furthermore, 58 children in the ASD groups were categorized as delay in sample collection (defined as samples collected at age older than 7 days). The median 25(OH)D3 level was not significantly different in the delayed samples group as compared to the normal group (17.8 nmol/L [IQR, 12.5 to 27.8 nmol/L] versus 17.5 nmol/L [IQR, 12.3 to 27.2 nmol/L]; $p = 0.403$).

The median 25(OH)D3 level was significantly lower in children with ASD as compared to controls (17.6 nmol/L [IQR, 12.4 to 27.4 nmol/L] versus 40.2 nmol/L [IQR, 27.7 to 48.8 nmol/L]; $Z = 11.967$; $p < 0.0001$; Fig. 2*A*). Similarly, the median 25(OH) D3 level was significantly lower in children who had an ID as compared with those who did not have an ID (17.3 nmol/L [IQR, 12.6 to 25.9 nmol/L] versus 40.5 nmol/L [IQR, 27.8 to 49.0 nmol/L]; $Z = 12.986$; $p < 0.0001$; Fig. 2*B*). Factors associated with ASDs risk in the univariate analysis were parental depression, breech presentation, fetal distress, transfer to NICU after delivery, winter birth, NBS positive results, sibling history of ASDs and low 25 (OH)D3 level (Table 2). For multivariate analysis, to best display the relationship between the variables of interests, neonates in the fourth quartiles were chosen as the reference category.

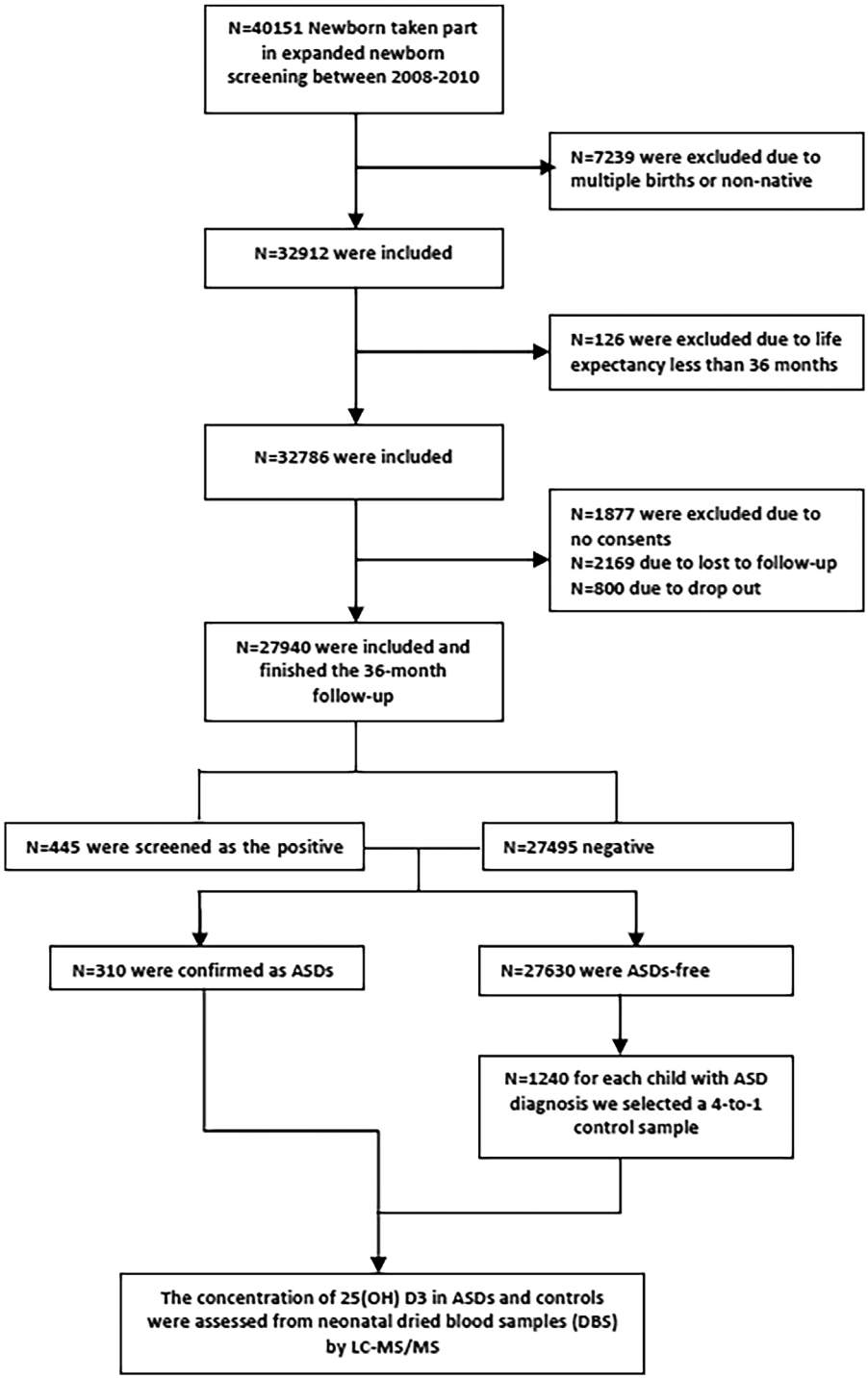

Fig. 1. Study population.

The relative risk of ASDs was significantly increased for neonates in each of the 3 lower quartiles of the distribution of 25(OH)D3. Compared with the reference category, neonates in the lowest quartile had a relative risk of 3.6 (95% CI, 1.8 to 7.2; $p < 0.001$), whereas those in the second and third quartiles had relative risks of 2.5 (95% CI, 1.4 to 3.5; $p = 0.024$) and 1.9 (95% CI, 1.1 to 3.3; $p = 0.08$), respectively (Table 3). For a more detailed exploration of the 25(OH)D3-ASD relationship, we also used multivariate analysis models to estimate adjusted OR and 95% CIs of ASD for 25(OH)D3 quintiles (with highest 25(OH)D3 quintile as reference versus quintile 1 to 4). Compared with the reference category (quintile 5), neonates in quintile 1 to 4 had a relative risk of 3.3 (95% CI, 1.9 to 6.8; $p < 0.001$) for ASD. Furthermore, ID was also considered as a covariate in multivariate analysis models. Compared with the reference category (quintile 5), neonates in quintile 1 to 4 had a relative risk of 2.8 (95% CI, 1.5 to 5.5; $p = 0.006$) for ASD.

Table 1. Baseline Characteristics of the Enrolled Children With ASDs and Controls

	Children with ASDs	Controls	p^a
n	310	1240	
Maternal age (years), mean ± SD	30.1 ± 5.3	30.3 ± 5.4	0.904
Han Chinese, %	93.5	91.9	0.894
Paternal age(years), mean ± SD	31.4 ± 5.5	31.6 ± 5.6	0.932
Gestational age at birth (days), mean ± SD	272 ± 15.0	270 ± 14.9	0.684
Parity, mean ± SD	1.10 ± 0.28	1.10 ± 0.28	1.000
Gravidity, mean ± SD	1.62 ± 0.34	1.65 ± 0.35	0.704
Birth weight (g), mean ± SD	3350 ± 612	3363 ± 615	0.683
Preterm birth (<37 weeks), %	11.3	8.1	0.072
Age at blood samples collected (days), mean ± SD	3.5 ± 1.8	3.3 ± 1.6	0.208
Month of birth, %			0.826
March–May	27.4	25.2	
June–August	23.5	24.5	
September–November	22.9	24.2	
December–February	26.1	26.1	
Assisted delivery, %	38.7	30.6	0.007
Preeclampsia, %	3.9	3.2	0.422
Parental depression, %	11.3	7.7	0.038
Chronic complication before pregnancy, %	10.3	11.3	0.576
Pregnancy-induced complication, %	12.3	11.9	0.308
Transfer to NICU, %	6.8	3.9	0.006
Sex of child, male, %	77.4	77.4	1.000
Autistic disorders, %	70.9	–	
Superior IQs, %	8.06	14.19	0.004
ID, %	32.58	2.26	<0.001
ASDs diagnosis confirmed or expected before follow-up (%)	24.19	–	
NBS positive, %	1.94	0.16	<0.001
Marital status, single, %	8.7	8.1	0.403
Family's socio-professional category, high, %	10.3	12.9	0.217
Family history of ASDs, %	9.4	0.65	<0.001
Mothers or infants with vitamin D supplements, %	85.2	88.7	0.086

[a]Values of p were compared by Student's unpaired t test or chi-square test as appropriate.

ASD = autism spectrum disorder; SD = standard deviation; NICU = neonatal intensive care unit; IQ = intelligence quotient; ID = intellectual disability; NBS = newborn screening.

A total of 239 children with autism suffered from vitamin D deficiency, giving a prevalence rate of 77.1% (95% CI, 72.4% to 81.8%). In contrast, 28.5% (353/1240; 95% CI, 26.0% to 31.0%) of the controls acknowledged vitamin D deficiency. The difference between groups was statistically significant (OR: 8.46; 95% CI, 6.32 to 11.33; $p < 0.001$). Furthermore, in multivariate analysis models, for vitamin D deficiency, the adjusted risk of ASD increased by 268% (OR: 3.68; 95% CI, 2.03 to 5.24, $p < 0.001$).

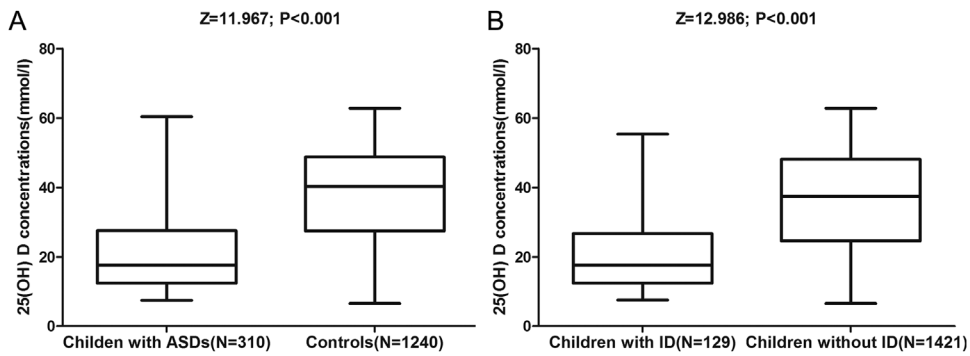

Fig. 2. The 25(OH)D3 level in different groups. (*A*) The median 25(OH)D3 level was significantly lower in children with ASDs as compared to controls (17.6 nmol/L [IQR, 12.4 to 27.4 nmol/L] versus 40.2 nmol/L [IQR, 27.7 to 48.8 nmol/L]; $Z = 11.967$, $p < 0.0001$). (*B*) The median 25(OH)D3 level was significantly lower in children who had an ID as compared with those who did not have an ID (17.3 nmol/L [IQR, 12.6 to 25.9 nmol/L] versus 40.5 nmol/L [IQR, 27.8 to 49.0 nmol/L]; $Z = 12.986$, $p < 0.0001$). ASD = autism spectrum disorder; 25(OH)D3 = 25 hydroxyvitamin D3.

Table 2. Conditional Logistic Regression Analysis of Factors Associated With ASDs at 3 Years

Factors	Crude OR	95% CI	*p*	aOR[a]	95% CI	*p*
Prolonged labor	1.52	0.89–3.06	0.36			
Parental depression	1.69	1.32–2.65	0.04	1.45	0.88–2.98	0.22
Chronic complication before pregnancy	1.75	0.24–12.55	0.57			
Pregnancy-induced complication	1.47	0.69–3.35	0.31			
Assisted delivery	2.44	1.31–4.40	0.007	1.50	0.68–3.48	0.33
Transfer to NICU after delivery	2.33	1.54–3.21	0.006	1.68	1.09–2.75	0.04
Breech presentation	2.81	1.55–4.02	0.005	1.44	1.09–1.84	0.03
Premature rupture of membranes	1.16	0.84–1.83	0.27			
Fetal distress	0.73	0.30–1.89	0.49			
Meconium	0.49	0.14–2.01	0.32			
Birth injury or trauma	3.44	1.21–8.48	0.23			
Maternal hemorrhage	2.11	1.11–3.87	0.18			
Neonatal encephalopathy	1.92	1.22–3.21	<0.001	1.32	1.12–1.85	0.006
Decreased 5-min Apgar score	1.64	1.21–2.20	0.01	1.05	0.75–2.61	0.58
Preeclampsia	1.13	0.45–5.66	0.42			
Congenital infection	1.48	0.33–8.11	0.39			
Respiratory distress syndrome	2.72	0.70–10.53	0.47			
Assisted ventilation	1.06	0.60–3.40	0.62			
Congenital anomaly	1.94	0.52–7.25	0.56			
NBS positive results	2.88	1.65–4.14	<0.001	2.01	1.32–3.42	0.003
Family single structure	1.65	1.07–2.55	0.16			
College graduate or more	1.11	0.85–3.21	0.16			
Family high income	1.38	0.68–3.79	0.28			
Family history of ASDs	14.46	7.56–24.15	<0.001	6.55	2.77–12.54	<0.001
25(OH)D3	0.82	0.77–0.89	<0.001	0.89	0.86–0.93	<0.001

[a]Adjusted for maternal age, paternal age, gestational age, parity, prolonged labor, parental depression, chronic complication before pregnancy, pregnancy-induced complication, assisted delivery, baby sex and weight, day and hospital of birth, breech presentation, premature rupture of membranes, transfer to NICU after delivery, fetal distress, meconium, birth injury or trauma, maternal hemorrhage, neonatal encephalopathy, decreased 5-min Apgar score, preeclampsia, respiratory distress syndrome, assisted ventilation, congenital infection, NBS positive results, family single structure, family income and history of ASDs.

ASD = autism spectrum disorder; OR = odds ratio; aOR = adjusted OR; CI = confidence interval; NICU = neonatal intensive care unit; NBS = newborn screening; 25(OH)D3 = 25 hydroxyvitamin D3.

Figure 3*A* shows that the nonlinear nature of the exposure–risk relationship was more prominent when the data were assessed in deciles (based on the control 25(OH)D3 values). The gray line shows the best fit analysis for the continuous data (a second-degree fractional polynomial with orders 3 and 3). This polynomial had a significantly better fit compared with both a straight line ($p = 0.032$) and the null model ($p = 0.006$). This model predicted the lowest relative risk of ASDs in the 76rd percentile (corresponding to 49.1 nmol/L of 25(OH)D3). Similarly, Fig. 3*B* shows that the nonlinear nature of the ID–risk relationship was more prominent when the data were assessed in deciles. This model predicted the lowest relative risk of ID in the 72rd percentile (corresponding to 48.1 nmol/L of 25(OH)D3). In addition, breech presentation, transfer to NICU after delivery, NBS-positive results, neonatal encephalopathy, and sibling history of ASDs were predictors of ASDs in the multivariate analysis, and none of these analyses could account for the nonlinear exposure–risk relationship.

Table 3. Multivariate Logistic Regression Model for Level of 25(OH)D3 Measured by the Quartiles of the Distribution of 25(OH)D3 Using ASDs as the Dependent Variable

Model ASDs	OR (95% CI)	*p*
25(OH)D3 (1st quartile)	3.6 (1.8–7.2)	<0.001
25(OH)D3 (2nd quartile)	2.5 (1.4–3.5)	0.024
25(OH)D3 (3rd quartile)	1.9 (1.1–3.3)	0.082
25(OH)D3 (4th quartile)	Reference	

Adjusted for maternal age, paternal age, gestational age, parity, prolonged labor, parental depression, chronic complication before pregnancy, pregnancy-induced complication, assisted delivery, baby sex and weight, day and hospital of birth, breech presentation, premature rupture of membranes, transfer to NICU after delivery, fetal distress, meconium, birth injury or trauma, maternal hemorrhage, neonatal encephalopathy, decreased 5-min Apgar score, preeclampsia, respiratory distress syndrome, assisted ventilation, congenital infection, NBS positive results, family single structure, family income and history of ASDs.

25(OH)D3 = 25 hydroxyvitamin D3; ASD = autism spectrum disorder; NICU = neonatal intensive care unit; NBS = newborn screening.

Discussion

To date, no study has directly examined the association between neonatal vitamin D status and risk of ASDs in a Chinese population. We had the opportunity to examine this hypothesis in a large, population-based Chinese case-control study. We found that neonatal vitamin D status was significantly associated with the risk of ASDs and ID in China children. The nature of those relationships was nonlinear. The association between newborn vitamin D and ASD might be driven primarily by an association between newborn vitamin D and developmental delay or ID. However, in the multivariate analysis

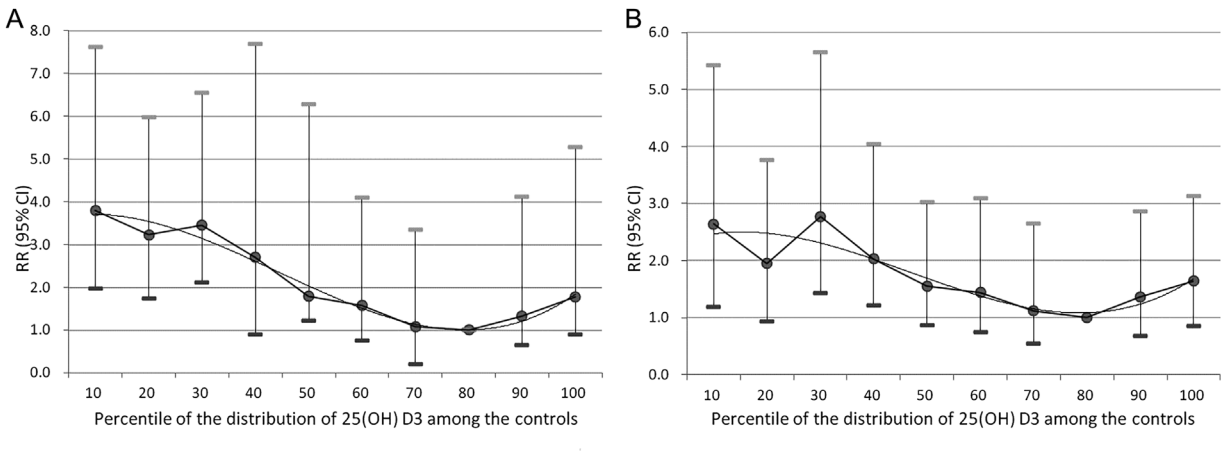

Fig. 3. RR of ASDs or ID according to the percentile of the distribution of 25(OH)D3. (*A*) RR of ASDs according to the percentile of the distribution of 25 (OH)D3. Vertical lines indicate 95% CIs. Newborns in the 70th to 80th percentile of the distribution were chosen as the reference category for the categorical analyses. The gray line shows the best fit analysis for the continuous data (a second-degree fractional polynomial with orders 3 and 3). This polynomial had a significantly better fit compared with both a straight line ($p = 0.032$) and the null model ($p = 0.006$). This model predicted the lowest relative risk of ASDs in the 76rd percentile (corresponding to 49.1 nmol/L of 25(OH)D3). (*B*) RR of ID according to the percentile of the distribution of 25 (OH)D3. Vertical lines indicate 95% CIs. Newborns in the 70th to 80th percentile of the distribution were chosen as the reference category for the categorical analyses. The gray line shows the best fit analysis for the continuous data (a second-degree fractional polynomial with orders 3 and 3). This polynomial had a significantly better fit compared with both a straight line ($p = 0.045$) and the null model ($p = 0.009$). This model predicted the lowest relative risk of ID in the 72rd percentile (corresponding to 48.1 nmol/L of 25(OH)D3). ASD = autism spectrum disorder; ID = intellectual disability; 25(OH) D3 = 25 hydroxyvitamin D3; CI = confidence interval; RR = relative risk.

adjusted by ID, 25(OH)D3 still was associated with ASD ($p = 0.006$). Furthermore, our study showed that the prevalence of ASDs in 3-year-old Chinese children was estimated to be 1.11% (boys: 1.65%; girls: 0.52%).

Nonlinear exposure-risk relationships have been described in studies examining neonatal vitamin D status and risk of schizophrenia[24] that were remarkably similar to the fractional polynomial shown in Fig. 3. Interestingly, J-shaped or U-shaped exposure-risk relationships have been previously suggested in studies examining maternal 25(OH)D3 status and neonatal growth outcomes.[25] However, in this study, concerning the deciles, while the point estimates are slightly above 1 in the two upper deciles, neither estimate differed significantly from the reference category. Thus, the data do not support a J-shaped exposure-risk relationship. Based on the continuous data, the polynomial fit explains more of the variance than a linear model; however, in the absence of a prior hypothesis, this is weak evidence to deduce that the relationship between the variables of interest is nonlinear. The data are inconclusive with respect to this issue—a larger sample may be able to more confidently draw this conclusion.

Interestingly, one of the recent studies demonstrating significantly lower vitamin D in young people with ASD in the Faroe Islands as compared to their parents, siblings, and healthy comparisons, demonstrated that there is arguably no apparent threshold for this association and hypothesized that some impairment of vitamin D metabolism potentially plays a part in the etiology of autism.[26] Furthermore, the article by Kaneko and colleagues[27] even included a discussion regarding the U-shaped response of gene induction by vitamin D that was very reminiscent of the nonlinear relationship observed in the current study. In that study, the authors implied that vitamin D affects brain serotonin concentrations, which may be relevant to psychiatric disorders, such as autism, and may control leptin levels and affect eating behavior.[27]

The prevalence of parent-reported ASD among children aged 6 to 17 years was 2.00% in 2011–2012, a significant increase from 2007 (1.16%) in school-aged US children.[28] In our study, we first reported that the prevalence of childhood autism in children aged 3 years in Beijing, China, was 1.11% (95% CI, 0.99% to 1.23%). Similarly, Baird and colleagues[29] found that the prevalence of childhood autism in children aged 9 to 10 years in South Thames, UK, was 38.9 per 10,000 (95% CI, 29.9 to 47.8) and that of other ASDs was 77.2 per 10,000 (95% CI, 52.1 to 102.3). Furthermore, the Centers for Disease Control and Prevention (CDC) estimate of the prevalence of ASD among children aged 8 years was 1 in 68 children in 2010 in the United States.[30]

There is now clear evidence that vitamin D is involved in brain development. Eyles and colleagues[31] suggested that vitamin D was a plausible biological risk factor for neuropsychiatric disorders and that vitamin D acts as a neurosteroid with direct effects on brain development. Furthermore, a study finished by Hawes and colleagues[32] showed that prenatal vitamin D deficiency leads to alterations in fetal mouse brain morphology and genes related to neuronal survival, speech and language development, and dopamine synthesis. It could be that ASD includes vitamin D deficiency as part of the syndrome etiology or they share some other etiology. Some possible mechanisms can be considered. First, Grant and Soles[33] reported that their findings were consistent with maternal vitamin D deficiency's being a risk factor for infantile autism disease (IAD), possibly by affecting fetal brain development as well as possibly by affecting maternal immune system status during pregnancy. Second, Patrick and Ames[34] presented evidence that vitamin D hormone (calcitriol) activates the

transcription of the serotonin-synthesizing gene tryptophan hydroxylase 2 (TPH2) in the brain at a vitamin D response element (VDRE) and represses the transcription of TPH1 in tissues outside the blood-brain barrier at a distinct VDRE. Third, because activated vitamin D, a secosteroid, upregulates DNA-repair genes, vitamin D deficiency during development may inhibit the repair of de novo DNA mutations in fetuses and infants and thus contribute to risk of autism. Vitamin D might also reduce the risk or severity of autism through its anti-inflammatory actions, anti-autoimmune effects, increasing seizure threshold, increasing T-regulatory cells, protecting the mitochondria, and upregulating glutathione, which scavenges oxidative byproducts and chelates (captures and excretes) heavy metals.[35]

Limitations

This study has a number of limitations. First, our sample is still relatively young, and some subjects may have ASDs that cannot be diagnosed at this early age (ie, Asperger syndrome). The relationship between neonatal vitamin D and later-onset ASDs will require revisiting in future years. In addition, the diagnosis of ASDs was confirmed by (ADI-R) and DSM-5 criteria, which was designed for school-aged children. Our young samples (3 years old) might cause bias. Second, this observational study cannot determine the causal relationship between 25(OH)D3 and ASDs. Third, the results from even well-designed observational studies can be influenced by residual confounding. Several potential confounders including maternal diet, breastfeeding status at time of DBS draw, and maternal air pollution exposure, were previously linked to ASD and could be linked to vitamin D status. However, in this study, we could not obtain that information. Thus, we could not determine the impact of those factors on ASD and vitamin D status. In addition, it remains a topic of debate as to whether neonates with vitamin D deficiency should be supplemented with vitamin D3, in order to maintain their 25(OH)D3 concentrations optimally to prevent ASDs. However, Adams and colleagues[36] suggested oral vitamin/mineral supplementation was beneficial in improving the nutritional and metabolic status of children with autism, including improvements in methylation, glutathione, oxidative stress, sulfation, adenosine triphosphate, and nicotinamide adenine dinucleotide phosphate. To confirm this, long-term controlled clinical trials with large sample sizes will be needed. Fourth, previous studies had suggested that paternal age maybe associated with ASDs risk.[37] However, we did not obtain this information. Thus, we could not determine the relationship between them. Last, the vitamin D was measured at 24 to 48 hours after delivery, and at no other time point. As such it is particularly noteworthy that it was reduced in a proportion of those individuals destined to show ASDs. However, it is not clear from a single time-point determination, whether vitamin D levels remained reduced in these individuals.

Conclusion

Neonatal vitamin D status was significantly associated with the risk of ASDs and ID. The nature of those relationships was nonlinear. Whether neonates with vitamin D deficiency should be supplemented with vitamin D3, in order to maintain their 25 (OH)D3 concentrations optimally to prevent ASDs requires further long-term controlled clinical trials.

Disclosures

All authors state that they have no conflicts of interest.

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Authors' roles: YLZ had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: DMW, XW, XRH, SW, YJW, MS, SHF, JZ, MQL, BH, CHS, YXB, JY, JL, and YLZ. Acquisition of data: SW, YJW, MS, SHF, and JZ. Analysis and interpretation of data: DMW, XW, XRH, SW, YJW, JL, and YLZ. Drafting of the manuscript: DMW, XW, XRH, SW, YJW, MS, SHF, and JZ. Critical revision of the manuscript for important intellectual content: MQL, BH, CHS, YXB, JY, JL, and YLZ. Administrative, technical, or material support: QS, MQL, BH, CHS, YXB, and JY. Study supervision: JL and YLZ. Obtained funding: DMW, XW, JL, and YLZ.

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