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Genetic Contributions to Maternal and Neonatal Vitamin D Levels

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ABSTRACT Vitamin D is essential for several physiological functions and biological processes. Increasing levels of maternal vitamin D are required throughout pregnancy as a unique source of vitamin D for the fetus, and consequently maternal vitamin D deficiency may result in several adverse outcomes in newborns. However, the genetic regulation of vitamin D in pregnancy and at birth is not yet well understood. We performed genome-wide association studies of maternal midgestational serum-derived and neonatal blood-spot-derived total 25-hydroxyvitamin D from a case-control study of autism spectrum disorder (ASD). We identified one fetal locus (rs4588) significantly associated with neonatal vitamin D levels in the *GC* gene, encoding the binding protein for the transport and function of vitamin D. We also found suggestive cross-associated loci for neonatal and maternal vitamin D near immune genes, such as *CXCL6-IL8* and *ACKR1*. We found no interactions with ASD. However, when including a set of cases with intellectual disability but not ASD (N = 179), we observed a suggestive interaction between decreased levels of neonatal vitamin D and a specific maternal genotype near the *PKN2* gene. Our results suggest that genetic variation influences total vitamin D levels during pregnancy and at birth via proteins in the vitamin D pathway, but also potentially via distinct mechanisms involving loci with known roles in immune function that might be involved in vitamin D pathophysiology in pregnancy.

KEYWORDS vitamin D; *GC* pregnancy; neonates; immune function; maternal and fetal genetics; GWAS; SNP-based heritability; early development; autism; intellectual disability

VITAMIN D is a steroid prohormone supplied to the body mainly through the transformation of 7-dehydrocholesterol in skin cells via sun exposure. The active form of vitamin D, calcitriol, interacts with vitamin D receptors (VDRs), transcription factors widely expressed in most tissues, including the placenta (Shin *et al.* 2010) and the brain, where vitamin D regulates a number of neurological functions (Eyles *et al.* 2013). In recent decades, increasing rates of vitamin D deficiency/insufficiency have been observed across populations with different countries of origin, ancestries, and by sex (Mithal *et al.* 2009; Patel *et al.* 2013; Weishaar *et al.* 2016).

In pregnancy, increasing levels of maternal vitamin D are required to be transferred via the placenta as a unique source of vitamin D for the newborn (Lee *et al.* 2007; Rodda *et al.* 2015; Larqué *et al.* 2018). In the first 12 weeks of pregnancy, the maternal serum concentrations of vitamin D are more than twice those of a nonpregnant adult (Hollis and Wagner 2017). Since evidence shows that vitamin D has antiproliferative and immunomodulatory functions (Hewison 2012; Ryynänen and Carlberg 2013; Jiang *et al.* 2018) at this early time point, vitamin D is proposed to act as an immune modulator involved in maternal tolerance to the foreign fetus (Hollis and Wagner 2017). Consequently, maternal vitamin D deficiency, occurring in $\leq$ 50% of pregnant women, has been

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associated with adverse complications (Urrutia and Thorp 2012) such as preeclampsia (Bodnar *et al.* 2007), gestational diabetes (Alzaim and Wood 2013; Lu *et al.* 2016), and detrimental outcomes in fetal and postnatal growth, fetal bone development (Javaid *et al.* 2006), and neurodevelopment (Eyles *et al.* 2003), increasing the risk of delayed cognitive impairment and neuropsychiatric diseases later in life (Eyles *et al.* 2018).

In recent decades, the increasing prevalence of neurodevelopmental disorders and, at the same time, of vitamin D deficiency in the prenatal and postnatal periods have generated strong interest in assessing vitamin D as a potential environmental risk factor for those disorders that have origins early in life, such as autism spectrum disorder (ASD) and/or intellectual disability (ID). However, contradictory results have been observed, likely due to underpowered studies, and few studies have measured vitamin D in these susceptibility windows (Windham *et al.* 2019).

Several studies have assessed a number of lifestyle factors related to the risk for vitamin D deficiency/insufficiency beyond sun exposure (Heidari and Mirghassemi 2012), such as latitude of residence, increasing age, and body mass index (Lagunova *et al.* 2009). However, based on evidence of heritability (Karohl *et al.* 2010; Orton and Ebers 2011), recent studies have also investigated the genetic regulation of interindividual variation in circulating vitamin D levels beyond the effects of genetic ancestry and skin color (Yuen and Jablonski 2010). Few loci have been associated with vitamin D at a genome-wide level, most encoding enzymes (*CYP2R1*, *CYP24A1*, *and DHCR7)* and receptors (*GC* and *VDR*) involved in its metabolic pathway (Benjamin *et al.* 2007; Ahn *et al.* 2010; Wang *et al.* 2010; Lasky-Su *et al.* 2012; Anderson *et al.* 2014; Sapkota *et al.* 2016). Recently, two novel loci, *AMDHD1* and *SEC23A*, have been associated with 25-hydroxyvitamin D levels in a large meta-analysis of 79,366 European-ancestry adults (Jiang *et al.* 2018), and two additional loci near *ANO6/ARID2* and *HTR2A* genes from a transethnic analysis including African Americans and Hispanic Americans (Hong *et al.* 2018).

Few human studies have been conducted in pregnancy, and existing studies mainly investigate the role of candidate loci in regulating the levels of gestational maternal vitamin D and vitamin D measured in the umbilical cord (Ramos-Lopez *et al.* 2008; Størdal *et al.* 2017; Shao *et al.* 2018). Human decidual and placental tissues synthesize the active form of vitamin D (Weisman *et al.* 1979), and recent work (Noyola-Martínez *et al.* 2014; Nguyen *et al.* 2015; Knabl *et al.* 2017) has shown that many candidate loci for vitamin D are expressed in the placenta and trophoblast cells. However, these studies are limited by *a priori* hypotheses based on candidate genes only, and no genome-wide association studies (GWAS) analyzing vitamin D in pregnancy and at birth have been performed.

Here, we take advantage of maternal and neonatal total 25-hydroxyvitamin D [sum of 25(OH)D2 and 25(OH)D3, hereinafter referred to as vitamin D] measured in mother–newborn pairs included in the Early Markers for Autism (EMA) case-control study (Tsang *et al.* 2013; Zerbo *et al.* 2016) to examine the relationship of vitamin D and ASD/ID (Windham *et al.* 2019). For most mother–newborn pairs, we investigated the genome-wide genetic control of maternal and neonatal total vitamin D during the critical period of neurodevelopment. We tested several genetic hypotheses. First, that maternal vitamin D levels may be under maternal genetic control. Second, that neonatal vitamin D levels may be under fetal genetic control. Third, that due to transfer of vitamin D from mother to child, neonatal vitamin D might be influenced by maternal genetics. Finally, based on our, and others', previous evidence of fetal genotype impacting maternal physiology and circulating maternal biomarkers during pregnancy (Petry *et al.* 2007, 2014; Liu *et al.* 2015; Traglia *et al.* 2017, 2018), we hypothesized that maternal vitamin D levels may be under fetal genetic control. To complement genome-wide analyses, we selected candidate vitamin D loci to be examined more carefully for their role in shaping risk for neurodevelopmental disorders.

Materials and Methods

Study subjects

The EMA project is a population-based nested ASD case-control study (Croen *et al.* 2008; Tsang *et al.* 2013) including pregnant women who were enrolled in the California Prenatal Expanded α Fetoprotein Screening Program (15–20 weeks gestation) in Orange, San Diego, and Imperial Counties, CA and who subsequently delivered a live birth in 2000–2003. The distributions of maternal race/ethnicity as documented on the birth certificates were as follows: 42% Hispanic, 35% non-Hispanic Caucasian, 15% East Asian, 3% South Asian, 3% African American, and 3% others. As we described previously (Tsang *et al.* 2013), maternal prenatal blood and neonatal blood-spot specimens were available from a biobank of the maternal–newborn pairs. Maternal blood was drawn midpregnancy (15–20 weeks gestation), and sera were stored in cryovials and cell pellets stored in serum separator tubes at −20°. Newborn blood spots are generally collected on filter paper 1–2 days after birth and stored at −20° by the Genetic Disease Screening Program, California Department of Public Health. The original diagnosis codes for ASD or ID were ascertained from the client files of two regional centers of the California Department of Developmental Services (DDS), and verified by study clinician expert review of records according to a previously developed protocol (Nonkin Avchen *et al.* 2006). During expert review, a total of 136 individuals originally identified at DDS as having ID with no known etiology (of 326 total) were reclassified as meeting ASD case definition (Diagnostic and Statistical Manual of Mental Disorders) (referred to as ID-ASD hereafter). The controls from the general population—frequency matched to the original ASD cases by sex, birth month, and birth year—were sampled from the birth certificate files of neonates with the same banked biospecimens (Tsang *et al.* 2013; Windham *et al.* 2019).

Measurement of maternal and neonatal vitamin D

Total 25-hydroxyvitamin D [sum of 25(OH)D2 and 25(OH)D3] was measured by a sensitive isotope dilution liquid chromatography-tandem mass spectrometry method for dried blood spots and for serum (University of Queensland, Australia) (Eyles *et al.* 2009; Kvaskoff *et al.* 2016). A hematocrit (Hct) correction was applied to levels in blood spots to obtain sera equivalent values, as is customary for the laboratory: 25(OH)D $\times$ (1/(1-Hct), where Hct = 0.61, as a standard neonatal capillary Hct (Windham *et al.* 2019). A summary of maternal and neonatal total 25-hydroxyvitamin D levels and covariates by outcome is reported in Supplemental Material, Table S1.

Genotyping

As previously described (Traglia *et al.* 2018), the QIAGEN (Valencia, CA) QIAamp 96 DNA Blood Kit was used to extract DNA from maternal and neonatal blood samples, and the Invitrogen (Carlsbad, CA) Quant-iT DNA Assay Kit to measure the DNA concentration by the biomedical laboratory at Utah State University. Maternal and neonatal samples were genotyped using the Affymetrix Axiom (Affymetrix 2011) EUR array by the Genomics Core Facility at the University of California, San Francisco, using standard protocols. Affymetrix Power Tools (thermofisher.com) was used for the genotype calling. Individual-based and marker-based quality controls were applied using PLINK (Purcell *et al.* 2007) as reported in Tsang *et al.* (2013). The maternal data set included 629,686 genotyped high-quality common SNPs [minor allele frequency (MAF) $\geq$ 1%] from 790 maternal samples defined as "case" and "control," based on original DDS offspring ASD diagnosis (390 mothers of ASD cases and 400 mothers of controls). The neonatal data set included 764 neonatal samples (385 ASD cases and 379 controls) and 622,716 genotyped common fetal markers. Herein after these are referred to as the "ASD/control discovery data set." This neonatal data set is included within the EMA epidemiological study of neonatal vitamin D recently reported in Windham *et al.* (2019).

Covariates and ancestry

The levels of midgestational maternal and neonatal vitamin D were separately transformed with a square root transformation ($\sqrt{x}$) to normalize the distributions. In maternal and neonatal samples, respectively, we applied a threshold of 3.5 and 3 SD from the mean to exclude two and three outliers due to noncontinuous values, or values in the extreme tails of maternal or neonatal distributions (Figure S1). We analyzed the effects of potential confounding factors (Table S1) on maternal and neonatal vitamin D levels with linear regression models in the genotyped mothers and newborns using R 3.3.3 (R Core Team 2016). We included confounding factors already known to influence vitamin D, such as season of blood draw or birth (four categories) for maternal and neonatal vitamin D, respectively. In both maternal and neonatal analysis, we included the genetic ancestry [the first 10 maternal or fetal coordinates as reported before (Traglia *et al.* 2017, 2018)], parity (nulliparous up to five liveborn children), and some variables used as matching factors in the case-control study design, such as ASD outcome and year of birth (2000–2003). We also included potential data set-specific quantitative confounding factors that were nominally associated with maternal vitamin D levels in the univariate analysis, such as maternal weight at screening and neonate birth weight. For neonatal vitamin D, we additionally included sex, used for matching controls. We assessed the linear correlation between maternal levels after covariate adjustment and neonatal adjusted levels using Spearman's test implemented in R 3.3.3 (R Core Team 2016).

To confirm our transethnic approach adjusting for population-specific principal components (PCs), we used PC1–2 to identify more homogeneous, nonoverlapping subgroups of East Asian, Hispanic, and Caucasian individuals, as previously described (Traglia *et al.* 2018). For our top associations, we performed a three-population meta-analysis of these homogeneous groups. We found consistent direction of association across ancestry groups for our top results (Table S2).

Heritability estimation

We computed a genetic relationship matrix across the maternal and neonatal ASD/control discovery data set separately, using the entire set of genotyped markers after previously described quality control (Tsang *et al.* 2013). We performed the heritability estimation (genetic variance/total phenotypic variance or σ_g/σ_p) for vitamin D levels after covariate adjustment with GCTA (Yang *et al.* 2011), applying two restricted maximum likelihood models. First, we calculated (i) the maternal genetic additive contribution to the maternal vitamin D variance after the adjustment for the confounding factors, and likewise (ii) the neonatal genetic additive contribution to the neonatal-adjusted vitamin D variation. When testing the individual-specific set of SNPs, we used the corresponding set of principal coordinates. In the results and tables, we used the term fetal genetics/SNP to emphasize the genetic control happening during gestation on maternal phenotype and on neonatal phenotype to avoid any confusion, using neonatal genetics for influence exerted during pregnancy.

GWAS and selection of candidate loci

We assessed genetic association between the entire set of common variants and levels of vitamin D after covariate adjustment in the ASD/control discovery data set. Four GWAS analyses were performed to detect: (i) maternal SNPs affecting maternal vitamin D levels, (ii) fetal SNPs affecting neonatal vitamin D levels, (iii) maternal SNPs affecting neonatal vitamin D levels, and (iv) fetal SNPs affecting maternal vitamin D levels. A cut-off set at $P < 5 \times 10^{-8}$ was used for multiple test correction to account for approximate independent common polymorphism testing per GWAS (Risch and Merikangas 1996; Pe'er *et al.* 2008). Because of the low statistical power but the unique characteristics of our study, we

also used a lenient suggestive threshold ($P < 5 \times 10^{-6}$). We assessed whether functional evidence for the top hits or their proxies [$r^2 \geq 0.8$; using LDproxy (dlink.nci.nih.gov) and FUMA GWAS (fuma.ctglab.nl/)] has been reported in the ENSEMBL (ensembl.org), GTEx (gtexportal.org), and Encyclopedia of DNA Elements databases (Dunham *et al.* 2012) (Table S3).

From each of the four types of analyses, we selected a set of candidate SNPs for a follow-up analysis: SNPs in/near loci in the vitamin D pathway and top independent SNPs at the genome-wide significance threshold. Additionally, for both maternal and neonatal vitamin D, we selected one independent maternal and/or neonatal SNP with the best nominal *P*-value ($P < 0.01$) among those located within ± 5 kb of loci with a biological function in vitamin D metabolism, such as *GC*, *VDR*, *CYP2R1*, *CYP24A1*, *LGMN*, *CYP27B1*, *DHCR7*, *CUBN*, or *LRP2*. In our analyses, we did not find any nominal contribution ($P < 0.01$) to vitamin D from SNPs in *CYP24A1*, *LGMN*, *CYP27B1*, and *DHCR7*, which were consequently excluded from further analysis.

Conditional model for independent maternal and fetal genetic contribution

When a SNP showed association at a more-significant *P*-value in maternal or fetal genetics, and a less-significant *P*-value in the other, we disentangled whether the contributions from maternal and fetal genetics were independent or explained by the relatedness of mother–newborn pairs. We performed conditional modeling with both maternal and fetal SNP genotypes as covariates, as previously described (Traglia *et al.* 2018).

Genotyping of candidate markers in additional ASD and ID samples

Thanks to additional funding, we were able to genotype the specimens of 179 ID (without ASD) and 131 ID-ASD child–mother pairs with measured vitamin D for a subset of candidate markers using TaqMan SNP Genotyping Assays, according to the manufacturer's protocol for 384-well plate reactions. The amplification was performed using a thermal cycler and the intensity was measured using ViiA 7 allelic discrimination software (Applied Biosystems, Foster City, CA) and visually refined. We replaced two SNPs (rs2544385 and rs72650824) that did not have available assays with two SNPs in high–moderate linkage disequilibrium (LD) showing nominal *P*-values in our GWAS (rs830958 and rs17202249). Three markers were excluded. The marker rs2228171 was not amplified when we repeated the analysis with a second primer set. Two additional SNPs (rs205761 and rs1550598) were excluded because of the poor quality of the discrimination plot. Finally, for each remaining SNP, individual samples showing poor amplification or those not clearly clustered were set as missing, and excluded from the marker-specific analysis.

Imputation of 10 principal coordinates for the additional samples

For the additional ID and ID-ASD maternal and neonatal samples, only 22 high-quality TaqMan-genotyped SNPs were available, thus we were not able to estimate accurate ancestry PCs. Based on the array genome-wide PCs and the reported race/ethnicity data available for the array-genotyped data set, we inferred PC values for the additional ID and ID-ASD samples. After calculating the Spearman's correlation coefficient between maternal and fetal PC1–2, and the Euclidean distance $\left(\sqrt{(fetal\ PC1 - maternal\ PC1)^2 + (fetal\ PC2 - maternal\ PC2)^2}\right)$ for each mother–neonate pair (results are shown in Figure S2), we used maternal reported race/ethnicity to form four groups: Hispanic, non-Hispanic Caucasian, Asian, and African American/Other/Unknown. Then, we used the array-genotyped individuals in each ancestry-specific cluster that had principal coordinate values to calculate the median of each principal coordinate (PC1–10). We obtained four ancestry-specific values for each PC. Then, we assigned the ancestry-specific median values to each individual of the additional ID and ID-ASD samples in the same ancestry-specific cluster (Figure S2). The distribution of the additional samples by ancestry is 60% Hispanic, 22% non-Hispanic Caucasian, 11% East Asian, and 6% African American/Other/Unknown. We used those PC1–10 covariates to combine array-genotyped ASD/control and TaqMan-genotyped ID and ID-ASD samples (full ASD/ID/control data set) for further genetic analysis.

Genetic association of candidate SNPs with vitamin D including the additional samples

We extracted the genotypes for 22 markers for all the mothers and neonates in the ASD/control discovery data set used for the GWAS, and we merged them with the 22 genotypes in the additional 131 ID-ASD and 179 ID mothers and neonates using PLINK (–merge) (Purcell *et al.* 2007). Thus, we obtained a full ASD/ID/control data set of 1069 mothers and 1058 neonates with vitamin D measurement for candidate testing. We reanalyzed the genetic association between the 22 markers and total vitamin D in the mothers and neonates in the full data set. We also performed the same analyses in the "full ASD/control" data set ($N = 507$ ASD and ID-ASD cases; $N = 389$ controls), excluding $N = 179$ ID subjects. To take into consideration whether potential differences in results between the full ID/ASD/control data set and the full ASD/control data set were caused by outcome or sample size after the inclusion of 131 ID-ASD cases, we replaced the additional $N = 131$ reclassified ASD subjects (ID-ASD) with the same number of children randomly sampled 100 times from the ID data set. We combined the permuted cases with the original array-typed ASD subjects and controls to assess the genetic association between the tested markers and total vitamin D. The empirical *P*-value was calculated by assessing the number of combined shuffled case-control data sets that returned the same or better association compared to the full ASD/control data set ($N = 507$ *vs.* $N = 389$).

Vitamin D association with outcome

We tested the distribution of vitamin D levels for a difference-by-child outcome after covariate adjustment (excluding ASD

Table 1 Maternal and fetal genome-wide significant, and suggestive, association of maternal and neonatal transformed 25-hydroxyvitamin D levels

Chromosome	SNP	Gene	Vitamin D	Genetics	β	SE	*P*
12	rs4149056	*SLCO1B1*	Maternal	Maternal	−0.58	0.12	2.7×10^{-7}
10	rs11528045	*HTR7*	Maternal	Maternal	0.53	0.11	5.4×10^{-7}
11	rs6486205	*CYP2R1*	Maternal	Maternal	0.40	0.09	1.9×10^{-6}
4	**rs4588**	***GC***	**Neonatal**	**Fetal**	**−0.62**	**0.11**	$\mathbf{3.1 \times 10^{-8}}$
2	rs11690195	*MMADHC-RND3*	Neonatal	Fetal	−0.47	0.10	2.3×10^{-6}
13	rs9527875	*DIAPH3 - PCDH17*	Neonatal	Maternal	0.59	0.11	6.9×10^{-8}
4	rs72650824	*CXCL8-CXCL6*	Neonatal	Maternal	1.53	0.30	6.7×10^{-7}
7	rs205761	*in LINC00513, near MKLN1*	Neonatal	Maternal	0.50	0.10	$7.6.10^{-7}$
16	rs2541497	*TEKT5*	Neonatal	Maternal	0.53	0.11	1.4×10^{-6}
18	rs1550598	*DOK6*	Neonatal	Maternal	0.53	0.11	2.6×10^{-6}
8	rs7837124	*c8orf34*	Neonatal	Maternal	1.61	0.34	2.7×10^{-6}
1	rs490379	*PKN2*	Neonatal	Maternal	0.75	0.16	4.1×10^{-6}
1	rs17666424	*ACKR1*	Maternal	Fetal	0.59	0.13	2.9×10^{-6}
3	rs2336664	*STIMATE- TMEM110*	Maternal	Fetal	0.42	0.09	2.0×10^{-6}
8	rs7015627	*MED30*	Maternal	Fetal	0.66	0.14	1.9×10^{-6}

Vitamin D levels were normalized with a square root transformation, and adjusted for maternal and neonatal confounding factors. The analysis was performed in the autism spectrum disorder/control discovery sample. Genome-wide significantly associated SNPs are shown in bold.

or ID status) using a two-sample Mann–Whitney Wilcoxon test in R 3.3.3 (R Core Team 2016). We compared the vitamin D levels measured in mothers of controls ($N = 389$) to: (i) vitamin D measured in mothers of ASD cases from the discovery sample ($N = 376$), (ii) vitamin D in mothers of all ASD cases ($N = 507$), and (iii) vitamin D in mothers of ID cases ($N = 179$).

Candidate SNPs for vitamin D and association with ASD/ID risk

We analyzed whether each of the 22 maternal and fetal candidate markers for vitamin D was associated with ASD and/or ID risk by itself. We applied logistic models implemented in PLINK (Purcell *et al.* 2007) using the sex and PC1–10 covariates. We extracted the candidate SNPs showing $P < 0.05$ in association with $N = 507$ ASD or $N = 179$ ID cases. For each candidate SNP–vitamin D association, we tested an interaction model between total vitamin D, SNP genotype, and ASD or ID status with *ad hoc* R 3.3.3 scripts (R Core Team 2016), adding an interaction term ("outcome × genotypes") in the linear regression, and we visualized the interactions using a boxplot function in R 3.3.3 (R Core Team 2016). We also performed the same interaction model between total vitamin D, SNP genotype, and ID status using a subset of controls that were matched by ancestry to ID individuals and observed similar results.

Data availability

Our samples were obtained by the state of California for prenatal and newborn screening, and the state specifically prohibits the release of individual-level data. Investigators wishing to use similar state resources can apply to the California Biobank Program (https://www.cdph.ca.gov/Programs/CFH/DGDS/Pages/cbp/default.aspx). The full genome-wide summary-level results for each of the maternal vitamin D GWAS with maternal genetics, maternal vitamin D GWAS with fetal genetics, neonatal vitamin D GWAS with fetal genetics, and the neonatal vitamin D GWAS with maternal genetics, as well as the summary statistics for the additional 22 SNPs in the follow-up data set, are available in four compressed tables deposited at Zenodo with the assigned DOI: https://doi.org/10.5281/zenodo.3592044. Supplemental material available at figshare: https://doi.org/10.25386/genetics.11445132.

Results

Maternal and neonatal vitamin D levels and predictors

The maternal levels of total vitamin D measured in midgestation varied in the range of 9.6–259.0 nmol/liter and showed a slightly right-skewed distribution. We obtained residuals after transformation, outlier exclusion, and adjustment for potential maternal and neonatal confounding factors, including matching factors such as offspring ASD outcome according to the study case-control design (see *Materials and Methods*). The maternal levels of vitamin D were mainly influenced by ancestry, blood-draw season, maternal weight, and birth year (Table S4). Similarly, the neonatal levels of vitamin D (9.4–309.4 nmol/liter) were transformed and cleaned of outliers. The covariates with the strongest association with neonatal vitamin D levels were birth season and ancestry ($P < 10^{-5}$; Table S4). After covariate adjustment, vitamin D levels showed unimodal distributions in mothers and neonates. We observed a modest but significant positive correlation ($\rho = 0.35$; Pearson's test $P < 10^{-16}$) between maternal and neonatal levels, which were obtained 5–6 months apart.

Suggestive loci associated with maternal vitamin D

We estimated the proportion of genetic contribution of common frequency variants to maternal vitamin D levels, but the maternal heritability estimate was very low ($h^2_g = 2\%$ SE = 0.33, $P = 0.47$). Then, we conducted a genome-wide analysis using maternal autosomal genetic markers and maternal

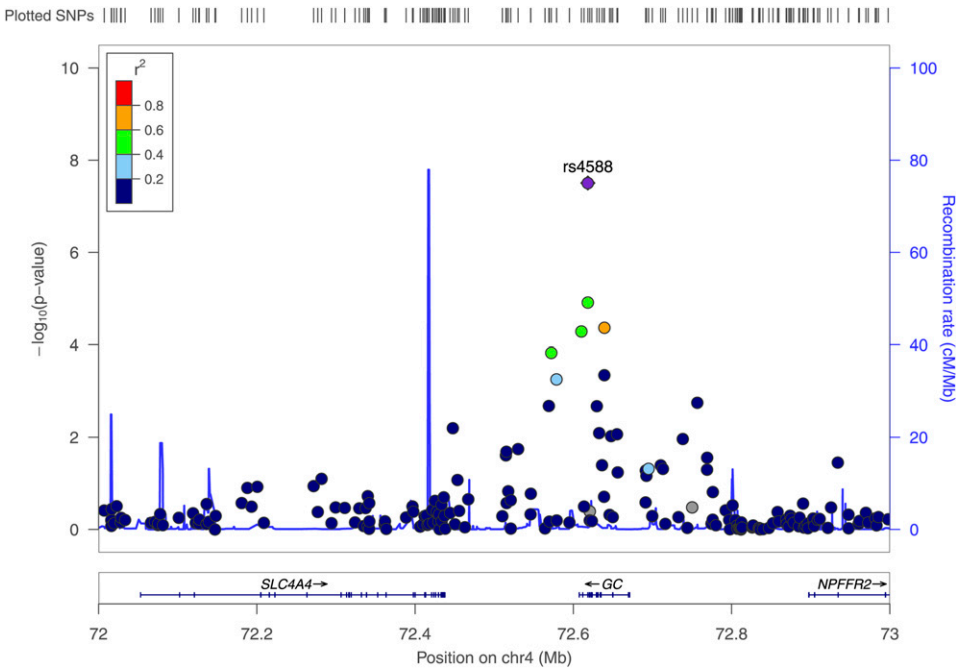

Figure 1 Linkage disequilibrium regional genomic plot of fetal genome-wide associated rs4588 in *GC* with neonatal levels of 25-hydroxyvitamin D in autism spectrum disorder/control discovery sample. Each dot represents one SNP in the genomic region based on linkage disequilibrium with rs4588 (r^2 in the range 0–1). The *x*-axis represents the genomic position; the *y*-axis shows the negative logarithm of the observed association *P*-value for each tested SNP. Plotted with the Locuszoom tool (Pruim *et al.* 2010). chr4, chromosome 4.

midgestational levels of vitamin D in the discovery sample. We found three independent novel suggestive maternal loci (Figure S3, A–C and Table 1) at the lenient *P*-value threshold ($P < 5 \times 10^{-6}$). On chromosome 12, a missense variant rs4149056 (Val174Ala, MAF = 0.12, β = −0.58, SE = 0.12, $P = 2.7 \times 10^{-7}$) is located in *SLCO1B1*, encoding a transporter for a vitamin D metabolite in the liver. One of the other two loci is on chromosome 10 near *HTR7* (rs11528045, MAF = 0.20, β = 0.53, SE = 0.11, $P = 5.4 \times 10^{-7}$), encoding one of the serotonin receptors. The other was on chromosome 11 in the intron of the candidate gene *CYP2R1*, rs6486205 (MAF = 0.35, β = 0.40, SE = 0.09, $P = 1.9 \times 10^{-6}$).

GC locus associated with neonatal vitamin D

The fetal heritability estimation of neonatal vitamin D was $h^2_g = 42\%$ (SE = 0.39); however, this did not reach significance ($P = 0.14$). A genome-wide significant fetal missense variant (rs4588 or Thr436Lys, MAF = 0.26, β = −0.62, SE = 0.11, $P = 3.1 \times 10^{-8}$) mapping in the candidate gene *GC* on 4q12-q13 emerged associated with neonatal vitamin D (Figure 1 and Table 1). A suggestive locus emerged on chromosome 2 (rs11690195, MAF = 0.42, β = −0.47, SE = 0.10, $P = 2.3 \times 10^{-6}$; Figure S3D) between *MMADHC*, encoding a mitochondrial protein fundamental for the conversion and transportation of vitamin B12, and *RND3*, encoding a small GTPase protein that interacts with p190 RhoGAP through lipid rafts (Oinuma *et al.* 2012).

Cross-genetic regulation of vitamin D at immune function loci

Since mothers supply vitamin D to the fetus throughout gestation, we also expected to observe some maternal genetic contribution to the levels of vitamin D measured in neonates at birth. At a suggestive level, we found numerous novel maternal SNPs in a so-called "cross-genetic contribution" (Figure S3, E–K and Table 1). These SNPs map in a genomic region not previously associated with vitamin D or near genes encoding proteins directly involved in the vitamin D pathway. The first SNP is near genome-wide significance level (rs9527875, MAF = 0.30, β = 0.59, SE = 0.11, $P = 6.9 \times 10^{-8}$) on chromosome 13, located between *DIAPH3*, diaphanous-related formin 3, and *PCDH17*, a calcium-dependent cell-adhesion protocadherin. On chromosome 4, the SNP rs72650824 (MAF = 0.07, β = 1.53, SE = 0.30, $P = 6.7 \times 10^{-7}$, Table 1), maps between *CXCL8/IL-8* and *CXCL6*, encoding interleukin-8 and GCP-2, two mediators of the inflammatory response (Mittal *et al.* 2008). On chromosome 16, the SNP rs2541497 (MAF = 0.42, β = 0.53, SE = 0.11, $P = 1.4 \times 10^{-6}$), is located in an intron of *TEKT5*, which encodes a protein controlling tubulin stability and promoting sperm morphogenesis (Aoki and Matsui 2019). The SNP rs1550598 on chromosome 18 (MAF = 0.38, β = 0.53, SE = 0.11, $P = 2.6 \times 10^{-6}$) maps in *DOK6*, a downstream tyrosine kinase/docking protein involved in neuronal development through Ret signaling (Crowder *et al.* 2004). The SNP rs490379 (MAF = 0.11, β = 0.75, SE = 0.16, $P = 4.1 \times 10^{-6}$) is near *PKN2*, encoding a protein kinase C (PKC)-related serine/threonine protein kinase and effector of Rho GTPases in cellular pathways such as MAPK signaling. The maternal rs7837124 (MAF = 0.02, β = 1.61, SE = 0.34, $P = 2.7 \times 10^{-6}$) is located near *C8ORF34*. Finally, rs205761 on chromosome 7 (MAF = 0.48, β = 0.50, SE = 0.10, $P = 7.6 \times 10^{-7}$), is located in *LINC00513*, a noncoding RNA near *MKLN1*, encoding an intracellular multidomain protein involved in actin binding, protein degradation, and nucleocytoplasmic signaling (Valiyaveettil *et al.* 2008).

Since the fetus may exert a genetic influence on maternal physiology, we next analyzed whether midgestational maternal levels of vitamin D might be influenced by fetal genetics. Similarly, we found three fetal loci suggestively ($P < 5 \times 10^{-6}$) associated with maternal vitamin D levels in novel regions (Figure S3, L–N and Table 1). One SNP (rs17666424, MAF = 0.13, β = 0.59, SE = 0.13, $P = 2.9 \times 10^{-6}$) on chromosome 1 is located near *ACKR1*, a chemokine receptor. A second SNP on chromosome 3 (rs2336664, MAF = 0.44, β = 0.42, SE = 0.09, $P = 2.0 \times 10^{-6}$) maps in the *STIMATE (TMEM110)* gene, which encodes the store-operated calcium entry regulator at the ER–plasma membrane. Finally, on chromosome 8, rs7015627 (MAF = 0.12, β = 0.66 SE = 0.14 $P = 1.9 \times 10^{-6}$) maps near *MED30*, a thyroid hormone receptor-associated protein, part of the mediator complex, also called vitamin D receptor-interacting protein (Rachez *et al.* 1999; Oda *et al.* 2012).

Association of candidate genes in the vitamin D pathway

Interestingly, except *GC* and *CYP2R1*, the predetermined set of genes encoding major proteins for vitamin D conversion and tissue distribution did not show any significant/suggestive association ($P < 5 \times 10^{-6}$) with vitamin D levels in pregnancy or neonatal time points in the discovery data set. We only observed maternal and/or fetal SNPs associated at nominal significance levels ($P < 0.05$) in *LRP2*, *CUBN*, *CYP24A1*, and *VDR* (Table S5). Additionally, the only fetal candidate locus genome-wide associated with neonatal vitamin D (*GC*) did not show significant association with maternal vitamin D levels ($P = 0.5$). The same maternal SNP, rs4588, in *GC* showed a nominal contribution to neonatal vitamin D levels (β = −0.46 SE = 0.11, $P = 3.8 \times 10^{-5}$) and to maternal vitamin D levels (β = −0.24 SE = 0.09, $P = 0.01$). Only the fetal genetic contribution remained significant in a neonatal vitamin D model including both maternal and fetal rs4588 genotypes (maternal $P = 0.11$ and fetal $P = 5.6 \times 10^{-4}$).

Selection of candidate genes and follow-up in the ID EMA cohort

To increase our sample size for follow-up of top signals, we reanalyzed the maternal or fetal genetic association with 22 candidate SNPs in a larger data set, including additional genotyped ID and ASD samples with measured vitamin D levels (see *Materials and Methods*). Overall, the SNP associations with vitamin D observed in this larger ASD/ID/control sample were estimated to have somewhat smaller effect sizes, in the same direction as the discovery sample (Table S6). A subset of 10 SNPs showed stronger effects in the larger sample (*P*-value change of at least 10-fold magnitude and > 50% decrease in β), but considering only the total ASD and control samples (ASD = 507, controls = 389), the effects were similar to the discovery sample examined in GWAS (Table S6). We found that the differences in vitamin D association were due to the 179 ID subjects included in the larger sample (empirical $P < 0.1$ for 8 out of 10 SNPs; see *Materials and Methods* and Table S6).

Vitamin D candidate genes in ASD/ID cases vs. controls

In the larger ASD/ID/control sample, as well as in the ASD/control discovery data set, we found no significant associations between ASD status and vitamin D. When comparing ID and control groups, contrary to expectation, we found a borderline increase ($P = 0.07$) in neonatal vitamin D in ID-affected individuals. We assessed the genetic contribution of the 22 vitamin D candidate SNPs to ASD and/or ID risk and, after multiple test correction, we did not find any significant maternal/fetal SNPs that might act as risk or protective factors for ASD or ID outcome itself (Table S7).

We performed interaction models to test the hypothesis that the levels of vitamin D might vary in a genotype-specific way within ASD or ID cases and controls. We observed a few nominal interactions between vitamin D, genetic variants, and ID outcome. The most suggestive interaction involves decreasing levels of neonatal vitamin D, ID outcome, and the maternal SNP rs490379, where the minor allele C was positively associated with neonatal vitamin D levels (β = 0.74, SE = 0.16, $P = 4.0 \times 10^{-6}$), and the SNP was a nominal protective factor for ASD (odds ratio = 0.66, 95% C.I. (0.48–0.89), $P = 7.3 \times 10^{-3}$). We observed that the ID-affected neonates showed nominally significant decreased levels of vitamin D depending on maternal risk alleles for rs490379 near *PKN2* ($P_{int} = 1.2 \times 10^{-5}$; Figure S4) compared to controls, but this interaction needs to be replicated in a larger ID sample. To exclude any potential technical issues in comparing ID cases entirely genotyped with TaqMan and controls entirely genotyped with chip arrays, we compared TaqMan-genotyped ID cases to the TaqMan genotyped ID-ASD neonates (here used as controls) to replicate the interactions and we observed the same trend, albeit less significant ($P = 0.01$).

Discussion

This is the first study of the genetic impact on levels of total 25-hydroxyvitamin D (D_2 and D_3) in a cohort of maternal–neonatal pairs. We were able to account for maternal genetic control of midgestational vitamin D levels and fetal genetic control of neonatal vitamin D levels at birth. Additionally, due to our unique cohort, we were able to investigate cross-genetic models to determine whether maternal genetics influence neonatal vitamin D and whether fetal genetics might play a role in maternal vitamin D physiology.

When using a conventional $P < 5 \times 10^{-8}$ threshold for multiple test correction to account for approximate independent common polymorphism testing per GWAS, we observed only one genome-wide significant variant, rs4588 ($P = 3.0 \times 10^{-8}$), a common missense change with fetal-specific impact on neonatal vitamin D levels. This SNP is in the *GC* gene, encoding the vitamin D-binding protein (DBP) for transport in the bloodstream to the liver. The missense variant was

previously shown to affect circulating DBP as well as vitamin D in infants (Carpenter *et al.* 2013), and several rs4588 proxies were associated with vitamin D in adults (Jiang *et al.* 2018). Surprisingly, we observed no independent contribution of the same maternal locus to neonatal vitamin D, and nominal contribution to midgestational maternal vitamin D independent of fetal genotype ($P = 0.02$). We identified SNPs in moderate LD ($r^2 = 0.4$–0.6) with rs4588 in the *GC* locus that were highly associated in adults in Jiang *et al.* (2018). Among them, we observed two SNPs (rs222047 and rs7041) having stronger associations with maternal vitamin D than rs4588 [albeit still with nominal independent significance ($P = 10^{-3}$)]. However, these SNPs also show similar or slightly larger effect sizes in neonatal vitamin D. We hypothesize that different alleles of *GC* may be important in adults, in pregnancy, and in neonates. Alternatively, we might have increased power to detect genetic associations in the neonatal period if there is less environmental contribution to variation in vitamin D compared with (pregnant) adults. No other known candidate genes from previous GWAS (Jiang *et al.* 2018) were found to contribute to midgestational maternal vitamin D or neonatal vitamin D at the genome-wide significance level.

We did not find genome-wide maternal-specific loci impacting midgestational vitamin D levels, but we found suggestive evidence for a missense variant rs4149056 ($P = 2.7 \times 10^{-7}$) located in *SLCO1B1*, encoding OATP1B1, a transmembrane receptor involved in the drug uptake and hepatic transport of a major conjugative metabolite of vitamin D in the liver (Gao *et al.* 2017). From the summary statistics of a large GWAS of vitamin D in adults (Jiang *et al.* 2018), we extracted several SNPs in moderate/high r^2 and D' with rs4149056 that showed nominal *P*-values in this locus (top hit: rs4149048, $P = 7.8 \times 10^{-4}$).

We also found suggestive evidence for a SNP (rs11528045, $P = 6 \times 10^{-7}$) near *HTR7*, encoding one of the serotonin receptors. This SNP may be a proxy for rs7896173, which overlaps candidate *HTR7* regulatory elements in fetal cell types (Table S3) (Dunham *et al.* 2012). A SNP in moderate LD with this locus ($r^2 = 0.54$) showed nominal association ($P = 0.03$) with vitamin D in Jiang *et al.* (2018). Interestingly, in a recent transethnic study on vitamin D, an association between vitamin D levels and the serotonin receptor *HTR2A* was observed (Hong *et al.* 2018). Serotonin has several functions, including being a regulator of immune and inflammatory responses exerted through the engagement of serotonin receptors.

When comparing our GWAS results with other vitamin D associations published after our study was underway, we did not replicate any SNPs near the additional associated loci *SEC23A* and *AMDHD1* in European adults (Jiang *et al.* 2018). Similarly, we did not replicate the novel loci from the Hong *et al.* (2018) transethnic analysis or *SSTR4/FOXA2* from Sapkota *et al.* (2016) in an Indian population. Our results from additional vitamin D candidate loci are discussed below.

When assessing the maternal cross-genetic contribution of neonatal vitamin D levels, we found a set of suggestively associated loci mapping near genes not previously associated with vitamin D, but (surprisingly, given the presumed large role of maternal transfer) no SNPs at a genome-wide significance level. Interestingly, we found a locus (rs72650824, $P = 6.7 \times 10^{-7}$) mapping to the region near *CXCL8(IL-8)* and *CXCL6*, encoding two fundamental inflammatory chemokines with chemotactic properties. In pregnancy, *IL8* promotes/inhibits trophoblast cell migration and invasion in the human first-trimester placenta, whereas *CXCL6* is expressed in several cells at the human maternal–fetal interface (Zhang *et al.* 2013). Thus, rather than a direct influence of the vitamin D pathway in mothers, perhaps placental function is more impactful in determining newborn vitamin D.

Since the placenta is a fetomaternal organ, and has a major role in fetal and maternal pathophysiology, interest has emerged in dissecting the maternal and fetal genetic contributions in adverse pregnancy outcome as well as in maternal physiology (Petry *et al.* 2007, 2014; Warrington *et al.* 2018). Recently, we showed fetal-specific loci contributing to midgestational circulating immune biomarkers (Traglia *et al.* 2018) and chemicals (Traglia *et al.* 2017). We hypothesized that fetal-derived placental genotype may determine how much vitamin D is transported or stored, and therefore removed from maternal circulation. When we investigated potential fetal-specific loci independently impacting maternal vitamin D levels, we did not find genome-wide significant loci, but we found a suggestive locus ($P = 3 \times 10^{-6}$) near the gene *ACKR1*, encoding a member of the "receptor for multiple chemokines" family, previously significantly associated with CCL2 (Ahola-Olli *et al.* 2016). Recent evidence from single-cell RNA sequencing profiles from human first-trimester villi and decidua samples showed that the decidual vascular endothelial cells, responsible for the vasculature of the placenta, expressed *ACKR1* and *PCDH17* (Suryawanshi *et al.* 2018), two of the maternal genes near loci suggestively cross-associated with neonatal vitamin D. We hypothesize that the observed suggestive maternal/fetal cross-genetic control of vitamin D implicating genes with immune function might act via placenta-specific regulatory mechanisms beyond the classic regulators of the vitamin D pathway. This suggestion of immune system gene association is particularly intriguing because prior evidence in animal models and genome-wide analyses suggests a role for vitamin D in the innate immune system response, as well as the expression of vitamin D activating enzymes by macrophages, dendritic cells, and lymphocytes (Adams and Hewison 2010; Chun *et al.* 2014; Corripio-Miyar *et al.* 2017). Our association results imply that immune genes influence vitamin D levels as well. Further investigations are required to clarify the interaction between vitamin D and the immune system. For example, a study with placental samples might be well positioned to investigate the roles of maternal and fetal-derived placental tissues in some of our results.

When genotyping a selected set of 22 candidate SNPs (from the vitamin D pathway and our GWAS loci) in the additional samples, including a larger number of ASD- and ID-affected subjects from the same EMA cohort, we did not find any genetic variants passing the GW threshold in association with vitamin D levels. Although a previous study showed genetic contribution to ASD risk from one paternal and one neonatal SNP in the *GC* and *VDR* genes (Schmidt *et al.* 2011), when we tested our maternal/fetal SNPs mapping near the vitamin D metabolism genes, we did not find any significant variants that might act as risk or protective factors for ASD or ID outcome. These inconsistent results are likely due to the low statistical power of both studies. However, examining interactions of vitamin D, offspring outcome, and significant/suggestive associated SNPs, we observed a nominal interaction ($P = 10^{-5}$) between ID, neonatal vitamin D levels, and maternal rs490379 genotypes near *PKN2*, a PKC-related serine/threonine protein kinase involved in specific signal transduction responses in the cell. However, this finding would need to be replicated.

Limitations

The main limitation of this genetic study is the small sample size. Particularly when assessing categorical disorder status for ASD or ID, we have very low power and no replication data available, which is why this was a minor analysis in our study, with the main aims being to assess vitamin D regulation by maternal and fetal genetics. Our study design allowed us to detect only transpopulation effects on vitamin D present in individuals of Caucasian, Hispanic, and Asian ancestry, but not ancestry-specific effects in groups not well represented in our study such as South Asians and African Americans. However, we were able to show a genome-wide significant association and some additional suggestive associations that require independent replication. We were able to increase the sample size by including ID-affected and additional ASD-affected individuals to follow up a subset of 22 candidate SNPs genotyped via a different methodology. However, this enlarged sample did not strengthen our results for the candidate SNPs. The inclusion of additional genotyped individuals with TaqMan may also lead to some technical biases. The additional individuals did not include any control individuals, who were all genotyped with the Affymetrix array. This could have influenced the suggestive interactions between vitamin D and ID risk we observed, although we performed a control analysis among the TaqMan data, and we replicated the interaction and the direction with a nominal *P*-value. Additionally, the controls were originally matched with the ASD cases used in the first genotyping set, so may be slightly less-well suited for the ID cases. Finally, the vitamin D levels measured and tested in this study may be influenced by non-genetic sources (*e.g.*, sunlight, diet, and supplement intake) about which we have no information, potentially reducing our power to detect heritability and genetic association. However, Jiang *et al.* (2018) did not find any relationship between vitamin D intake and their GWAS SNPs, and no novel association signals when adjusting the marginal genetic effects for vitamin D intake.

Conclusions

Overall, we investigated genetic control of gestational and neonatal 25-hydroxyvitamin D levels in mother–neonate pairs in the EMA study, and found evidence for a genome-wide significant missense variant located in the *GC* gene, previously shown to be fundamental for the vitamin D pathway, influencing neonatal vitamin D levels. Novel loci from maternal and fetal genetics emerged at suggestive levels near genes important for immune function, potentially influencing vitamin D via distinct regulatory mechanisms at the placenta. However, these findings need to be replicated. Finally, the observation of an association between decreased neonatal vitamin D levels in ID-affected children from mothers with a specific genotype suggests that cross-genetic contribution in pregnancy might have a role in early risk for neurodevelopmental disorders.

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