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Vitamin D genes influence MS relapses in children

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Abstract

Objective: The aim of this study was to determine whether a vitamin D genetic risk score (vitDGRS) is associated with 25-hydroxyvitamin D (25(OH)D) level and multiple sclerosis (MS) relapses in children.

Methods: DNA samples were typed for single nucleotide polymorphisms (SNPs) from four genes previously identified to be associated with 25(OH)D levels. SNPs with strong associations with 25(OH)D after multiple comparison correction were used to create a genetic risk score (vitDGRS). Cox regression models tested associations of vitDGRS with relapse hazard.

Results: Two independent SNPs within or near *GC* and *NADSYN1/DHCR7* genes were strongly associated with 25(OH)D levels in the discovery cohort ($n=182$) after Bonferroni correction. The vitDGRS of these SNPs explained 4.5% of the variance of 25(OH)D level after adjustment for genetic ancestry. Having the highest versus lowest vitDGRS was associated with 11 ng/mL lower 25(OH)D level (95% confidence interval (CI) = −17.5, −4.5, $p=0.001$) in the discovery cohort. Adjusting for ancestry, sex, disease-modifying therapy (DMT), and *HLA-DRB1*15* carrier status, the highest versus lowest vitDGRS was associated with 2.6-fold (95% CI = 1.37, 5.03, $p=0.004$) and 2.0-fold (95% CI = 0.75, 5.20, $p=0.16$) higher relapse hazard in the discovery and replication cohorts, respectively.

Conclusion: The vitDGRS identifies children at greater risk of relapse. These findings support a causal role for vitamin D in MS course.

Keywords: Genetics, epidemiology, vitamin D, pediatric multiple sclerosis

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Introduction

Hypovitaminosis D has been associated with increased risk for multiple sclerosis (MS).[1,2] Low 25-hydroxyvitamin D (25(OH)D) level has also been associated with active disease in MS patients, including higher relapse rate, new T2 lesion formation, and increased disability.[3–6] However, concerns regarding unmeasured confounders and reverse causation still remain for this putative, modifiable risk factor. Genetic instrumental variables (IVs) can directly address these concerns by serving as a proxy of the environmental or modifiable risk factor of interest upstream of any confounding effects.[7] A genetic IV facilitates a Mendelian randomization experiment for the risk factor of interest.[7] For example, such variables have been used to support a causal association between fasting glucose, type 2 diabetes, and coronary artery disease[8] and between obesity and MS.[9,10] A number of previous studies including genome-wide association studies (GWAS) have examined the associations of individual single nucleotide polymorphisms (SNPs) in the vitamin D pathway with levels of vitamin D in healthy controls and subjects with various diseases and ancestries.[11–18]

Common genetic variants associated with the vitamin D pathway have emerged in GWAS for MS,[19,20] further supporting a role for vitamin D in the risk of disease. However, it is not clear if these particular variants directly affect vitamin D levels in patients or

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if additional genetic interrogation of the vitamin D pathway will reveal more sensitive proxies for vitamin D level effects in MS. Previous studies have shown that genetic IVs for low vitamin D level are also associated with risk to have MS,[21–23] but it is not known if genetic factors driving vitamin D level are associated with the clinically pressing question of disease course.

We sought to establish a genetic risk score for vitamin D pathway polymorphisms associated with serum vitamin D levels in pediatric MS patients and to determine whether that risk score could replicate the association of vitamin D level with disease activity, specifically relapse rate, in pediatric MS.

Materials and methods

Subjects

Consecutive pediatric subjects presenting for care and meeting criteria for MS or clinically isolated syndrome (CIS) with high risk of MS were offered enrollment into the US Network of Pediatric MS Centers registry. For the discovery cohort, subjects were enrolled from University of California, San Francisco, and Stony Brook, New York, from 2006 to 2011 and prospectively followed for relapses. For the replication cohort, subjects were recruited from nine centers across the United States from 2011 to 2016 (none overlapping with the discovery set) as part of an investigation of risk factors in pediatric MS (R01NS071463, PI Waubant). Most centers have large catchment areas and provide care to families with and without private health insurance. Parents provided written consent and age-appropriate children provided assent to participate in the study. Blood samples were collected upon enrollment. Parents reported race and ethnicity according to the National Institutes of Health (NIH) standards. Disease-modifying therapy (DMT) use was recorded in both cohorts.

Vitamin D levels

Serum measures of 25(OH)D for all subjects were determined by chemiluminescence assay as previously described (Heartlands, ARUP Laboratories).[3] Assays were performed in batch within each cohort group.

Relapses

Clinical events had to meet the following standard criteria to be considered a clinical MS relapse: new or significantly worsened neurological symptoms lasting greater than 24 hours, no evidence of infection or fever, and no exposure to extreme heat (pseudoexacerbation excluded).

Genotyping

DNA samples were prepared from whole blood using standard procedures. The Infinium 660K BeadChip or HumanOmniExpress BeadChip was used to genotype each study participant as previously described[19] for SNPs associated with four vitamin D pathway genes of interest (*GC, CYP2R1, DHCR7*, and *NADSYN1*), which have previously been shown in GWAS and other studies to be associated with vitamin D levels.[11,14,24–27] The Illumina GenomeStudio software was employed to perform standard quality control assessments including overall sample and SNP call rates ($<$90%), sex discrepancies, reproducibility of replicates, and checks for Mendelian inheritance using CEPH control trios. Quality control measures and comparison of sample genotypes across the two abovementioned Illumina platforms were performed using PLINK v.1.07.[28]

We employed IMPUTE2 for genotype imputation using 1000 Genomes Phase 3 reference haplotypes. To be used in the analyses, SNPs had to have high imputation quality ("info" score $>$ 0.3). SNPs that were associated with being genotyped on a particular array were removed, imputed in fewer than 99% of the individuals, or deviated from the Hardy–Weinberg equilibrium in the available controls. SNPs were also excluded if the minor allele frequency (MAF) was less than 0.05.

Ancestral estimates were generated as previously described using default parameters within STRUCTURE v2.3.1 and five ancestral populations (European, African, Central Asian, American, and East Asian).[29,30]

Linkage disequilibrium (LD) analysis is implemented by PLINK, and using data of 1000 genomes 32 haplotype blocks were identified within genomic regions on chromosomes 4, 11, and 12 containing the genes of interest: *GC, CYP2R1, DHCR7*, and *NADSYN1*. Full variation within each gene, including the SNPs previously identified through GWAS,[11,14,24–27] was investigated for association with vitamin D level. SNPs associated with the genes *VDR* and *CYP24A1* were not included in these analyses, as they have been identified to be direct risk factors for MS in large GWAS, limiting their ability to be used in an IV for vitamin D level.

Table 1. Participant characteristics.

	Discovery set ($n=182$)	Replication set ($n=110$)
Age at baseline, mean years (±SD)	13.1 (±4.2)	13.7 (±3.5)
Median follow-up, months (range)	34 (2–174)	32 (1–69)
Patient-years of follow-up	611	221
Median relapses over follow-up period (range)	1 (0–12)	0 (0–7)
Total relapses in cohort	408	130
White (%)	120 (65.1)	70 (65.4)
Hispanic (%)	55 (30.3)	29 (26.4)
Female (%)	124 (65.8)	71 (64.5)
Median 25(OH)D, ng/mL (range)	23 (2–63)	25 (4–66)
*HLA-DRB1*15:01/03* positive (%)	74 (41.4)	39 (35.4)
DMT exposure (%)	131 (72)	90 (81)

25(OH)D: 25-hydroxyvitamin D; DMT: disease-modifying therapy; SD: standard deviation; vitDGRS: vitamin D genetic risk score.

Carrier status for *HLA-DRB1*15:01/03* allele was determined by direct sequencing. Participants were noted as carriers versus non-carriers of *HLA-DRB1*15*.

Statistical analysis

Mean values, medians, or frequencies were used to describe patient characteristics as appropriate. Additive genotype models using linear regression were used to test association between each SNP and vitamin D level. To address concern for false positives resulting from multiple comparisons, a Bonferroni correction was applied for the 32 regions of DNA tested ($p<0.0016$). SNPs meeting these stringent criteria were then used to construct an unweighted risk score for low vitamin D level (vitamin D genetic risk score (vitDGRS)). To assess whether vitDGRS and vitamin D levels approximated a normal distribution, the quantiles of the risk score or vitamin D level were plotted against the quantiles of a normal distribution (qnorm function, STATA v12). Linear regression models compared vitDGRS to 25(OH)D level. To determine the variance of vitamin D level explained by the risk score, we started first with a model only adjusting for genetic ancestry and then a second model with ancestry and the vitDGRS. We examine the additional variance of the outcome (vitamin D level) explained by the model by adding the vitDGRS variable.

Survival analysis (Cox proportional hazards regression) was employed to determine the association of vitDGRS with relapse hazard. The model was tested for proportional hazard assumption using Schoenfeld residuals. Potential confounding was assessed for genetic ancestry, sex, and *HLA-DRB1*15:01/03* carrier status (yes/no). While DMT use would not be expected to be a confounder, as it is not associated with genotype, treatment would be expected to have strong effects on the outcome and thus the standard errors of the models. Therefore, we included DMT use as a time-varying covariate. Start and stop dates of DMT were used to determine the amount of exposure. All analyses were performed using STATA 15 (StataCorp, TX, USA).

Results

Characteristics of the 182 pediatric subjects in the discovery cohort and 110 subjects in the replication cohort are described in Table 1. Briefly, in both groups roughly 65% of the subjects were female, 65% were White, and 30% were Hispanic. In the more recent replication cohort, a higher percentage of patients had been exposed to DMT by the time of enrollment (81% vs 72%). Mean 25(OH)D levels in both patient cohorts were near lower limit of normal range (20 ng/mL) and the distribution of levels approximated a normal distribution (data not shown). There was no difference in vitamin D levels between girls and boys ($p=0.44$). Degree of European ancestry was associated with vitamin D level (explained 13% variance of 25(OH)D level). For those with at least 50% European ancestry compared to non-European ancestry, vitamin D levels were 7.9 ng/mL higher on average (95% confidence interval (CI) 5.02, 10.81, $p<0.001$).

Derivation of the vitDGRS. A total of 32 independent genomic regions of DNA on chromosomes 4, 11, and 12, containing the four genes of interest (*GC, CYP2R1, DHCR7*, and *NADSYN1*) from previous GWAS, were evaluated for association with vitamin D levels in the discovery cohort of pediatric MS subjects (Figure 1). Of these, two independent regions were found to be

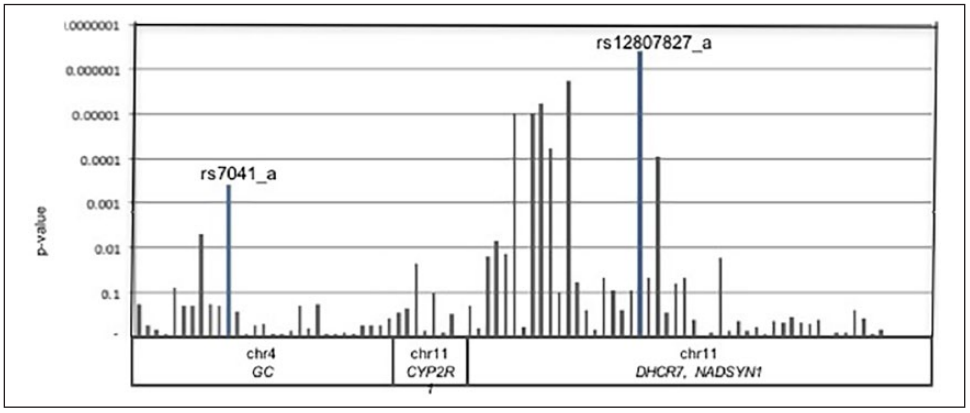

Figure 1. Association of SNPs related to *GC, CYP2R1, NADSYN1*, and *DHCR7* genes and 25(OH)D level in children with MS.
Manhattan plot of the *p*-value (*y*-axis) of the association between vitamin D pathway SNPs (*x*-axis) and 25(OH)D level is generated. Two final SNPs selected from independent linkage disequilibrium blocks are indicated by the blue lines (rs7041_a (*GC*) and rs12807827_a (*NADSYN1*).

associated with 25(OH)D levels in the discovery cohort that survived correction for multiple comparisons ($p < 0.0016$). SNPs in these regions with the lowest *p*-values were selected to represent these LD blocks: rs7041_a (*GC*) and rs12807827 (*NADSYN1/DHCR7*). For the two loci, there were additive effects for the risk allele. An unweighted genetic risk score of the alleles at these loci was normally distributed in the discovery cohort (data not shown).

For the discovery cohort univariate analysis, one unit of the vitDGRS was associated with 3.9 ng/mL lower 25(OH)D level (95% CI = −5.21, −2.61, $p = 2.95 \times 10^{-9}$). Having the highest vitDGRS versus carrying no risk alleles was associated with 15.6 ng/mL lower 25(OH)D level on average (95% CI = −20.8, −10.4, $p = 2.95 \times 10^{-9}$) in the discovery cohort. Adjusting for genetic ancestry, the highest versus lowest vitDGRS was associated with 11 ng/mL lower 25(OH)D level (95% CI = −17.5, −4.5, $p = 0.001$). The vitDGRS explained 4.5% of the variance of 25(OH)D level after adjustment for genetic ancestry. If the analysis was restricted only to those of European descent, similar results were observed ($n = 107$ participants): the highest vitDGRS versus the lowest was associated with 13.5 ng/mL lower vitamin D level on average (95% CI = −22.1, −5.1, $p = 0.002$). Adjustments for sex, *HLA-DRB1*15:01/03* status (yes/no), or DMT use did not change the results (data not shown).

The vitDGRS was associated with serum 25(OH)D levels in the replication cohort. Each unit of the score was associated with 3.2 ng/mL lower 25(OH)D level and the highest versus lowest vitDGRS with 12.8 ng/mL lower level in this independent data set (Table 2). After adjusting for genetic ancestry, the highest versus lowest vitDGRS was associated with 5.7 ng/mL lower 25(OH)D level (95% CI = −16.3, 4.8, $p = 0.28$). When the analysis was limited to those of European descent, similar point estimates to unadjusted were observed; though given limited sample size ($n = 70$), the results did not reach nominal statistical significance: the highest vitDGRS versus the lowest was associated with 11.6 ng/mL lower vitamin D levels (95% CI = −23.9, 0.74, $p = 0.065$).

Association between vitDGRS and relapses

Of important potential clinical significance and to support a causal role for vitamin D in disease course, we examined whether the vitDGRS was associated with relapses in children. In a multivariable Cox regression model adjusting for ancestry, sex, DMT use, and *HLA-DRB1*15:01/03* status, one unit of the vitDGRS was associated with 27% higher hazard to relapse in the discovery cohort (Table 3). Comparing the highest to the lowest vitDGRS score, there was a 2.6-fold (260%) higher hazard to relapse (Table 3). Similar results were observed in the replication cohort, but in this smaller sample of participants with greater DMT exposure and fewer overall relapses this result did not reach nominal statistical significance ($p = 0.16$). For each unit of vitDGRS, there was 19% higher hazard to relapse and for highest versus lowest vitDGRS score, a twofold (200%) higher hazard to relapse (Table 3). Furthermore, even though the primary multivariable analysis for the replication cohort did not reach nominal statistical significance there was a clear dosage effect of increasing vitDGRS score in this cohort (e.g. having two risk alleles = 11%

Table 2. Vitamin D genetic risk score association with 25(OH)D level.

	Discovery ($n=182$)		Replication ($n=110$)	
	β (95% CI)	*p*-value	β (95% CI)	*p*-value
Unadjusted				
Per unit vitDGRS*	–3.90 ng/mL (–5.21, –2.61)	2.95×10^{-9}	–3.2 ng/mL (–5.44, –0.95)	6.0×10^{-3}
4 units vitDGRS	–15.6 ng/mL (–20.8, –10.4)	2.95×10^{-9}	–12.8 ng/mL (–21.8, –3.78)	6.0×10^{-3}
Adjusted for genetic ancestry				
Per unit vitDGRS*	–2.74 ng/mL (–4.38, –1.11)	1.0×10^{-3}	–1.43 ng/mL (–4.07, 1.21)	2.8×10^{-1}
4 units vitDGRS	–11.0 ng/mL (–17.5, –4.50)	1.0×10^{-3}	–5.7 ng/mL (–16.3, 4.8)	2.8×10^{-1}

25(OH)D: 25-hydroxyvitamin D; CI: confidence interval.
*vitDGRS—unweighted 25(OH)D genetic risk score derived from number of risk genotypes from rs7041 (*GC*) and rs12807827 (*DHCR7/NADSYN1*).

Table 3. Vitamin D genetic risk score association with relapse rate.

	Discovery ($n=182$)		Replication ($n=110$)	
	HR (95% CI)	*p*-value	HR (95% CI)**	*p*-value
Univariate models				
Per unit vitDGRS*	1.27 (1.08, 1.50)	0.004	1.17 (0.98, 1.41)	0.079
4 units vitDGRS	2.62 (1.37, 5.03)	0.004	1.91 (0.93, 3.92)	0.079
Multivariable models**				
Per unit vitDGRS*	1.27 (1.08, 1.50)	0.004	1.19 (0.93, 1.51)	0.16
4 units vitDGRS	2.62 (1.37, 5.03)	0.004	1.98 (0.75, 5.20)	0.16

25(OH)D: 25-hydroxyvitamin D; CI: confidence interval; HR: hazard ratio.
*vitDGRS—unweighted 25(OH)D genetic risk score derived from number of risk genotypes from rs7041 (*GC*) and rs12807827 (*DHCR7/NADSYN1).*
**Adjusted for ancestry, sex, disease-modifying therapy use, and *HLA-DRB1*15* carrier allele status.

increased relapse hazard, three risk alleles = 34% increase, and four risk alleles = 44% increased relapse hazard).

Restricting the analyses to those of European descent, there was 38% higher relapse hazard per unit of vitDGRS (hazard ratio (HR) = 1.38, 95% CI = 1.12, 1.67, $p = 0.003$) in discovery set and 18% higher hazard (HR = 1.18, 95% CI = 0.87, 1.59, $p = 0.29$) in the replication data set. Comparing highest to lowest vitDGRS, there was 3.6-fold (360%) higher relapse hazard (95% CI = 1.53, 8.55, $p = 0.003$) in the discovery set and 1.9-fold (190%) higher hazard (95% CI = 0.58, 6.42, $p = 0.29$) in the replication data set.

Discussion

We show for the first time that polymorphisms in the vitamin D pathway are associated with 25(OH)D levels and relapse rate in pediatric MS patients. A vitDGRS captures lower levels of 25(OH)D in children with MS. Importantly, we demonstrate that a high-risk score was associated with a clinically relevant increased relapse rate, supporting a causal role of vitamin D in disease course. It is of future interest to determine how the risk score may affect subgroup responses in treatment trials of vitamin D supplementation.

The two polymorphisms in the risk score have strong rationale for driving vitamin D levels and relapses. The rs7041 SNP is within the protein encoded by *GC*. This protein binds vitamin D and transports it to various targets.[31] The second SNP (rs12807827) in the risk score is annotated as tagging *NADSYN1*, the gene product of which is glutamine-dependent NAD(+) synthetase. It is an enzyme that catalyzes the final step in the synthesis of nicotinamide adenine dinucleotide. However, according to HaploReg (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) and as illustrated in the figure, rs12807827 also tags some variants in *DHCR7*. This latter gene encodes an enzyme that acts on 7-dehydrocholesterol, precursor to 25(OH)D. The enzyme is thought to play

a critical role as a switch between cholesterol and vitamin D production.[32]

Conclusive evidence that vitamin D supplementation improves clinical outcomes in MS is still pending in large ongoing clinical trials;[33] in light of this evidence gap, our results further support a causal role for vitamin D level and the related pathway and disease course in MS. Our data are consistent with some of the completed vitamin D pilot trials. One of the largest trials to date, SOLAR, with over 200 participants randomized, demonstrated a lower annualized relapse rate in those receiving add-on high-dose vitamin D3 compared to those only receiving interferon (annualized relapse rate (ARR) 0.28 vs 0.41), though this was a secondary aim of the study and did not reach statistical significance.[34] In the CHOLINE study of 63 patients, high-dose vitamin D3 was associated with decreased relapses in those who completed the trial, but not in the intention-to-treat analysis.[35] EVIDIMS, a small pilot trial of 53 MS patients with primary outcome reported in 2018, examined high-dose versus low-dose vitamin D supplementation on new T2 lesion development and did not find a significant difference between groups.[36] However, the sample size was small and even the control low-dose arm had an average increase of 8 ng/mL in vitamin D level from below to above the normal cutoff value for vitamin D according to the US Food and Drug Administration (FDA) (20 ng/mL).

Previous IVs for vitamin D and obesity (high body mass index (BMI)) have demonstrated association with risk to have MS, but were not compared to measure serum 25(OH)D levels or BMI in pediatric MS patients.[21] The use of genetic IVs, which drive the levels of environmental risk factors, reduces concerns about confounding and reverse causation. In addition, it has thus far not been extensively explored what the heterogeneity may be in the role of vitamin D in MS across patients who carry different genotypes and how polymorphisms may affect vitamin D supplementation response. Further study of vitamin D genetic polymorphisms may help clarify these clinically relevant questions.

Strengths of this study include the following: (1) the use of very well-phenotyped cohorts of pediatric MS patients with prospective relapse capture, (2) replication with similar effect sizes of the association of the vitDGRS with vitamin D levels and relapses in an independent cohort of children, and (3) rigorous analytical methods. We demonstrate that the genetic IV is associated with disease course, and the magnitude of this association is clinically relevant. Limitations include the possibility of violations of the assumptions underlying use of genetic IVs, that the genes studied have (unknown) direct links to relapses independent of vitamin D level or pathway, and possible exclusion of the other SNPs that may contribute, but due to sample size did not survive the conservative Bonferroni correction for multiple comparisons. The results in the replication data set did not all reach nominal statistical significance, but the effect sizes were reassuringly similar to the discovery set and there was a linear dosage effect on relapse hazard. The replication data set had decreased statistical power due to smaller N and fewer relapses with greater use of and more potent DMTs. Finally, studies of Mendelian randomization in which the IV may be associated with both disease incidence and course have the possibility of collider bias in which unmeasured factors associated with incidence and disease course could drive an association between the IV and disease course variable.[37]

In summary, a genetic IV captures risk for lower 25(OH)D serum levels and is associated with relapses in pediatric MS patients. Future work will determine whether these particular SNPs have an impact on vitamin D supplementation success with respect to increasing levels, decreasing MS activity, or affecting immunological function.

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