

Title: Maternal *VDR* variants rather than 25-hydroxyvitamin D concentration during early pregnancy are associated with type 1 diabetes in the offspring

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Abstract

Aims/hypothesis

We investigated whether 25-hydroxyvitamin D concentration associated SNPs in the metabolic pathway of vitamin D show different genotype distributions between Finnish families with an offspring with type 1 diabetes (cases) and families with a healthy offspring (controls).

Methods

31 SNPs in 8 genes were studied in case and control mothers and family members (offspring with type 1 diabetes and healthy siblings, healthy control children and fathers) ($n=2854$). 25-hydroxyvitamin D concentration was studied in 474 case and 348 matched control mothers during pregnancy.

Results

Genotype distributions of 13 SNPs (in the following genes: 7-dehydrocholesterol reductase *NADSYN1/DHCR7*, vitamin D receptor *VDR*, group-specific component *GC* and *CYP27A1*) that showed a nominal association with 25-hydroxyvitamin D concentration ($p<0.05$) were compared between case and control families. SNPs in the *VDR* had different genotype distributions between case and control mothers (rs1544410 $p=0.007$, rs731236 $p=0.003$, rs4516035 $p=0.015$), of which two (rs1544410 and rs731236) remained significant after correction for multiple testing with false discovery rate (FDR). Mean 25-hydroxyvitamin D concentrations during pregnancy in case and control mothers did not differ.

Conclusions/interpretation

Our preliminary results suggest that maternal genotypes of SNPs in the *VDR* may influence the in-utero environment and thus contribute to the early programming of type 1 diabetes in the fetus. It is possible that the effects are only relevant in the presence of vitamin D insufficiency.

Keywords

Type 1 diabetes, vitamin D, 25-hydroxyvitamin D, *VDR*, pregnancy, in-utero, maternal genotype

Abbreviations

CUBN	cubilin gene
CYP	cytochrome p450
DBP	vitamin D binding protein
NADSYN1/DHCR7	7-dehydrocholesterol reductase
GC	group-specific gene
VDR	Vitamin D receptor
25OHD	25-hydroxyvitamin D

Introduction

Vitamin D deficiency, which is most commonly defined as serum 25-hydroxyvitamin D (25OHD) concentration lower than 50 nmol/l, may increase the risk of autoimmune diseases, diabetes, cancer and cardiovascular diseases [1]. The existing evidence on the association between vitamin D and type 1 diabetes is inconsistent. Vitamin D supplementation during infancy has been connected with decreased risk of type 1 diabetes in several [2-4], but not in all studies [5]. While it has been suggested that vitamin D supplementation during pregnancy associates with lower risk of type 1 diabetes, and that lower 25OHD concentrations during pregnancy associates with higher type 1 diabetes risk in the offspring [6,7], some studies have not been able to confirm this [8,9]. The prevalence of vitamin D deficiency has been shown to be higher in children with multiple pancreatic islet autoantibodies compared with autoantibody-negative children, although type 1 diabetes did not progress faster in the vitamin D deficient group [10]. In another study, 25OHD concentration in children was not associated with islet autoimmunity or progression of type 1 diabetes [11]. Several genes important to the metabolic pathway of vitamin D have been robustly associated with 25OHD concentrations, islet autoimmunity or type 1 diabetes [12-17].

The metabolism of vitamin D consists of several hydroxylation reactions catalysed by the members of the cytochrome p450 (CYP) family. In the skin, vitamin D₃ (cholecalciferol) is synthesized from 7-hydrocholesterol (7-dehydrocholesterol reductase *NADSYN1/DHCR7* catalyses the conversion of 7-dehydrocholesterol to cholesterol). Vitamin D₃ is hydroxylated in the liver to 25OHD (*CYP2R1* and *CYP27A1*). Vitamin D₃ and 25OHD are transported in the circulation bound to the vitamin D binding protein (DBP; encoded by the group-specific component *GC* gene). 25OHD is hydroxylated to the active form of vitamin D, 1,25-dihydroxyvitamin D (1,25OHD₂) in the kidney or target cells (*CYP27B1*). Cubilin (encoded by the cubilin gene *CUBN*) is needed for the absorption of 25OHD-DBP complex into the proximal tubules of the kidney. 1,25OHD₂ is further hydroxylated by 24-hydroxylase (*CYP24A1*) to the inactive form of vitamin D [18]. The active form of vitamin D acts through vitamin D receptor (*VDR*), which is a nuclear transcription factor that regulates transcription of vitamin D sensitive genes. *VDRs* have been detected in most tissues and it has been estimated that *VDRs* can regulate the expression of as many as 500 genes [19].

The aim of the study was to compare the genotype distributions of the vitamin D metabolism related SNPs that show the strongest association with 25OHD concentration in the Finnish population between case (offspring with type 1 diabetes) and control families (non-diabetic offspring).

Methods

Study Population

A detailed description of the study population of pregnant women (including the eligibility and matching criteria for case and control mothers) and the serum samples, as well as 25OHD analysis, has been previously published (8). Initially, 751 families with offspring with type 1 diabetes and 751 control families around Finland were invited to participate in the study. A saliva sample was collected for DNA extraction from all participants. DNA sample was derived from altogether 2854 individuals (512 case

mothers, 470 case fathers, 534 case children, 238 healthy siblings, 379 control mothers, 340 control fathers and 381 control children). Of the 512 case mothers and of the 379 control mothers that had a DNA sample available, 474 and 348 had a 25OHD concentration available, respectively. The main reasons for missing 25OHD concentrations were that there was no sample, or no sample left in the Finnish Maternity Cohort sample collection, and that the mother did not give a permission to use the stored serum sample for 25OHD analysis. The mean age at diagnosis of children with type 1 diabetes was 3.4 years (range 0-7 years). Written informed consent was collected from all participants. Ethical committee of the Hospital District of Helsinki and Uusimaa approved the study.

Single Nucleotide Polymorphism Selection and Genotyping

DNA was isolated from 2854 individuals who submitted saliva samples using Oragene® kits (DNA Genotek Inc., Canada). 31 single nucleotide polymorphisms (SNPs) in 8 candidate genes were genotyped using TaqMan; *VDR* (rs731236, rs1544410, rs7975232, rs2228570, rs4516035, rs10783219); *GC* (rs4588, rs7041, rs12512631, rs2282679, rs3755967, rs17467825, rs2298850); *CYP2R1* (rs10741657, rs2060793, rs1993116, rs7116978, rs12794714, rs10500804); *CYP27B1* (rs108770112, rs4646536); *CYP24A1* (rs6013897); *CYP27A1* (rs17470271); *CUBN* (rs3740165); *NADSYN1/DHCR7* (rs12785878, rs3829251, rs7944926, rs12800438, rs3794060, rs4945008, rs4944957). All SNPs were in genes associated with the vitamin D pathway and previously associated with either serum 25OHD concentrations or type 1 diabetes [16,17,20-27].

Statistical methods

All statistical analyses were performed using Intercooled Stata10 for Windows (StataCorp. 2007. *Stata Statistical Software: Release 10*. College Station, TX: StataCorp LP). A linear regression was used to analyse the association between 25OHD concentrations and vitamin D metabolism related SNPs. Due to the known seasonal variation of 25OHD concentrations in Finland, the analyses were adjusted for month of sample collection. Pearson's χ^2 test was used for differences in proportions of case and control mothers by genotype. The sample size was derived from power calculations from our original paper (8). In SNP genotype distribution comparison multiple testing was controlled for using the false discovery rate (FDR) method (step-up procedure described by Benjamini and Hochberg [28] (0.05 as the criterion). If the original *p* value was smaller than the Benjamini-Hochberg critical value, the difference in genotype distribution was considered statistically significant.

Results

Genotyping

All SNPs were in Hardy-Weinberg equilibrium with the exception of *VDR* SNP rs7975232, which was removed from further analyses. 95-99% of samples were successfully genotyped and 100% of genotypes were concordant in 120 duplicate samples.

From the 31 SNPs we selected those that had the strongest association with 25OHD (with a nominal *p* value of <0.05) in the Finnish population (13 SNPs in 4 genes; *NADSYN1/DHCR7*, *VDR*, *GC* and *CYP27A*; table 1), and then compared the genotype

frequencies between case and control families (table 2). Three SNPs demonstrated different genotype distributions between mothers of children with type 1 diabetes and control mothers; all were located to the *VDR* (rs1544410 $p=0.007$, rs731236 $p=0.003$, and rs4516035 $p=0.015$). Two SNPs (rs1544410 and rs731236) remained statistically significant after correction for multiple testing with FDR. The difference in minor allele proportion between case and control mothers was not statistically significant (table 2).

There were no significant differences observed between allele frequency of the 13 studied SNPs in fathers, between case and control children, or between case children and their healthy siblings. Similarly, in case families no allele of the 13 studied SNPs was preferentially transmitted to the child with type 1 diabetes.

Maternal serum 25OHD concentrations

Mean 25OHD concentrations in case (44.9 nmol/l $n=474$) and control mothers (43.7 nmol/l $n=348$) did not differ significantly when adjusted for month of sample collection. 69.1% of all mothers had vitamin D deficiency (25OHD concentration <50 nmol/l). Only 3.7% of case mothers and 1.7% of control mothers had optimal vitamin D status (>75 nmol/l) in the first trimester of pregnancy. 25OHD concentrations changed almost 2 fold by season (March 34.3 ± 11.0 nmol/l and in August 61.2 ± 20.8 nmol/l; figure 1).

Discussion

In this study we investigated the genotype distributions of vitamin D metabolism related SNPs between families with type 1 diabetes offspring (cases) and families with healthy offspring (controls). Our results suggest that certain maternal *VDR* variants are associated with type 1 diabetes risk of the child independent of the child's genotype, and may thus influence the in-utero environment and contribute to the early programming of type 1 diabetes in the fetus.

In our previously published study [8] we did not find a difference in 25OHD concentrations between pregnant women in the first trimester who gave birth to child with type 1 diabetes when strictly matched with control families with no diabetic children, or between 25OHD concentrations during pregnancies of children with type 1 diabetes and their healthy siblings. This is in contrast with a smaller study in Norwegian women [7] in which low 25OHD concentrations (mainly in the last trimester) were associated with an increased risk of type 1 diabetes. However, more than 70% of the Norwegian women had 25OHD >50 nmol/l compared with 31% in Finnish women.

At this point we can only speculate the possible biological mechanisms that seem to connect certain maternal *VDR* variants with type 1 diabetes risk in the child. The 13 SNPs that we used to compare the genotype frequencies between case and control mothers were selected on basis of a nominal association with 25OHD concentration. However, based on the data provided in the present study, there is no reason to assume that the connection of the *VDR* variants with maternal 25OHD concentration would necessarily explain the increase in the risk of type 1 diabetes risk in children of the mothers carrying these *VDR* variants. The association of the *VDR* variants with 25OHD concentration can rather be seen as a marker of an (unknown) effect of the variants to

the maternal vitamin D metabolism and/or *VDR* function that further associate with type 1 diabetes risk in the child.

VDR is a transcription factor with hundreds of target genes [19]. We have previously reported that genetic variation of the *VDR* is a determinant of the expression of the *VDR* [29]. Thus it is possible that the genetic variation of the maternal *VDR* modifies the genetic effects of vitamin D of which some may, possibly together with vitamin D deficiency, contribute to the autoimmune process in the developing fetus and thus to the pathogenesis of type 1 diabetes. This would be consistent with the Diabetes Autoimmunity Study in the Young (DAISY) study, that demonstrated an interaction between *VDR* and protein tyrosine phosphatase, non-receptor type 2 gene (*PTPN2*) affecting the risk of progression to type 1 diabetes [13]. The fact that in the present study no difference in the genotype distributions of SNPs of the *VDR* was seen between children with type 1 diabetes and control children suggests that the effect is associated with maternal in utero environment and developmental processes during pregnancy.

The *VDR* is expressed in placenta and its expression levels rise in late pregnancy compared with mid-pregnancy [30]. Since the genomic actions of ligand binding to the *VDR* include modulation of the immune system with a shift to a T helper type 2 cytokine response pattern [31], the genomic actions of the maternal *VDR* may assume a greater role in the presence of a reduced supply of 25OHD to the fetus determined by both environment and genotype.

The focus in the genetics studies of type 1 diabetes has been on the affected child, and the effect of maternal genotype independent of the child's genotype has not been described as a determinant of type 1 diabetes risk in the child before. However, the maternal genotype has been previously associated with for example child's brain morphology [32], cognitive development [33], risk of autism [34] and atopic dermatitis [35]. The fact that type 1 diabetes associated autoantibodies that precede the diagnosis of type 1 diabetes can appear only months after birth [36], suggests a fetal programming of the disease.

The clinical significance of our finding is not clear. Several genes contribute jointly to the development of type 1 diabetes and thus a certain genetic marker alone may have only minor impact on the disease susceptibility. Our results require independent validation in a larger well characterised data set of cases (mothers of children with early-onset type 1 diabetes) and well-matched controls and in relatively homogenous populations and in whom 25OHD are recorded and have genotype data available. Also it would be important to investigate whether genetic variation of the *VDR* affects 25OHD concentrations in the fetus for example by collecting cord blood samples. Our results highlight the importance of investigating factors that affect the in-utero environment and that may thus contribute to the early programming of type 1 diabetes.

Although the present study had adequate power to detect the effect of certain maternal SNPs of the *VDR* on the type 1 diabetes risk in the child, the effect of other SNPs may not be seen in the present study due to limited power. If our results can be validated then this will be the first time that the maternal gene to environment interaction will have been demonstrated to influence autoimmunity in-utero which together with child's high risk type 1 diabetes associated genotype determines susceptibility to type 1 diabetes.

The results of the present study emphasize that the in-utero environment should be a line of investigation when trying to find means for primary prevention of type 1 diabetes.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript. The sponsors had no role in the design, data collection, data analysis, data interpretation, or writing or revisions of the report. The corresponding author had full access to all data in the study and had final responsibility to submit for publication.

Contribution statement

Professors Hitman and Tuomilehto had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Hitman, Tuomilehto, Lamberg-Allardt and Harjutsalo.

Acquisition of data: Miettinen, Kinnunen, Surcel, Smart (genotyping), Tuomilehto

Analysis and interpretation of data: Smart, Miettinen, Hitman, Tuomilehto, Mathews, Harjutsalo

Drafting of the manuscript: Smart, Miettinen, Hitman, Tuomilehto

Critical revision of the manuscript for important intellectual content: Kinnunen, Mathews, Surcel, Lamberg-Allardt

Statistical analysis: Smart, Mathews, Harjutsalo.

Administrative, technical, or material support: Mathews

Study supervision: Hitman, Tuomilehto.

All authors gave their final approval of the version to be published.

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Tables

25-hydroxyvitamin D concentrations (nmol/l) ^a by genotype												
SNP	Gene	11			12			22			<i>p</i>	<i>n</i>
		Mean	LCI	UCI	Mean	LCI	UCI	Mean	LCI	UCI		
rs4945008	<i>NADSYN1/DHCR7</i>	42.88	41.18	44.66	41.07	39.97	42.19	39.33	37.28	41.48	0.029	766
rs12785878	<i>NADSYN1/DHCR7</i>	42.94	41.25	44.69	41.03	39.95	42.14	39.21	37.19	41.34	0.021	768
rs4944957	<i>NADSYN1/DHCR7</i>	42.69	40.95	44.51	41.06	39.93	42.22	38.70	37.38	41.71	0.055	728
rs7944926	<i>NADSYN1/DHCR7</i>	42.97	41.24	44.78	41.04	39.94	42.17	39.20	37.16	41.35	0.022	757
rs3794060	<i>NADSYN1/DHCR7</i>	42.74	41.01	44.54	40.70	39.59	41.84	38.77	36.71	40.95	0.016	733
rs12800438	<i>NADSYN1/DHCR7</i>	42.98	41.29	44.74	41.12	40.03	42.23	39.34	37.31	41.47	0.024	775
rs4516035	<i>VDR</i> Prom	43.03	41.22	44.92	41.23	40.17	42.32	39.50	37.69	41.40	0.022	764
rs731236	<i>VDR</i> Taq1	40.29	38.86	41.78	42.12	40.91	43.36	44.02	41.54	46.66	0.025	769
rs1544410	<i>VDR</i> Bsm1	40.25	38.81	41.75	42.01	40.78	43.27	43.84	41.33	46.51	0.033	747
rs10783219	<i>VDR</i>	39.93	38.43	41.48	41.75	40.60	42.92	43.64	41.32	46.10	0.022	751
rs12512631	<i>GC</i>	40.23	38.72	41.80	41.94	40.79	43.12	43.71	41.43	46.11	0.029	755
rs4588	<i>GC</i> ; T436K	42.10	40.72	43.52	40.23	38.78	41.72	38.44	35.64	41.45	0.050	737
rs17470271	<i>CYP27A1</i>	42.04	40.71	43.42	40.59	39.15	42.09	39.19	36.38	42.22	0.030	750

Table 1: Association of mother's genotype with 25-hydroxyvitamin D concentrations. ^aadjusted for month serum sample taken; LCI: Lower Confidence Interval; UCI: Upper Confidence Interval; 11: Common Homozygotes; 12: Heterozygotes; 22: Rare Homozygotes.

SNP	Case mothers	Control mothers	OR (95% CI)	<i>p</i>	Benjamini-Hochberg critical value ^a
rs1544410				0.007**	0.008
Genotypes <i>n</i> (%)					
AA	48 (10.2)	42 (12.0)	0.85 (0.58-1.26)	0.41	
AG	227 (48.1)	130 (37.0)	1.30 (1.10-1.53)	0.0016	
GG	197 (41.7)	179 (51.0)	0.81 (0.70-0.94)	0.0062	
Alleles <i>n</i> (%)					
A	323 (34.2)	214 (30.5)	1.19 (0.96-1.46)	0.11	
G	621 (65.8)	488 (69.5)			
rs731236				0.003**	0.004
Genotypes <i>n</i> (%)					
CC	49 (9.9)	43 (12.3)	0.80 (0.55-1.18)	0.26	
CT	243 (49.0)	130 (37.2)	1.32 (1.12-1.55)	0.0007	
TT	204 (41.1)	176 (50.4)	0.82 (0.70-0.95)	0.0075	
Alleles <i>n</i> (%)					
C	341 (34.4)	216 (30.9)	1.17 (0.95-1.44)	0.14	
T	651 (65.6)	482 (69.1)			
rs4516035				0.015	0.012
Genotypes <i>n</i> (%)					
CC	124 (25.1)	63 (18.4)	1.37 (1.04-1.79)	0.0215	
CT	225 (45.5)	189 (55.1)	0.83 (0.72-0.95)	0.0066	
TT	145 (29.4)	91 (26.5)	1.11 (0.89-1.38)	0.37	
Alleles <i>n</i> (%)					
C	473 (47.9)	315 (45.9)	1.08 (0.89-1.32)	0.43	
T	515 (52.1)	371 (54.1)			

Table 2: Genotype distribution of 3 VDR SNPs in case and control mothers.

^amultiple testing correction with false discovery rate (FDR) approach (Benjamini-Hochberg step-up procedure). If the original p value is less than the Benjamini-Hochberg critical value, it is considered statistically significant

^{**}statistically significant after multiple testing correction with FDR approach.

Legends for figures

Figure 1: Maternal 25-hydroxyvitamin D concentrations by case (black) / control (white) status and month of sampling.