Genetic Variation of the Vitamin D Binding Protein Affects Vitamin D Status and Response to Supplementation in Infants

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Short title: *GC* genotype affects vitamin D response in infants

Precis: In this vitamin D intervention study on 913 infants *GC* genotype, diplotype and haplotype affected vitamin D status and response to supplementation in participants randomized to receive 30 µg/day.

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Abstract

Context: Single nucleotide polymorphisms (SNPs) of the vitamin D binding protein encoding *GC* (group component) gene affect 25-hydroxyvitamin D (25OHD) concentrations but their influence on vitamin D status and response to vitamin D supplementation in infants is unknown.

Objective: To study *GC* genotype-related differences in 25OHD concentrations and response to supplementation during a vitamin D intervention study in infants.

Design: In this randomized controlled trial, healthy term infants received 10 or 30 μg vitamin D_3/day from 2 weeks to 24 months of age. *GC* SNPs rs2282679, rs4588, rs7041 and rs1155563 were genotyped, rs4588/7041 diplotype and haplotypes of rs2282679, rs4588 and rs7041 ($Haplo_{3SNP}$) and of all four SNPs ($Haplo_{4SNP}$) were determined.

Main outcome measures: 25OHD measured in cord blood at birth and at 12 and 24 months during intervention.

Results: Altogether 913 infants were included. Minor allele homozygosity of all studied *GC* SNPs, their combined haplotypes and rs4588/rs7041 diplotype 2/2 were associated with lower 25OHD concentrations at all time points in one or both intervention groups (analysis of covariance $p<0.043$), with the exception of rs7041 which did not affect 25OHD at birth. In the high-dose supplementation group, receiving 30 μg vitamin D_3/day, but not in those receiving 10 μg/day, genotype of rs2282679, rs4588 and rs7041, diplotype and $Haplo_{3SNP}$ significantly affected intervention response (repeated measurement analysis of covariance $p_{interaction}$ <0.019). Minor allele homozygotes had lower 25OHD concentrations and smaller increase in 25OHD throughout intervention.

Conclusions: In infants, vitamin D binding protein genotype affects 25OHD concentration and efficiency of high-dose vitamin D_3 supplementation.

Key words: Vitamin D, infant, intervention, *GC* genotype, supplementation response, randomized trial

1. Introduction

Vitamin D insufficency is common worldwide (1), and in response, many countries have implemented recommendations of vitamin D supplementation and vitamin D fortification of food products (2-4). Supplementation can be considered particularly important during infancy and early childhood, when vitamin D supply from diet and sunlight may be scarce and growth and development are rapid. Vitamin D insufficiency in this age-group can have lifelong skeletal and possibly extraskeletal effects (1,5-7).

Concentration of 25-hydroxyvitamin D (25OHD) is an acknowledged marker of vitamin D status. Optimal 25OHD concentration is still unclear, but in children concentrations above 50 nmol/L are generally considered sufficient. Serum 25OHD concentrations have been found to show notable individual variation, partly due to genetic factors (8-10). Previous reports and genome wide association studies (GWAS) have identified the *GC* (group component) gene, encoding the vitamin D binding protein (DBP), as one of the genes associated with differences in 25OHD concentrations and with individual risk of vitamin D insufficiency (8,11-13). DBP is a 52-59 kDa protein of the albumin gene family, which in the circulation binds and transports up to 90% of vitamin D and its metabolites (14,15). The *GC* gene has been found to be greatly polymorphic, with over 120 described variants, some resulting in distinct structural phenotypes of DBP (14,16). The distribution of these variants differs between ethnic groups (14).

Two of the most studied genetic variants of the *GC* gene, single nucleotide polymorphisms (SNPs) rs4588 (NM_000583.3 (GC): c.1307C>A, p.Thr436Lys) and rs7041 (c.1296T>G, p.Asp432Glu), have been repeatedly shown to be linked to differences in 25OHD concentrations. In adults and older children, associations have been demonstrated for both genotypes of the SNPs and their six diplotypes, reflecting the combinations of the three common phenotypic variants of the DBP (1S, 1F and 2) (9,17-19). Among other identified polymorphisms of the *GC* gene, several adult studies have shown the intronic SNPs rs2282679 and rs1155563 to be associated with differences in 25OHD concentrations (8,11,20-22). For rs2282679, genotype-related differences in 25OHD concentrations have also been found in infants at birth (23).

In addition to associations with 25OHD concentration, some previous studies have also shown possible genotype-related differences in response to vitamin D supplementation in adults, including pregnant women (24-27), but these differences have not been studied in children.

Potential associations of the *GC* SNPs with vitamin D supplementation response in infants are unclear. As 25OHD concentrations and response to vitamin D supplementation show individual variation, the optimal dose for vitamin D supplementation in infants may also be genotype dependent (8,17,24,25,27,28). Our study examined how genetic variation in four single nucleotide polymorphisms of the DBP encoding *GC* gene impacts 25OHD concentrations and response to two different vitamin D supplementation doses in infants from 2 weeks to 24 months of age.

2. Methods

A. Participants and follow-up

This study is a part of the randomized, double-blinded and controlled Vitamin D Intervention in Infants (VIDI) trial, of which protocol, inclusion and exclusion criteria have previously been described (29,30). Ethical approval for the study was granted by the Research Ethics Committee of the Hospital District of Helsinki and Uusimaa (107/13/03/03/2012) and the study was performed in accordance with the principles of the Helsinki Declaration. The trial protocol is registered in ClinicalTrials.gov (NCT01723852). Parents of participants gave written informed consent at recruitment.

Altogether 987 healthy infants of mothers of Northern European origin, born at term and with birthweight appropriate for gestational age, participated in the VIDI trial performed at the Kätilöopisto Maternity Hospital, in Helsinki, Finland, between January 2013 and June 2016. The participants were randomized to receive daily vitamin D_3 supplementation of either 10 μg (400 IU) (Group10), the standard recommended supplementation for this age-group in Finland (4,31), or 30 μg (1200 IU) (Group30) from age 2 weeks to 24 months.

Baseline data on infant birth, maternal background and use of vitamin D supplementation during pregnancy were collected retrospectively from medical records and by questionnaires. Umbilical cord blood samples collected at birth were used for genomic DNA and to assess baseline 25OHD concentrations.

At 12 and 24 month study visits venous blood samples were obtained for analyses of 25OHD concentrations and weight and length of the participants were measured and transformed into standard deviation scores (SDS) using Finnish pediatric growth references (32).

Adherence to the intervention D_3 supplement was calculated from study-diaries in which administration of supplement was recorded daily by the parents of the participating child. Duration of breastfeeding was also reported in the diaries. The study-diaries were collected and reviewed every 3-6 months during the trial (29,30).

VIDI trial participants, who were later found not to fulfil the initial inclusion criteria (N=12), were diagnosed with basic pathologies (N=8), or lacked genotype data were excluded from analyses. The final study cohort included a total of 913 participants with available genotyping results for one or more of four selected *GC* SNPs (rs2282679, rs4588, rs7041 and rs1155563) in addition to baseline data.

B. Genotype analysis

Genomic DNA was extracted from cord blood samples, in the laboratory of the Finnish National Institute for Health and Welfare, using automated Chemagen MSM1 extraction (PerkinElmer Inc., Chemagen Technologie GmbH, Baesweiler, Germany) or the Gentra Puregene - kit (Qiagen GmgH, Hilden, Germany), in accordance with the manufacturers' instructions.

The studied SNPs were previously selected from the HapMap project database (33), preferring functional polymorphisms with high heterozygosity levels and previously shown associations with 25OHD concentrations (34). Genotyping of SNPs rs2282679, rs4588, rs7041 and rs1155563 was performed using TaqMan Assays (Thermo-Fisher, Waltham, MA, USA) (Taqman SNP Assay ID: C__26407519_10, C__8278879_10, C__3133594_30 and C__8278782_20 respectively) and the qPCR Bio-Rad CFX384 C1000 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) or the qPCR ABI Prism

7900HT system (Applied Biosystems, Foster City, CA, USA), according to manufacturers' instructions. Amplification was performed by protocols of 95 °C for 3 or 10 minutes, followed by 39 or 40 cycles of 15 seconds at 92 °C or 95 °C and 1 minute at 60 °C, respectively. Results were determined using end-point protocol-analysis by CFX Manager 3.1 (BioRad, Hercules, CA, USA) or SDS 2.3 (Applied Biosystems, Foster City, CA, USA) software. Previously genotyped samples from adult controls (4%) as well as randomly chosen duplicate internal (3%) and negative controls (2%) were used to validate the obtained genotyping results.

The obtained genotypes of SNP rs4588/rs7041 were combined into six known diplotypes, representing the six structural phenotype variants of the DBP protein (1S/1S, 1S/1F, 1F/1F, 1S/2, 1F/2, 2/2) (9,35). The genotypes of the studied SNPs were also combined into haplotypes including all four ($Haplo_{4SNP}$) and three ($Haplo_{3SNP}$) (excluding rs1155563) SNPs. Haplotypes were determined and linkage disequilibrium (LD) and Hardy-Weinberg equilibrium evaluated using Haploview 4.2 (Broad Institute, Boston, MA, USA) software. Haplotype homozygotes were identified and used in analyses.

C. Biochemical analyses

Concentrations of 25OHD at baseline (cord blood), 12 and 24 months were analyzed at the Pediatric Research Center, University of Helsinki, using a fully automated IDS-iSYS immunoassay system with chemiluminescence detection (Immunodiagnostic Systems Ltd., Bolton, UK). As previously reported (30), cord plasma 25OHD concentrations were adjusted to be comparable with serum 25OHD concentrations and further corrected due to changes in the IDS-iSYS system, in accordance with the manufacturers' instructions.

Intra-assay variation for 25OHD concentrations was <13% for cord blood, and <5% for the 12-month and 24-month samples. The quality and accuracy of the used analyses were validated by participation in the vitamin D External Quality Assessment Scheme (DEQAS, Charing Cross Hospital, London, UK). The method used showed a <8% positive bias when compared to the NIST (National Institute of Standards and Technology) Reference Measurement Procedure.

D. Statistical methods

Results are given in means and standard deviations (SD) or 95% confidence intervals (95%CI) for adjusted means. The normality of distribution within variables was visually evaluated. Logarithmic conversion (Ln) was used for non-normally distributed variables. Differences in normally distributed variables were studied using independent samples T-Test, while Mann-Whitney U-test was used when normal distribution was not obtained by logarithmic conversion. Chi-Square test was used for comparisons of categorical variables between intervention groups.

Analysis of variance (ANOVA) and covariance (ANCOVA) were used to evaluate the impact of SNP genotypes, diplotypes and haplotype homozygotes on serum 25OHD concentrations at birth, 12 and 24 months. Maternal and infant-related factors showing significant independent associations with S25OHD concentrations (maternal vitamin D supplementation during pregnancy, season, length-adjusted weight SDS, duration of breastfeeding, intervention group and adherence to intervention vitamin D_3 supplementation) were used as covariates. Bonferroni or Tamhane adjustments were used for multiple comparisons. Linear regression analysis was used to evaluate mean allelic effect on 25OHD concentration, of the studied polymorphisms.

Temporal change in 25OHD concentration during the intervention and modifying effects of the studied SNPs were analyzed using linear mixed models for repeated measurements (repeated measurements analysis of covariance) including all three time points (baseline, 12 and 24 months). Analyses were first performed for all participants, and secondly, as intervention group showed significant interaction with temporal change of 25OHD, separately within intervention groups. Non-genetic factors affecting temporal change of 25OHD concentration (season of birth, length-adjusted weight SDS at 24 months, duration of breastfeeding, adherence to intervention vitamin D_3 supplementation and interaction between adherence and temporal change) were used as covariates. As duration of breastfeeding was a significant covariate only in Group10, it was not included in the model when analyzing the higher-dose intervention group (Group30). To further evaluate intervention response, analyses were also performed in the subset of participants with >80% adherence to intervention D_3 supplement.

In participants with adherence >80%, the mean changes in 25OHD concentrations at 24 months of intervention (Δ25OHD = 25OHD concentration at 24 months - baseline 25OHD concentration) by geno-, diplo- and haplotype were also calculated and evaluated by ANOVA and ANCOVA, adjusting for season of birth, length-adjusted weight SDS at 24 months and adherence to supplementation. For variables with variances that were not equal, Welch test of equality of means was used to further evaluate differences between variants.

IBM SPSS Statistics 24 (IBM, Armonk, NY, USA) software was used for data analyses. A p-value <0.05 was considered statistically significant. Missing values were excluded analysis-by-analysis.

3. Results

A. Participants and distributions of genotypes, diplotypes and haplotypes

A total of 913 infants (49.7% girls) were included in this study. Participant details are described in Table 1. Baseline characteristics did not differ between intervention groups.

Table 1. Characteristics of study participants.

	All	Group10	Group30	P *
Baseline				
Number of participants (% girls)	913 (49.7%)	459 (49.7%)	454 (49.8%)	0.974
Duration of gestation (weeks)	40.2 (1.1)	40.1 (1.1)	40.3 (1.1)	0.076
Weight at birth (kg)	3.5 (0.4)	3.5 (0.4)	3.6 (0.4)	0.058
Length-adjusted weight at birth (SDS)	0.1 (1.0)	0.1 (0.9)	0.1 (1.0)	0.240
Maternal vitamin D supplement during pregnancy (µg/day)	15.3 (15.5)	16.1 (17.8)	14.5 (12.9)	0.147
12-month follow-up				
Number of participants (% girls)	816 (50.7%)	409 (51.1%)	407 (50.4%)	0.834
Weight at 12 months (kg)	9.8 (1.1)	9.8 (1.2)	9.8 (1.2)	0.359
Length-adjusted weight at 12 months (SDS)	0.0 (1.0)	-0.0 (1.0)	0.0 (1.0)	0.911
Adherence 0-12 months (%)	89.2 (11.4)	89.3 (11.8)	89.1 (10.9)	0.570
Adherence 0-12 months >80% (%)	84.9	86.1	83.5	0.306
24-month follow-up				
Number of participants (% girls)	776 (50.3%)	384 (50.3%)	392 (50.3%)	0.999
Weight at 24 months (kg)	12.5 (1.4)	12.5 (1.3)	12.6 (1.4)	0.317
Length-adjusted weight at 24 months (SDS)	-0.1 (1.0)	-0.1 ((1.0)	0.0 ((1.0)	0.084
Duration of breastfeeding (months)	10.7 (5.6)	10.5 (5.7)	10.9 (5.5)	0.285
Adherence 0-24 months (%)	88.0 (12.6)	88.7 (11.8)	87.3 (13.4)	0.349
Adherence 0-24 months >80% (%)	84.0	86.4	81.6	0.070
25OHD concentration				
25OHD at baseline (UCB) (nmol/L)	81.3 (25.9)	81.4 (27.8)	81.2 (23.8)	0.883
25OHD at 12 months (nmol/L)	98.6 (28.8)	82.7 (20.0)	114.4 (27.6)	**<0.001**
25OHD at 24 months (nmol/L)	102.4 (27.8)	86.7 (19.8)	117.8 (25.8)	**<0.001**

Values are reported as means and standard deviations (SD)

* Independent-samples T-test for analyses of differences between intervention groups for anthropometric and biochemical variables. Pearson Chi-Square for number of participants. Mann-Whitney U-test for adherence.

Number of subjects if data available for <95% at follow-up:
Data on maternal vitamin D supplementation during pregnancy (N=813).
Data on serum 25-hydroxyvitamin D (25OHD) concentration at 12 months (N=757).

SDS = standard deviation score
25OHD = Serum 25-hydroxyvitamin D concentration (nmol/L)

Genotype call rates varied between 92-99%, with consistent negative and positive controls. Genotype was determined for all four studied SNPs in 89% of participants. The distributions of the studied genotypes were in line with available previously reported genotype data (36-38). The obtained genotyping results were in Hardy-Weinberg equilibrium. The studied SNPs were in strong linkage disequilibrium (r^2 >0.8).

Three different combinations of haplotype homozygotes were identified for both the haplotype including three SNPs rs2282679, rs4588 and rs7041 ($Haplo_{3SNP}$) (*TGC*, combined major alleles; *TGA* and *GTA*, combined minor alleles) and the haplotype including all four studied SNPs ($Haplo_{4SNP}$) (*TGCT*, combined major alleles; *TGAT* and *GTAC*, combined minor alleles). Genotype, diplotype and haplotype distributions (Table 2) differed in the two intervention groups.

Table 2. Genotype, diplotype and haplotype distributions and results for analyses of differences in distributions between intervention groups.

	Variant	All	Group10	Group30	P*
rs2282679	***TT***	571 (64.2%)	267 (60.3%)	304 (68.2%)	**0.009**
(Genotyped N=889)	***GT***	283 (31.8%)	152 (34.3%)	131 (29.4%)	
	GG	35 (3.9%)	24 (5.4%)	11 (5.4%)	
rs4588	***GG***	568 (63.6%)	266 (59.8%)	302 (67.4%)	**0.025**
(Genotyped N=893)	***GT***	290 (32.5%)	156 (35.1%)	134 (29.9%)	
	TT	35 (3.9%)	23 (5.2%)	12 (2.7%)	
rs7041	***CC***	366 (40.5%)	166 (36.5%)	200 (44.5%)	**0.040**
(Genotyped N=904)	***AC***	440 (48.7%)	233 (51.2%)	207 (46.1%)	
	AA	98 (10.8%)	56 (12.3%)	42 (9.4%)	
rs1155563	***TT***	523 (62.3%)	242 (57.8%)	281 (66.7%)	**0.047**
(Genotyped N=840)	***CT***	279 (33.2%)	155 (37.0%)	124 (29.5%)	
	CC	38 (4.5%)	22 (5.3%)	16 (5.3%)	
Diplotype	***1S/1S***	366 (41.3%)	166 (37.6%)	200 (45.0%)	0.069
(N=886)	***1F/1S***	180 (20.3%)	91 (20.6%)	89 (20.0%)	
	1F/1F	16 (1.8%)	6 (1.4%)	10 (2.3%)	
	1S/2	247 (27.9%)	134 (30.3%)	113 (25.5%)	
	1F/2	43 (4.9%)	22(5%)	21 (4.7%)	
	2/2	34 (3.8%)	23 (5.2%)	11 2.5%)	
$Haplo_{3SNP}$	***TGC***	364 (88.1%)	164 (85.0%)	200 (90.9%)	**0.035**
(N=413)	***TGA***	16 (3.9%)	6 (3.1%)	10 (4.5%)	
	GTA	33 (8.0%)	23 (11.9%)	10 (4.5%)	
$Haplo_{4SNP}$	***TGCT***	323 (91.0%)	145 (88.4%)	178 (93.2%)	0.062
(N=355)	***TGAT***	11 (3.1%)	4 (2.4%)	7 (3.7%)	
	GTAC	21 (5.9%)	15 (9.1%)	6 (3.1%)	

* Pearson Chi-Square

Diplotype= rs4588/rs7041 Diplotype
$Haplo_{3SNP}$ = Haplotype of rs2282679, rs4588 and rs7041
$Haplo_{4SNP}$ = Haplotype of all four studied single nucletotide polymorphisms

B. Biochemical variables

In accordance with the previously described outcomes of the VIDI trial (30), the mean 25OHD concentrations did not differ between intervention groups at baseline, but were significantly higher in Group30 at 12 and 24 months of intervention (Table 1). The majority of participants (>95.7%) were vitamin D sufficient, with a 25OHD concentration >50 nmol/L, throughout the trial. Concentrations of 25OHD were, at all time points, lowest in spring, when compared to other seasons (ANOVA $p<0.050$).

C. Associations of genotypes, diplotypes and haplotypes with 25OHD concentrations

Adjusted mean serum 25OHD concentrations by geno-, diplo- and haplotype and results for analyses of covariance during follow-up are presented in Table 3. Table 4 shows adjusted mean allelic effect sizes for genotypes, and effect of diplotype and haplotypes, with results for multivariate linear regression.

SNPs rs2282679, r4588 and rs1155563 were associated with 25OHD concentrations at all time points (Table 3). Common (major) allele homozygotes had the highest and rare (minor) allele homozygotes the lowest 25OHD concentrations. Rs7041 major allele homozygotes showed significantly higher 25OHD concentrations than minor allele homozygotes in Group10 at 12 months and in both intervention groups at 24 months. Mean allelic effect size (B) per one minor allele in the studied SNPs varied between -3.8 and -10.8 nmol/L, being greatest for rs2282679 (Table 4).

Diplotype and $Haplo_{3SNP}$ affected 25OHD concentrations at all studied time points. $Haplo_{4SNP}$ was associated with 25OHD concentrations at 12 months in both intervention groups and in Group10 at 24 months (Table 3). Major allele homozygote haplotypes and diplotype *1* (*1S/1S*, *1F/1S*, *1F/1F*) had the highest and minor allele homozygote haplotypes and diplotype *2* (*1S/2*,*1F/S* and *2/2*) the lowest 25OHD concentrations. Mean effect size (B) of diplotype *2* vs. *1* ranged from -4.4 nmol/L at baseline to -10.9 nmol/L at 24 months (Table 4). When comparing minor to major allele homozygotes of $Haplo_{3SNP}$ and $Haplo_{4SNP}$ the mean effect (B) ranged from -12.6 to -33.7 nmol/L and was significant at baseline and at 12 months in both intervention groups, and in Group10 at 24 months.

Table 3. Differences in 25-hydroxyvitamin D (25OHD) concentrations between variants during follow-up.
Adjusted mean 25OHD concentrations (nmol/L) by genotype, diplotype and haplotype at baseline, 12 and 24 months of intervention and results for analyses of covariance for differences between variants.

		Baseline		12 months				24 months			
		All		Group10		Group30		Group10		Group30	
	Variant	$25OHD_{Adj}$* (nmol/L)	P_{Adj}*	$25OHD_{Adj}$** (nmol/L)	P_{Adj}**	$25OHD_{Adj}$** (nmol/L)	P_{Adj}**	$25OHD_{Adj}$*** (nmol/L)	P_{Adj}***	$25OHD_{Adj}$*** (nmol/L)	P_{Adj}***
rs2282679	***TT***	84.5 (82.2,86.8)	**0.019**	87.0 (84.3,89.8)	**<0.001**	119.9 (116.6,123.2)	**<0.001**	92.5 (89.9,95.1)	**<0.001**	123.6 (120.5,126.6)	**<0.001**
	GT	82.0 (78.8,85.1)		80.1 (76.6,83.6)		109.0 (104.2,113.9)		84.5 (81.2,87.8)		112.8 (108.3,117.2)	
	GG	72.3 (63.5,81.1)		69.6 (60.9,78.3)		88.0 (70.5,105.5)		70.7 (62.4,79.0)		102.2 (86.0,118.4)	
rs4588	***GG***	84.5 (82.2,86.8)	**0.010**	87.7 (85.0,90.5)	**<0.001**	120.0 (116.6,123.3)	**<0.001**	92.6 (89.9,95.2)	**<0.001**	123.6 (120.6,126.6)	**<0.001**
	GT	81.6 (78.4,84.7)		80.1 (76.7,83.6)		109.4 (104.5,114.3)		84.7 (81.5,88.0)		112.5 (108.1,117.0)	
	TT	71.5 (62.7,80.3)		68.1 (59.3,77.0)		92.7 (76.1,109.4)		70.8 (62.3,79.4)		104.5 (89.2,119.8)	
rs7041	***CC***	83.2 (80.4,86.0)	0.078	89.1 (85.7,92.6)	**<0.001**	118.9 (114.7,123.1)	0.124	92.7 (89.4,95.9)	**<0.001**	125.2 (121.5,129.0)	**<0.001**
	AC	84.3 (81.7,86.9)		82.1 (79.3,85.0)		114.8 (110.8,118.8)		88.0 (85.2,90.7)		116.4 (112.9,120.0)	
	AA	77.5 (72.2,82.9)		74.0 (68.3,79.7)		110.1 (101.6,118.6)		76.9 (71.2,82.5)		110.6 (102.9,118.4)	
rs1155563	***TT***	85.3 (82.9,87.7)	**0.011**	87.1 (84.2,90.0)	**<0.001**	119.3 (115.9,122.8)	**<0.001**	91.2 (88.5,94.0)	**<0.001**	123.0 (119.8,126.2)	**<0.001**
	CT	80.8 (77.5,84.0)		80.1 (76.6,83.7)		111.3 (106.2,116.4)		85.0 (81.7,88.3)		113.7 (109.1,118.3)	
	CC	74.7 (65.9,83.4)		69.7 (60.3,79.1)		95.4 (81.5,109.2)		70.5 (61.8,79.2)		102.0 (88.5,115.4)	
Diplotype	***1S/1S***	83.1 (80.3,86.0)	**0.028**	89.2 (85.8,92.7)	**<0.001**	118.9 (114.8,123.0)	**<0.001**	92.9 (89.6,96.2)	**<0.001**	125.1 (121.4,128.9)	**<0.001**
	1F/1S	87.5 (83.5,91.6)		85.1 (80.5,89.8)		120.6 (114.7,126.5)		92.0 (87.6,96.5)		120.5 (115.1,126.0)	
	1F/1F	83.6 (69.9,97.4)		80.7 (63.4,97.9)		135.0 (118.5,151.5)		93.8 (77.2,110.4)		118.8 (103.5,134.1)	
	1S/2	81.8 (78.5,85.2)		80.5 (76.9,84.2)		109.8 (104.5,115.1)		85.6 (82.1,89.0)		113.2 (108.3,118.0)	
	1F/2	80.5 (72.5,88.5)		77.8 (68.7,86.9)		106.5 (94.8,118.2)		79.3 (70.5,88.1)		109.3 (98.1,120.5)	
	2/2	71.5 (62.7,80.3)		68.1 (59.3,76.9)		92.7 (76.2,109.2)		70.9 (62.3,79.4)		104.5 (89.1,119.9)	
$Haplo_{3SNP}$	***TGC***	83.9 (81.5,86.3)	**0.006**	89.0 (85.2,92.7)	**<0.001**	117.7 (113.4,122.0)	**0.001**	93.5 (90.0,97.0)	**<0.001**	124.6 (120.5,128.6)	**0.042**
	TGA	83.6 (72.4,94.8)		81.2 (62.4,99.9)		134.4 (117.8,150.9)		96.4 (78.4,114.3)		118.6,102.7,134.5)	
	GTA	71.4 (64.1,78.8)		67.7 (58.1,77.3)		87.0 (69.4,104.7)		69.6 (60.3,79.0)		102.8 (85.9,119.8)	
$Haplo_{4SNP}$	***TGCT***	83.9 (81.4,86.4)	0.059	89.4 (85.5,93.4)	**0.005**	118.3 (113.7,123.0)	**0.026**	92.1 (88.4,95.7)	**<0.001**	124.7 (120.3,129.0)	0.065
	TGAT	84.5 (71.0,98.1)		86.5 (65.4,107.7)		127.1 (107.8,146.4)		100.7(80.9,120.5)		125.3 (105.5,145.0)	
	GTAC	72.6 (63.5,81.6)		69.0 (57.5,80.4)		88.0 (64.8,111.3)		69.8 (59.2,80.3)		98.7 (77.2,120.2)	

Values are reported as adjusted mean serum 25-hydroxyvitamin D concentrations (nmol/L) and 95% confidence intervals (95% CI).

* Adjusted for: Season of birth, length-adjusted weight standard deviation score at birth and maternal vitamin D supplementation (µg/day) during pregnancy
** Adjusted for: Season of 12-month follow-up, adherence to intervention supplement (0-12 months) (%), length-adjusted weight standard deviation score at 12 months and duration of breastfeeding up to 12 months
*** Adjusted for: Season of 24-month follow-up, adherence to intervention supplement (13-24 months) (%) and length-adjusted weight standard deviation score at 24 months

Significant differences in multiple comparisons (Bonferroni adjusted p-values):
Baseline: rs2282679 and rs4588 major vs. minor homozygotes (p<0.024), Diplotype *2/2* vs. *1F/1S* (p=0.017), $Haplo_{3SNP}$ *TGC* vs. *GTA* (p=0.004).
12 months: rs2282679, rs4588 and rs1155563 major homozygotes vs. heterozygotes and minor homozygotes (p<0.040) in both intervention groups, rs7041 all comparisons (p<0.035) in Group10. Diplotype *1S/1S* vs. *1S/2* and *2/2* (p<0.012) and *1F/1S* vs. *2/2* (p=0.012) in Group10, *2/2* vs. *1S/1S*, *1F/1S*, *1F/1F* (p<0.038) in Group30. $Haplo_{3SNP}$ *TGC* vs. *GTA* (p<0.001) in Group 10, *TGC* vs. *TGA* and *GTA* (p<0.004) in Group30, $Haplo_{4SNP}$ *TGCT* vs. *GTAC* (p=0.003) in Group10, and *TGCT* vs. *TGAT* and *GTAC* (p<0.037) in Group30.
24 months: rs2282679, rs4588 and rs1155563 all comparisons (p<0.011) in Group10, major homozygotes vs. heterozygotes and minor homozygotes (p<0.050) in Group30. Rs7041 *AA* vs. *AC*, *CC* (p<0.002) in Group10 and *CC* vs. *AC*, *AA* (p<0.004) in Group30. Diplotype *2/2* vs. *1S/1S*, *1F/1S* ,*1S/2* (p<0.029) in Group10, *1S/1S* vs. *1S/2* (p=0.001) in Group30. $Haplo_{3SNP}$ *TGC* vs. *TGA* and *GTA* (p<0.030) in Group10, *TGC* vs. *GTA* (p=0.043) in Group30, $Haplo_{4SNP}$ *TGCT* vs. *TGAT* and *GTAC* (p<0.022) in Group10.

Number of subjects in analyses at baseline/12 months/24 months respectively:
Rs2282679 (N=783/710/731), rs4588 (N=789/716/733), rs7041 (N=800/725/741), rs1155563 (N=742/671/689), Diplotype (N=784/710/728), $Haplo_{3SNP}$ (N=366/319/338), $Haplo_{4SNP}$ (N=317/285/294).

Diplotype= rs4588/rs7041 Diplotype
$Haplo_{3SNP}$ = Haplotype of rs2282679, rs4588 and rs7041
$Haplo_{4SNP}$ = Haplotype of all four studied single nucletotide polymorphisms

Table 4. Mean allelic effects of variants on 25-hydroxyvitamin D (25OHD) concentrations during follow-up.
Adjusted mean allelic effects on 25OHD concentrations (nmol/L) for the studied SNPs and adjusted mean effect size of diplotype and haplotype during intervention. Results for multivariate linear regression analyses.

	Baseline		12 months				24 months			
	All		Group10		Group30		Group10		Group30	
Mean allelic effect size (nmol/L)	**B (95%CI)**	**P_{Adj}***	**B (95%CI)**	**P_{Adj}****	**B (95%CI)**	**P_{Adj}****	**B (95%CI)**	**P_{Adj}*****	**B (95%CI)**	**P_{Adj}*****
rs2282679 *T>G*	-3.8 (-6.9,-0.8)	**0.014**	-7.9 (-11.2,-4.5)	**<0.001**	-10.8 (-15.9,-5.8)	**<0.001**	-9.0 (-12.3,-5.8)	**<0.001**	-9.6 (-14.1,-5.0)	**<0.001**
rs4588 *G>T*	-4.2 (-7.3,-1.2)	**0.006**	-8.7 (-12.0,-5.3)	**<0.001**	-9.9 (-14.9,-4.9)	**<0.001**	-8.6 (-11.8,-5.3)	**<0.001**	-9.6 (-14.0,-5.1)	**<0.001**
rs7041 *C>A*	-1.7 (-4.3,0.9)	0.209	-7.6 (-10.6,-4.6)	**<0.001**	-3.1 (-7.3,1.2)	0.154	-6.7 (-9.7,-3.8)	**<0.001**	-6.9 (-10.6,-3.1)	**<0.001**
rs1155563 *T>C*	-4.4 (-7.5,-1.4)	**0.005**	-7.7 (-11.1,-4.2)	**<0.001**	-8.5 (-13.5,-3.6)	**0.001**	-8.0 (-11.3,-4.6)	**<0.001**	-8.9 (-13.3,-4.4)	**<0.001**
Diplotype *1>2*	-4.4 (-8.0,-0.9)	**0.015**	-9.0 (-13.1,-4.9)	**<0.001**	-10.3 (-16.0,-4.6)	**<0.001**	-8.8 (-12.8,-4.8)	**<0.001**	-10.9 (-16.0,-5.8)	**<0.001**
$Haplo_{3SNP}$ *TGC>GTA*	-13.8 (-23.1,-4.5)	**0.004**	-21.4(-31.0,-11.9)	**<0.001**	-30.1 (-48.3,-12.0)	**0.001**	-22.0 (-31.3,-12.7)	**<0.001**	-16.1 (-32.9,0.6)	0.059
$Haplo_{4SNP}$ *TGCT>GTAC*	-12.6 (-24.1,-1.1)	**0.033**	-22.5 (-34.2,-10.8)	**<0.001**	-33.7 (-57.2,-10.3)	**0.005**	-21.1 (-32.5,-9.8)	**<0.001**	-18.3 (-39.9,3.3)	0.096

Values are reported as B coefficients and 95% confidence intervals (95% CI).

* Adjusted for: Season of birth (spring vs. other), length-adjusted weight standard deviation score at birth and maternal vitamin D supplementation (µg/day) during pregnancy
** Adjusted for: Season of 12-month follow-up (spring vs. other), adherence to intervention supplement (0-12 months) (%), length-adjusted weight standard deviation score at 12 months and duration of breastfeeding up to 12 months
*** Adjusted for: Season of 24-month follow-up (spring vs. other), adherence to intervention supplement (13-24 months) (%) and length-adjusted weight standard deviation score at 24 months

Number of subjects in analyses:
Baseline: rs2282679 (N=790), rs4588 (N=796), rs7041 (N=807), 1155563 (N=748), Diplotype (N=791), $Haplo_{3SNP}$ (N=356), $Haplo_{4SNP}$ (N=310)
12 months: rs2282679 (Group10/Group30; N=382/387), rs4588 (N=385/389), rs7041 (N=393/391), rs1155563 (N=361/366), Diplotype (N=382/387), $Haplo_{3SNP}$ (N=157/186), $Haplo_{4SNP}$ (N=135/165)
24 months: rs2282679 (N=365/372), rs4588 (N=365/374), rs7041 (N=371/376), 1155563 (N=343/351), Diplotype (N=362/372), $Haplo_{3SNP}$ (N=150/177), $Haplo_{4SNP}$ (N=130/156)

Diplotype= rs4588/rs7041 Diplotype (1= 1S/1S, 1F/1S and 1F/1F to 2= 1S/2, 1F/2 and 2/2)
$Haplo_{3SNP}$ = Haplotype of rs2282679, rs4588 and rs7041 (major to minor homozygotes, *TGC>GTA*)
$Haplo_{4SNP}$ = Haplotype of all four studied single nucletotide polymorphisms (major to minor homozygotes, *TGCT>GTAC*)

D. Temporal change of 25OHD concentration and genotype, diplotype and haplotype

When examining the effects of genetic variants on temporal 25OHD change in a model including concentrations at baseline, 12 and 24 months, we found mean adjusted 25OHD concentrations to differ between genotype, diplotype and haplotype in both intervention groups ($p_{variant}$), but in Group10 these did not significantly affect intervention response. In contrast, in the intervention group receiving higher vitamin D_3 supplementation (Group30), we observed a significant interaction between variants and temporal change ($p_{interaction}$ <0.019), indicating differences in intervention response between variants of rs2282679, rs4588 and rs7041, diplotype and $Haplo_{3SNP}$ (Table 5). Minor allele homozygotes, diplotype 2/2 and haplotype homozygotes for the combination of minor alleles showed the smallest temporal increases in 25OHD. Differences in temporal change for $Haplo_{4SNP}$ were not statistically significant. Temporal change and results by intervention group and genotypes or diplotype and haplotypes are presented in Figure 1 and Figure 2 respectively.

When including only study subjects with >80% adherence to vitamin D_3 supplementation, the genotypes of rs2282679, rs4588, rs7041 and diplotype were still significantly associated with differences in temporal 25OHD changes in Group30 ($p_{interaction}$ <0.028), but the associations for $Haplo_{3SNP}$ did no longer reach significance ($p_{interaction}$=0.180).

Table 5. Temporal change of 25-hydroxyvitamin D (25OHD) concentrations (nmol/L) during follow-up in **Group30** by genotype, diplotype and haplotype.
Results of repeated measurement analysis of covariance for differences in adjusted mean 25OHD concentrations ($P_{variant}$) and differences in temporal change between variants, ie. interaction of variant and temporal change ($P_{interaction}$).

		Baseline$_{Group30}$	**12 months$_{Group30}$**	**24 months$_{Group30}$**	**Repeated measurement analysis of covariance**	
	Variant	**25OHD$_{Adj}$ (nmol/L)**	**25OHD$_{Adj}$ (nmol/L)**	**25OHD$_{Adj}$ (nmol/L)**	**$P_{variant}$ ***	**$P_{interaction}$**
rs2282679	***TT***	82.5 (79.5 85.4)	120.2 (116.8,123.6)	123.2 (120.3,126.2)	**<0.001**	**0.003**
(N=367)	***GT***	82.9 (78.5, 87.2)	109.7 (104.6,114.7)	112.3 (107.8,116.7)		
	GG	76.1 (61.0,91.2)	86.4 (68.4,104.5)	102.2 (86.1,118.3)		
rs4588	***GG***	82.4 (79.4,85.3)	120.2 (116.7,123.6)	123.3 (120.3,126.3)	**<0.001**	**0.005**
(N=369)	***GT***	82.9 (78.6,87.3)	110.1 (105.0,115.2)	112.0 (107.6,116.4)		
	TT	74.4 (60.1,88.7)	91.8 (74.7,109.0)	104.0 (88.7,119.3)		
rs7041	***CC***	82.0 (78.4,85.7)	119.2 (114.8,123.5)	125.0 (121.3,128.6)	**0.013**	**0.018**
(N=371)	***AC***	83.5 (80.0,87.0)	115.4 (111.2,119.6)	115.9 (112.3,119.5)		
	AA	81.0 (73.4,88.7)	110.1 (101.1,119.1)	110.2 (102.4,118.0)		
rs1155563	***TT***	84.0 (80.9,87.1)	119.8 (116.2,123.4)	122.8 (119.7,126.0)	**<0.001**	0.180
(N=346)	***CT***	80.9 (76.3,85.5)	112.3 (107.0,117.6)	113.3 (108.6,117.9)		
	CC	77.3 (65.0,89.6)	95.7 (81.4,110.0)	101.3 (88.0,114.7)		
Diplotype	***1S/1S***	81.9 (78.3,85.6)	119.0 (114.8,123.3)	124.9 (121.2,128.5)	**<0.001**	**0.008**
(N=367)	***1F/1S***	83.7 (78.3,89.1)	120.7 (114.4,126.9)	120.0 (114.6,125.5)		
	1F/1F	84.0 (68.0,100.0)	138.1 (121.0,155.1)	118.3 (103.0,133.7)		
	1S/2	82.9 (78.1,87.6)	111.1 (105.6,116.5)	112.5 (107.6,117.3)		
	1F/2	83.2 (72.2,94.2)	104.6 (91.9,117.4)	109.3 (98.1,120.5)		
	2/2	74.4 (60.1,88.8)	91.7 (74.7,108.8)	104.0 (88.7,119.3)		
Haplo$_{3SNP}$	***TGC***	81.6 (78.5,84.8)	118.6 (114.4,122.9)	124.5 (120.6,128.4)	**0.001**	**0.011**
(N=183)	***TGA***	84.4 (71.0,97.7)	138.2 (121.2,155.2)	118.5 (102.6,134.3)		
	GTA	74.4 (61.7,87.2)	85.4 (67.3,103.5)	101.8 (85.1,118.6)		
Haplo$_{4SNP}$	***TGCT***	82.0 (78.5,85.4)	119.6 (114.9,124.3)	124.8 (120.6,128.9)	**0.005**	0.314
(N=159)	***TGAT***	88.2 (70.6,105.7)	126.8 (140.5,148.3)	124.6 (104.8,144.4)		
	GTAC	73.5 (57.4,89.7)	84.8 (61.4,108.2)	97.4 (76.1,118.6)		

Values are reported as adjusted mean serum 25-hydroxyvitamin D (25OHD$_{Adj}$) concentrations (nmol/L) and 95% confidence intervals (95% CI).

Means are adjusted for season of birth, length-adjusted weight standard deviation score at 24 months, adherence to intervention supplementation (%) throughout the intervention (0-24 months) as well as interaction of adherence to supplementation and temporal change.

* Significant differences in multiple comparisons (Bonferroni adjusted p-values):
rs2282679: *TT* vs. *GT* and *GG* (p=0.001); rs4588: *GG* vs. *GT* and *TT* (p<0.003); rs7041: *CC* vs. *AC* (p=0.024); rs1155563: *TT* vs. *CT* and *CC* (p<0.004); Diplotype: *2/2* vs. *1S/1S*, *1S/1F* and *1F/1F* (p<0.037); Haplo$_{3SNP}$: *GTA* vs. *TGC* and *TGA* (p<0.003); Haplo$_{4SNP}$: *GTAC* vs. *TGCT* and *TGAT* (p<0.019)

Diplotype= rs4588/rs7041 Diplotype
Haplo$_{3SNP}$ = Haplotype of rs2282679, rs4588 and rs7041
Haplo$_{4SNP}$ = Haplotype of all four studied single nucleotide polymorphisms

Figure 1.

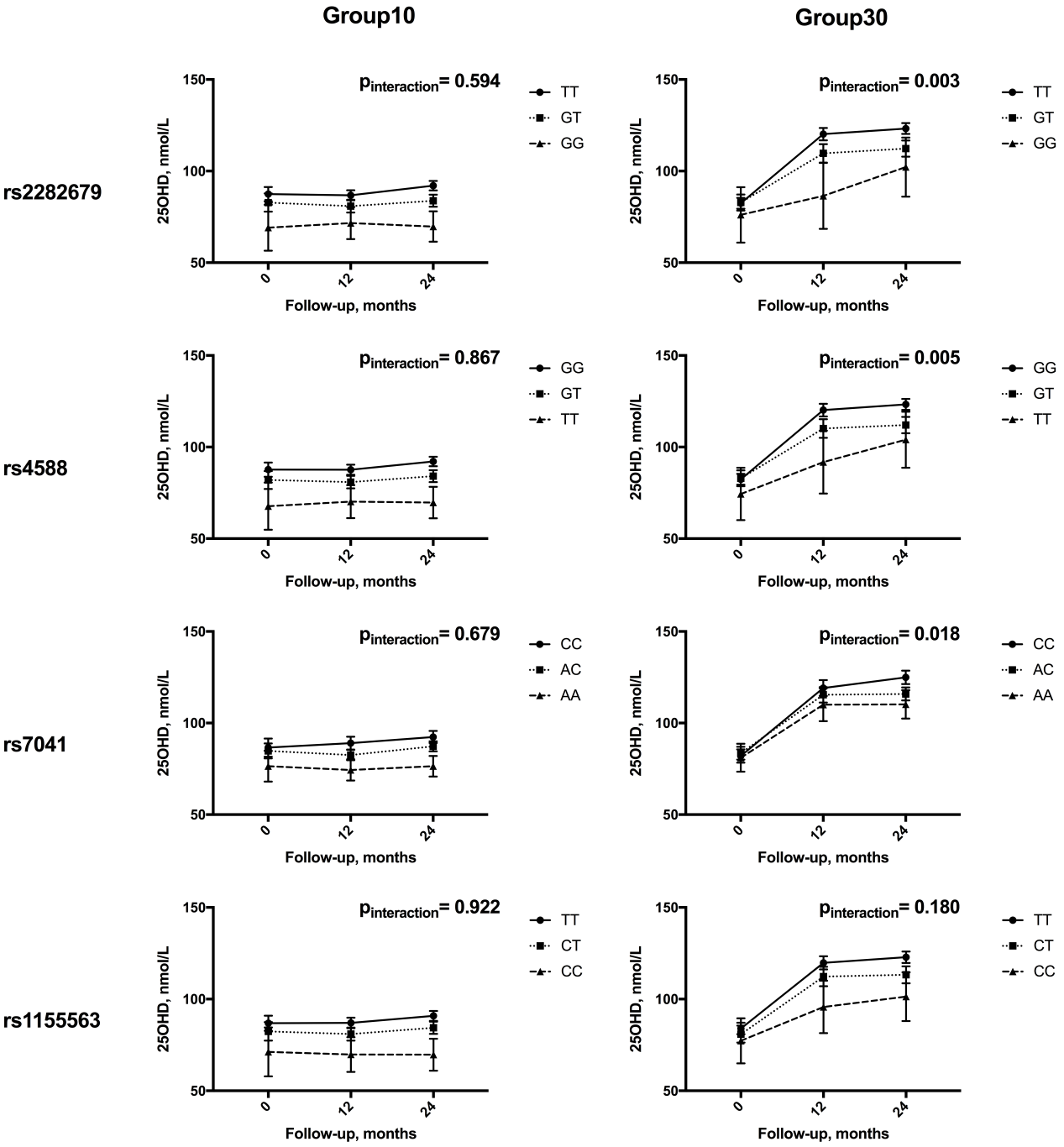

Figure 1.
Temporal change of mean serum 25-hydroxyvitamin D (25OHD) concentrations (nmol/L) during follow-up by genotype in the two intervention groups, Group10 and Group30, with results of interaction between variants and temporal change during follow-up in repeated measurement analysis of covariance ($P_{interaction}$).

In Group10 means are adjusted for season of birth, length-adjusted weight standard deviation score at 24 months, duration of breastfeeding (months), adherence to intervention supplementation (%) throughout the intervention (0-24 months) as well as interaction of adherence to supplementation and temporal change.

In Group30 means are adjusted for season of birth, length-adjusted weight standard deviation score at 24 months, adherence to intervention supplementation (%) throughout the intervention (0-24 months) as well as interaction of adherence to supplementation and temporal change, as breastfeeding was not a significant covariant in this group.

Figure 2.

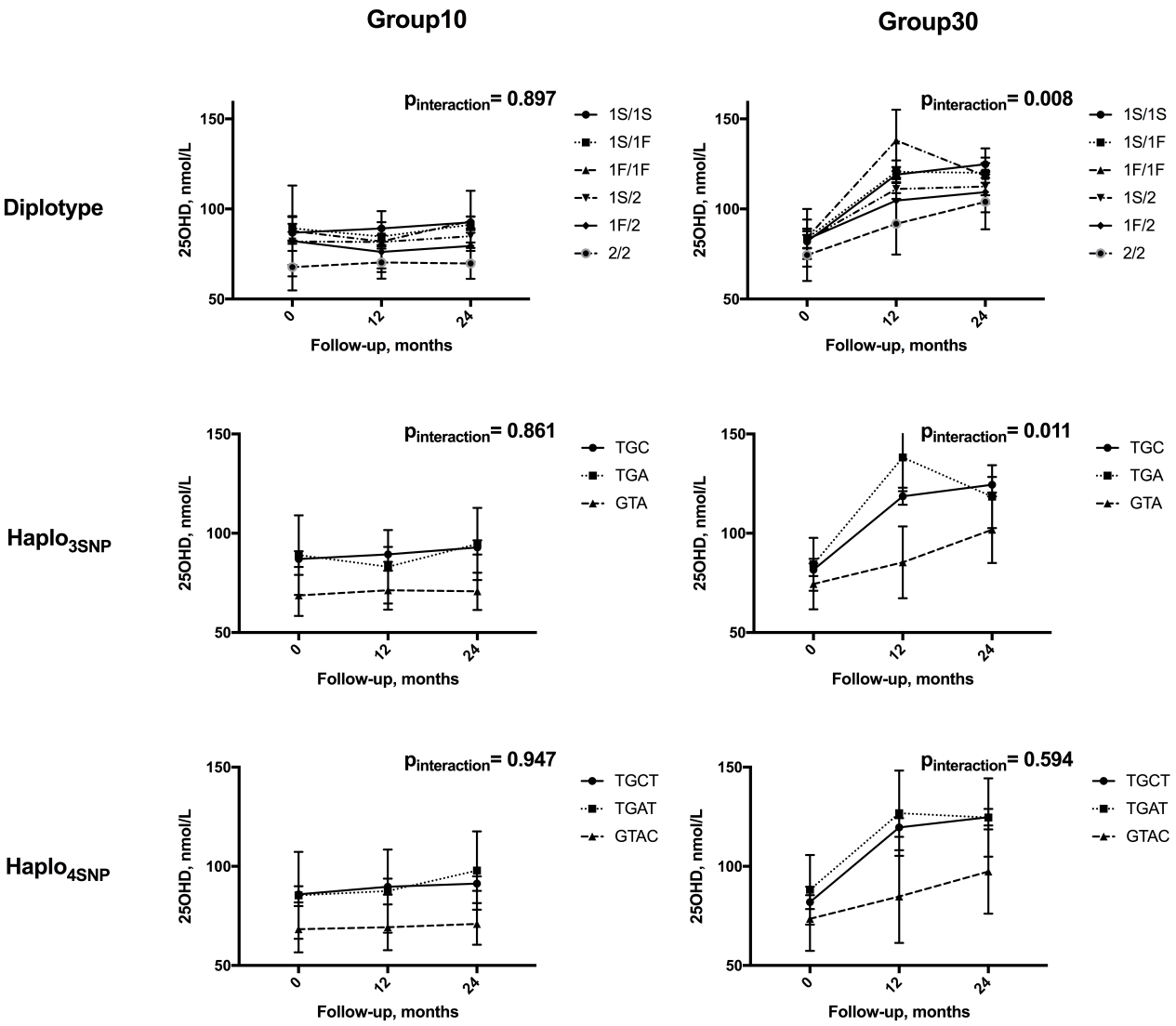

Figure 2.
Temporal change of mean serum 25-hydroxyvitamin D (25OHD) concentrations (nmol/L) during follow-up by diplo- and haplotypes in the two intervention groups, Group10 and Group30, with results of interaction between variants and temporal change during follow-up in repeated measurement analysis of covariance ($P_{interaction}$).

In Group10 means are adjusted for season of birth, length-adjusted weight standard deviation score at 24 months, duration of breastfeeding (months), adherence to intervention supplementation (%) throughout the intervention (0-24 months) as well as interaction of adherence to supplementation and temporal change.

In Group30 means are adjusted for season of birth, length-adjusted weight standard deviation score at 24 months, adherence to intervention supplementation (%) throughout the intervention (0-24 months) as well as interaction of adherence to supplementation and temporal change, as breastfeeding was not a significant covariant in this group.

In accordance with the results for temporal change, also the calculated mean change of 25OHD concentration (Δ25OHD) from baseline to 24 months of intervention, in participants with adherence >80%, differed significantly between genotypes of rs2282679, rs4588, rs7041 and diplotype in Group30 (Table 6). Significant differences in Δ25OHD ranged from 13 to 17 nmol/L between major and minor homozygotes and 15 nmol/L between diplotypes *1S/1S* and *1S/2*. For haplotypes, differences in Δ25OHD of up to 20 nmol/L between minor and major allele homozygotes were observed, but reached significance only for unadjusted means of $Haplo_{3SNP}$ (unequal variances, Welch test for equality of means p=0.008, Tamhane adjusted p=0.008). In Group10, Δ25OHD was small, and did not significantly differ between genotypes, but in both intervention groups Δ25OHD was greatest in major allele homozygotes, and smallest for genotypes and haplotypes of minor allele homozygotes. Figure 3 presents adjusted mean Δ25OHD from baseline to 24 months in both intervention groups by genotype, in participants with >80% adherence to intervention D_3 supplementation, and results for analysis of covariance.

Table 6. Mean change of serum 25-hydroxyvitamin D concentration (Δ25OHD) (nmol/L) by genotype, diplotype and haplotype from baseline to 24 months of intervention, in participants with >80% adherence to intervention vitamin D_3 supplementation. Results for analyses of variance and covariance for differences between variants.

		All (adherence >80%)			**Group10** (adherence >80%)			**Group30** (adherence >80%)		
	Variant	**Δ25OHD (nmol/L)**	**P** †	**P_{adj}** *††	**Δ25OHD (nmol/L)**	**P**	**P_{adj}** **	**Δ25OHD (nmol/L)**	**P** †††	**P_{adj}** **††††
rs2282679	***TT***	25.3 (38.7)	**0.001**	**0.016**	5.5 (36.5)	0.730	0.673	43.2 (31.3)	**0.006**	**0.004**
(N=603)	***GT***	15.0 (35.1)			2.4 (30.0)			30.6 (34.8)		
	GG	8.2 (19.7)			2.6 (18.8)			25.7 (10.3)		
rs4588	***GG***	25.3 (38.8)	**0.003**	**0.021**	5.5 (36.6)	0.859	0.771	43.2 (31.3)	**0.005**	**0.004**
(N=609)	***GT***	15.2 (34.8)			3.4 (29.9)			30.2 (34.9)		
	TT	11.6 (21.7)			4.0 (18.3)			31.2 (17.5)		
rs7041	***CC***	27.9 (35.4)	**0.001**	**0.010**	6.6 (29.8)	0.731	0.814	45.6 (29.4)	**0.002**	**0.002**
(N=614)	***AC***	16.9 (38.1)			3.7 (37.0)			33.5 (32.8)		
	AA	14.1 (34.1)			2.9 (24.5)			29.2 (39.5)		
rs1155563	***TT***	24.2 (39.4)	**0.011**	0.182	4.7 (37.1)	0.891	0.899	41.2 (33.0)	0.068	0.073
(N=566)	***CT***	15.9 (34.4)			3.9 (30.0)			33.0 (33.1)		
	CC	8.3 (24.3)			0.6 (20.6)			22.8 (25.2)		
Diplotype	***1S/1S***	27.9 (35.4)	**0.004**	**0.035**	6.6 (29.8)	0.682	0.631	45.6 (29.4)	**0.022**	**0.016**
(N=604)	***1F/1S***	18.7 (45.8)			2.5 (47.3)			36.9 (36.5)		
	1F/1F	31.8 (22.3)			24.0 (31.2)			36.3 (16.6)		
	1S/2	16.1 (32.8)			4.4 (29.9)			31.6 (30.2)		
	1F/2	10.2 (43.9)			-4.6 (26.4)			25.0 (53.1)		
	2/2	11.6 (21.7)			4.0 (18.3)			31.2 (17.5)		
$Haplo_{3SNP}$	***TGC***	27.9 (35.4)	**0.035**	0.401	6.6 (29.8)	0.448	0.304	45.6 (29.4)	0.188	0.253
(N=282)	***TGA***	31.8 (22.3)			24.0 (31.2)			36.3 (16.6)		
	GTA	9.4 (19.1)			4.0 (18.3)			25.7 (10.3)		
$Haplo_{4SNP}$	***TGCT***	27.7 (35.8)	0.076	0.449	5.3 (29.3)	0.129	0.082	45.8 (29.8)	0.354	0.468
(N=244)	***TGAT***	39.2 (14.7)			39.1 (9.2)			39.3 (18.3)		
	GTAC	10.0 (19.8)			5.3 (20.2)			25.4 (6.2)		

Values are reported as means and standard deviations (SD)

* Adjusted for: Season of birth, adherence to intervention supplement (0-24 months) (%), length-adjusted weight standard deviation score at 24 months and intervention group
** Adjusted for: Season of birth, adherence to intervention supplement (0-24 months) (%) and length-adjusted weight standard deviation score at 24 months

Significant differences in multiple comparisons:
† rs2282679: *TT* vs. *GT* and *GG* (Tamhane p<0.005), rs4588: *GG* vs. *GT* and *TT* (Tamhane p<0.022), rs7041: *CC* vs. *AC* and *AA* (Bonferroni p<0.020), rs1155563: *TT* vs. *CT* and *CC* (Tamhane p<0.033), Diplotype: *1S/1S* vs. *1S/2* and *2/2* (Tamhane p<0.029), $Haplo_{3SNP}$: *GTA* vs. *TGC* and *TGA* (Tamhane p<0.032); $Haplo_{4SNP}$: *GTAC* vs. *TGCT* and *TGAT* (Tamhane p<0.010; Welch test of equality of means p=0.002)
†† rs2282679: *TT* vs. *GT* (Bonferroni p=0.016), rs4588: *GG* vs. *GT* (Bonferroni p=0.018), rs7041: *CC* vs *AC* (Bonferroni p=0.020).
††† rs2282679: *TT* vs. *GT* (Bonferroni p=0.007), rs4588: *GG* vs. *GT* (Bonferroni p=0.005), rs7041: *CC* vs. *AC* and *AA* (Bonferroni p= 0.006, 0.039), Diplotype: *1S/1S* vs. *1S/2* (Bonferroni p=0.034), $Haplo_{3SNP}$: *GTA* vs. *TGC* (Tamhane p=0.008, Welch test of equality of means p=0.008).
†††† rs2282679: *TT* vs. *GT* (Bonferroni p=0.004), rs4588: *GG* vs. *GT* (Bonferroni p=0.003), rs7041: *CC* vs. *AC* and *AA* (Bonferroni p=0.005, 0.036), Diplotype: *1S/1S* vs. *1S/2* (Bonferroni p=0.021).

Diplotype= rs4588/rs7041 Diplotype
$Haplo_{3SNP}$ = Haplotype of rs2282679, rs4588 and rs7041
$Haplo_{4SNP}$ = Haplotype of all four studied single nucleotide polymorphisms

Figure 3.

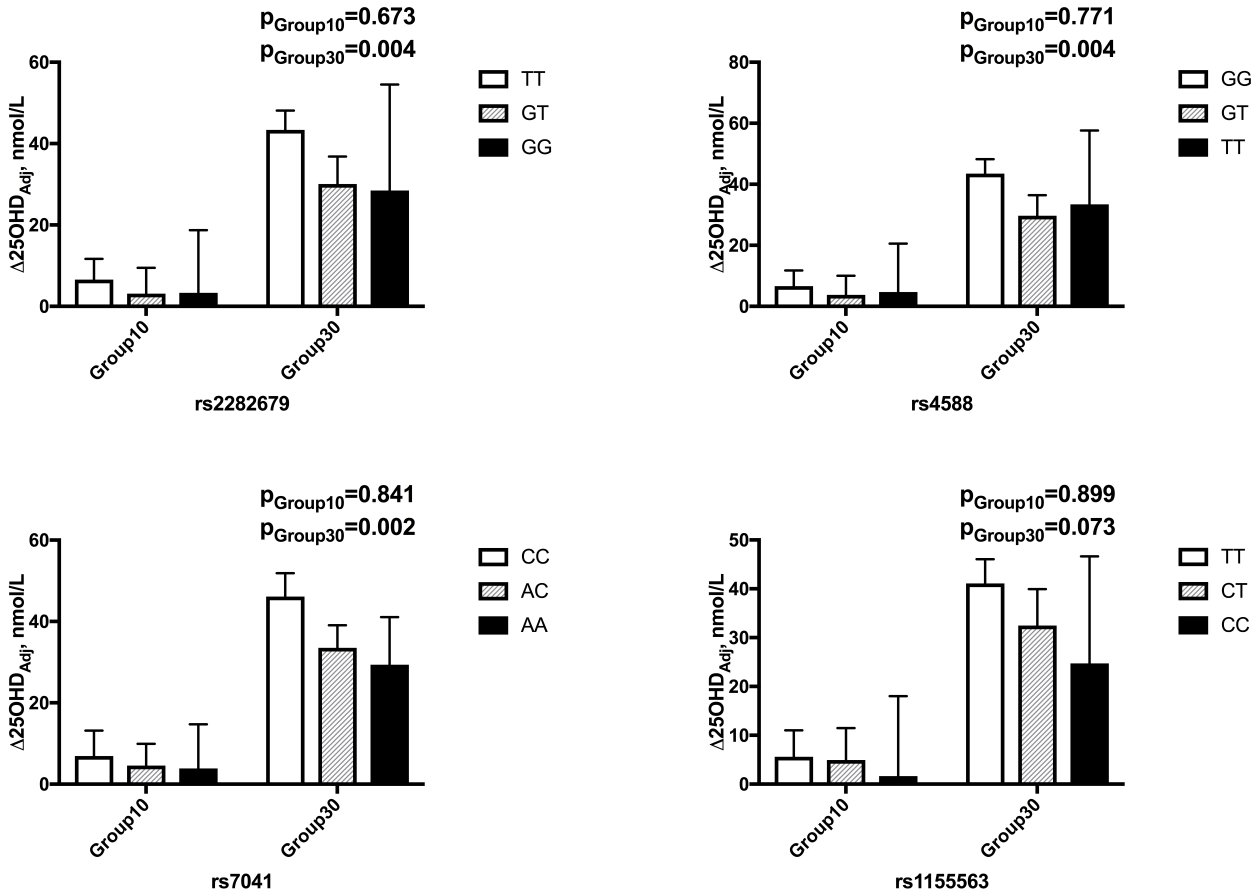

Figure 3.
Adjusted mean change of serum 25-hydroxyvitamin D concentration ($\Delta 25OHD_{Adj}$) (nmol/L) in the two intervention groups, Group10 and Group30, by genotype, diplotype and haplotype from baseline to 24 months of intervention, in participants with >80% adherence to intervention vitamin D_3 supplementation. Results for analyses of covariance for differences between variants.

Means are adjusted for season of birth, adherence to intervention supplementation (%) throughout the intervention (0-24 months) and length-adjusted weight standard deviation score at 24 months.

4. Discussion

The results of this randomized controlled trial in infants show that vitamin D binding protein genotype impacts vitamin D status and response to vitamin D supplementation. The key findings of this study are that in infants aged 24 months and younger, individual variation of the *GC* gene not only affects 25OHD concentrations from birth onwards, but also modifies temporal changes in 25OHD concentrations in response to high-dose vitamin D_3 supplementation. In our intervention group receiving 30 μg of vitamin D_3/day, participants homozygous for minor alleles of SNPs rs2282679, rs4588, rs7041, combined minor allele haplotype and participants with DBP phenotype 2 (*GC* diplotype *1S/2*, *1F/2* and *2/2*) had the lowest 25OHD concentrations and showed the smallest increase in 25OHD concentrations throughout intervention. Participants homozygous for the major alleles of these SNPs and their haplotype, as well as those with DBP phenotype 1 (*GC* diplotype *1S/1S*, *1S/1F* and *1F/1F*), showed higher 25OHD concentrations and greater intervention response.

Our findings support the previously reported cross-sectional associations of *GC* SNP genotype and rs4588/rs7041 diplotype with 25OHD concentrations in adults and children, linking minor alleles of rs2282679, rs4588, rs7041, rs1155563 and rs4588/rs7041 diplotype *2* with lower 25OHD concentrations. (8,11,18,21,23,39,40). In our study population these associations are evident already at birth, not only for rs2282679 as previously shown (23), but also for variants of rs4588, rs1155563 and rs4588/rs7041 diplotype. We also find that combined homozygote carriers of the minor alleles of SNPs rs2282679, rs4588 and rs7041 ($Haplo_{3SNP}$), and rs1155563 ($Haplo_{4SNP}$), show the lowest 25OHD concentrations during intervention.

Research on *GC* genotype related differences in response to vitamin D supplementation is, especially in a randomized controlled trial-setting, scarce and inconclusive, and studies in young children are lacking. In adult populations one study has reported minor allele rs4588 genotype to be linked with greater increase of 25OHD in response to vitamin D supplementation (28), while others reported no significant differences in dose-response between rs4588 and rs7041 genotypes in adults aged 45-75 (41) or 60-84 years (42) or in postmenopausal women (43). On the other hand, rs4588 major allele carriers have been reported to show greater increase of 25OHD in response to vitamin D supplementation in four adult studies (27,44-46). Four

studies have also found significant or indicative associations of major or minor alleles of rs7041 with respectively greater or smaller 25OHD increase in response to supplementation (25,27,45,46). One study of pregnant women showed rs2282679 major allele genotype to be associated with greater achieved 25OHD concentrations and change thereof (26), and two adult studies have reported minor allele genotype of rs2282679 to be related to smaller increase of 25OHD in response to vitamin D supplementation (25,44). Our study finds that in infants, major allele homozygotes of rs2282679, rs4588 and rs7041 as well as those homozygous for the major alleles of these three SNPs ($Haplo_{3SNP}$) show significantly greater supplementation responses to vitamin D_3 of 30 μg/day, when compared to minor allele homozygotes. Vitamin D binding protein phenotype 1 is also linked to greater, and phenotype 2 to smaller, increase in 25OHD during intervention. DBP 1 phenotypes have been shown to correspond with higher 25OHD concentrations than DBP 2 phenotype, as DBP 1 has a higher affinity for 25OHD, and is thought to prolong 25OHD halflife in plasma to a greater extent (17,19). It is plausible that the observed differences in supplementation response are similarly explained by DBP phenotype-related effects on 25OHD concentrations and free and bioavailable 25OHD. The effects of genotype on supplementation response are seemingly dose-dependent, with greater differences between variants seen at higher supplementation doses.

Our results are in line with the majority of adult studies on differences in response to vitamin D supplementation between variants in the *GC* gene (25-27,44-46). It has recently been suggested that genetic regulation of 25OHD concentration may be age-dependent, with stronger associations reported in adults aged ≤ 60 compared with those > 60 years, for some SNPs participating in vitamin D metabolism (47). It is possible that age-related differences in associations of genotype and response to supplementation could explain some of the differences in associations between our study and the minority of conflicting results found in older adult populations (28,41-43).

The randomized, double-blinded intervention trial setting and relatively large, homogenous study population, with uniform intervention and follow-up and on average very good compliance, are notable strengths of this study. Many previously reported studies of genotype-associated differences in vitamin D supplementation response have been performed in smaller study populations and/or by pooling data from several different trials. Although our study included 913 infants, some genotypes, and consequently diplo- and haplotypes,

are quite rare and the number of subjects proved to be a limitation in this study. To increase power, combinations of haplotypes of the three SNPs most consistently associated with 25OHD concentrations and temporal changes thereof (rs2282679, rs4588, and rs7041) were used.

We recognize some limitations in our study setting. It was not possible to obtain data on nutritional vitamin D intake, including more detailed information on total amount and vitamin D-contents of breastmilk for the entire 24 month follow-up period. Due to the randomized study setting, nutritional vitamin D intake did not differ between our intervention groups at 12 months of age (30). Data on DBP concentrations were not available for this study. *GC* genotype has been reported to affect 25OHD concentrations through both quantitative differences of DBP and genotype-associated functional differences of the binding protein (48). Supplementation dose has, however, previously been reported not to affect DBP concentration (18).

Optimal vitamin D supplementation and 25OHD concentrations in infants are still under discussion with some international guidelines currently recommending higher doses of up to 25 μg/day for children >1 year of age (1,2,49,50). Although genotype does not seem to affect response to current 10 μg/day supplementation, the observed differences between genotype-defined “poor” and “good” responders are of significance at higher supplementation doses, and should be considered when evaluating changes to supplementation guidelines in the studied age-group. Whether or not our findings could have bearing in tailoring individual treatment of vitamin D deficiency, where notably greater vitamin D doses are used, requires further studies.

In line with recent findings in Finnish adults, showing a clear decrease in vitamin D deficiency following increased fortification and supplementation guidelines in Finland (4,51), our study population was mainly vitamin D sufficient at all time-points, in both intervention groups. It is therefore difficult to draw conclusions on the consequences of our findings, or potential genotype-associated differences in response to current supplementation, in vitamin D deficient populations. In light of the observed differences in vitamin D supplementation response, it seems feasible that vitamin D deficient minor allele homozygotes of the studied *GC* variants could require higher supplementation doses in order to achieve optimal 25OHD concentrations and to avoid skeletal and extraskeletal effects of vitamin D deficiency. This should, however, be evaluated by separate prospective studies, in which intervention participants are stratified by genotype of the vitamin D binding protein.

We have previously reported that there was no significant difference in parent-reported infections or in bone strenght between the two intervention groups of the VIDI trial (30). Genotype of *GC* variants have been associated with differences in bone strength (rs4588) (19) and extraskeletal effects including effects on inflammation and immunity (rs4588/rs7041 diplotype) (52). Whether or not *GC* genotype related differences in supplementation response translates into differences in vitamin D dependent outcomes, such as bone strength and inflammation, warrants further studies, possibly with a wider spectrum of variants of the *GC* gene.

In conclusion, our study involving infants from birth to 24 months, found that in addition to associations between *GC* SNPs and 25OHD, the haplotypes of rs2282679, rs4588, rs7041 and rs1155563 significantly affected 25OHD concentrations. Genotype of rs2282679, rs4588 and rs7041, their haplotype and rs4588/rs7041 diplotype also significantly modified response to 24-month high-dose supplementation of 30 μg vitamin D_3/day. Genetic predisposition in the *GC* and other genes of vitamin D metabolism may have a notable impact on individual 25OHD concentrations and response to vitamin D supplementation. Further studies are warranted for a more complete understanding of the effects of genetic variation of the vitamin D binding protein on the response to supplementation and consequences thereof, as well as possible identification of those in need of greater supplementation doses.

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Author contributions

MEC, SA, OM and MP designed the study, MEC, LK, SA, OM and MP were responsible for conducting the research, MEC and MP analysed the data, MEC wrote the first draft of the manuscript; MEC, LK, EHS, HHA, JR, SV, OH, TH, HV, SA, OM and MP took part in the writing and editing of the report, with MEC, SA, OM and MP having the primary responsibility for the final content. All authors read and approved the final version of the manuscript.

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