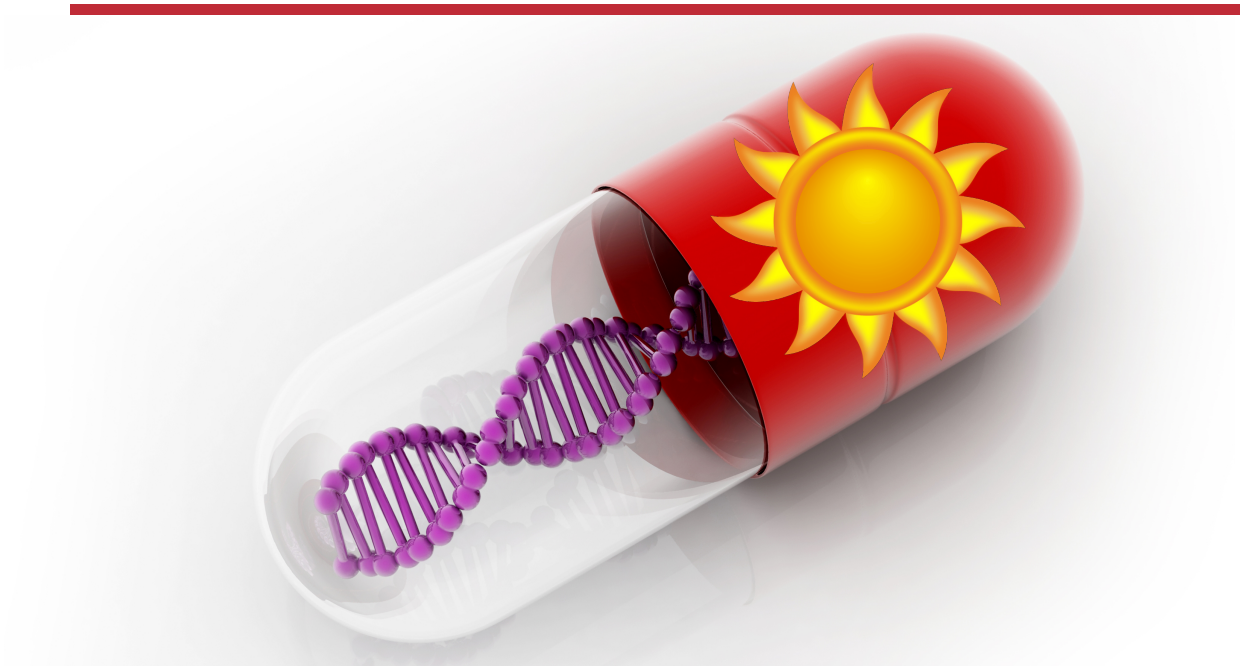
Common genetic variations in the *CYP2R1* and *GC* genes are determinants of vitamin D status in Danes



Janna Nissen
PhD Thesis
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DTU Food
National Food Institute

Common genetic variations in the *CYP2R1* and *GC* genes are determinants of vitamin D status in Danes

PhD thesis

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The National Food Institute

Technical University of Denmark

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Preface

This PhD thesis was performed at the National Food Institute, Technical University of Denmark between September 2010 and July 2015 including 2 maternity leaves. The project was financially supported by a Mobility PhD grant (*0601-01440B*) from the Danish Council of Research and Innovation.

The thesis is based on the following published publications, which are referred to in the text by their respective Roman numerals:

I. **Nissen J**, Rasmussen LB, Ravn-Haren G, Andersen EW, Hansen B, Andersen R, Mejborn H, Madsen KH, Vogel U.
Common variants in *CYP2R1* and *GC* genes predict vitamin D concentrations in healthy Danish children and adults.
PLoS One. 2014 Feb 27;9(2):e89907.

II. **Nissen J**, Vogel U, Ravn-Haren G, Andersen EW, Nexø BA, Andersen R, Mejborn H, Madsen KH, Rasmussen LB.
Real-life use of vitamin D_3-fortified bread and milk during a winter season: the effects of *CYP2R1* and *GC* genes on 25-hydroxyvitamin D concentrations in Danish families, the VitmaD study.
Genes Nutr. 2014 Jul;9(4):413.

III. **Nissen J**, Vogel U, Ravn-Haren G, Andersen EW, Madsen KH, Nexø BA, Andersen R, Mejborn H, Bjerrum PJ, Rasmussen LB, Wulf HC.
Common variants in *CYP2R1* and *GC* genes are both determinants of serum 25-hydroxyvitamin D concentrations after UVB irradiation and after consumption of vitamin D_3-fortified bread and milk during winter in Denmark.
Am J Clin Nutr. 2015 Jan;101(1):218-27.

A reprint of the publications in enclosed in the Appendix section.

Related publications

The following publications are related to the PhD-project, but not a part of the thesis:

IV. Madsen KH, Rasmussen LB, Andersen R, Mølgaard C, Jakobsen J, Bjerrum PJ, Andersen EW, Mejborn H, Tetens I.
Randomized controlled trial of the effects of vitamin D–fortified milk and bread on serum 25-hydroxyvitamin D concentrations in families in Denmark during winter: the VitmaD study.
Am J Clin Nutr. 2013 Aug;98(2):374-82.

V. Madsen KH, Rasmussen LB, Mejborn H, Andersen EW, Mølgaard C, **Nissen J**, Tetens I, Andersen R.
Vitamin D status and its determinants in children and adults among families in late summer in Denmark.
Br J Nutr. 2014 Sep;112(5):776-84.

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Finally, I also express my gratitude to the assessment committee for evaluating this thesis.

Vallensbaek, July 2015

Janna Nissen

[1] **2015** Abstract award held by Danish Nutrition Society, Copenhagen, Denmark
2015 Trainee Travel Award for the 18th Workshop on Vitamin D, Delft, the Netherlands.
2014 Young Investigator Award at the 2014 Vitamin D and Human Health meeting –from the gamete to the grave, London, United Kingdom

Summary

Vitamin D is considered a key fat-soluble vitamin critically important for good bone- and overall health throughout life. Vitamin D deficiency increases the risk of developing rickets, osteomalacia and osteoporosis, and moreover increases the risk of various non-skeletal adverse health outcomes including cardiovascular diseases, autoimmune diseases, some cancers and overall mortality. In humans, vitamin D is mainly synthesized in the skin after solar exposure and only a small amount is obtained through the diet.

An inter-individual variation in vitamin D status exists, which may be explained by genetic variation in vitamin D modulating genes. Twin and family-based studies indicate that genetic variation may have an appreciable influence on vitamin D status. Moreover, several candidate gene studies including two genome-wide association studies (GWAS) have found single nucleotide polymorphisms (SNPs) in *CYP2R1, CYP24A1, CYP27B1, C10orf88, DHCR7/NADSYN1, GC* and *VDR* genes to be associated with vitamin D status. The main hypothesis of this work was that genetically determined variation in vitamin D metabolism would influence the effect of vitamin D sources (vitamin D-supplementation and ultraviolet (UV)-B) on vitamin D status.

This was done by assessing the association between 25 SNPs located in the *CYP2R1, CYP24A1, CYP27B1, C10orf88, DHCR7/NADSYN1, GC* and *VDR* genes and vitamin D status in 756 participants in the VitmaD study in late summer (**paper I**), at the end of a winter season (**paper II**), after 6 months intake of vitamin D_3-fortified bread and milk (**paper II**) and in 92 participants in the VitDgen study after artificial UVB irradiation during winter (**paper III**).

Common genetic variations in the *CYP2R1* and *GC* genes were found to be important determinants of vitamin D status in three out of four scenarios: in late summer, after 6 months intake of vitamin D_3-fortified bread and milk and after artificial UVB irradiation, but not at the end of winter when no artificial vitamin D sources (vitamin D_3-fortification or UVB irradiation) had been given.

Overall, a general negative gene-dose dependent relationship was observed between increasing numbers of risk alleles of *CYP2R1* and *GC* and lower vitamin D status, and moreover an additive effect of *CYP2R1* and *GC* polymorphisms on vitamin D status was observed. Genetically predisposed individuals carrying all risk alleles of *CYP2R1* and *GC* had the lowest vitamin D status in late summer, the largest decrease in vitamin D status after intake of vitamin D_3-fortified bread

and milk during winter and the smallest increase in vitamin D status after artificial UVB irradiation compared to individuals carrying fewer or no risk alleles of *CYP2R1* and *GC*.

Based on the studies included in this thesis, it is concluded that genetically predisposed individuals, with a genetic profile of *CYP2R1* and *GC* leading to low vitamin D status, had the lowest vitamin D status in late summer and responded the least to increased exposure of the vitamin D sources, vitamin D_3-fortification and UVB irradiation. Genetically determined variation in *CYP2R1* and *GC* may potentially be used as a biomarker to identify at-risk individuals who have substantially increased risk of having low vitamin D status.

Dansk resumé (summery in Danish)

D-vitamin er et vigtigt fedtopløseligt vitamin der livet igennem har stor betydning for opretholdelsen af stærke knogler og for den generelle sundhed. D-vitamin-mangel øger ikke kun risikoen for at udvikle rakitis, osteomalaci og osteoporose, men også ikke-knoglerelateret sygdomme såsom hjerte-kar sygdomme, autoimmune sygdomme, visse kræftformer samt total dødelighed. D-vitamin dannes primært i huden efter soleksponering i sommermåneder og kun en lille mængde D-vitamin optages gennem kosten.

Der ses en inter-individuel forskel i D-vitamin status, som måske kan forklares af genetisk variation i D-vitamin modulerende gener. Tvillinge- og familiebaserede studier har vist at genetisk variation kan have mærkbar indflydelse på D-vitamin status. Derudover har kandidat-gen studier, herunder to genom-wide association studier (GWAS), fundet en sammenhæng mellem enkelt nukleotid polymorfier (SNP) i *CYP2R1, CYP24A1, CYP27B1, C10orf88, DHCR7/NADSYN1, GC* and *VDR* generne og D-vitamin status. I denne afhandling var den overordnet hypotese at genetisk bestemt variation in D-vitamin metabolismen ville influere effekten af D-vitamin kilder (D-vitamin berigelse og ultraviolet (UV)-B) på D-vitamin status.

Dette blev undersøgt ved at vurdere association mellem 25 SNPs lokaliseret i *CYP2R1, CYP24A1, CYP27B1, C10orf88, DHCR7/NADSYN1, GC* og *VDR* generne og deres indvirkning på D-vitamin status hos 756 deltagere i VitmaD studiet i sensommeren (**artikel I**), i slutningen af vinteren (**artikel II**), efter 6 måneders indtagelse af D_3-vitaminberiget brød og mælk (**artikel II**) og hos 92 deltagere i VitDgen studiet efter kunstig UVB-bestråling (**artikel III**).

Almindelige forekomne genetiske variationer i *CYP2R1* og *GC* generne var vigtige determinanter for D-vitamin status i tre ud af fire scenarier: i sensommeren, efter 6 måneders indtag af D_3-vitaminberiget brød og mælk, og efter kunstig UVB-bestråling, men ikke i slutningen af vinteren, hvis D-vitamin ikke blev kunstigt tilført (D_3- vitamin berigelse eller UVB-bestråling).

Overordnet var der en generel negativ gen-dosis afhængig sammenhæng mellem stigende antal risiko alleler af *CYP2R1* og *GC* og lavere D-vitamin status. Ydermere sås en additiv effekt af *CYP2R1* og *GC* på D-vitamin status. Genetisk disponerede individer som var bærere af alle risiko-alleler af *CYP2R1* og *GC,* havde den laveste D-vitamin status i sensommeren, det største fald i D-vitamin status efter indtagelse af vitamin D_3-beriget brød og mælk i løbet af vinteren, og den

mindste stigning i D-vitamin status efter kunstig UVB-bestråling i forhold til individer, som var bærere af færre eller ingen risiko-alleler af *CYP2R*1 og *GC*.

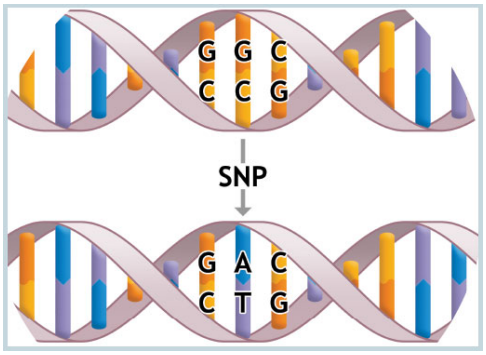
På grundlag af de undersøgelser, som indgår i denne afhandling, kan det konkluderes, at genetisk disponerede individer, med en genetisk profil i *CYP2R1* og *GC,* som fører til lav D-vitamin status, havde den laveste D-vitamin status i sensommeren og reagerede mindst på en øget D-vitamin eksponering, D_3-berigelse og UVB-bestråling. Genetisk bestemt variation i *CYP2R1* og *GC* generne kan potentielt anvendes som biomarkør til at identificere udsatte personer, der har en væsentligt forhøjet risiko for at udvikle lav D-vitamin status.

Abbreviations

ACADSB	Acyl-Coenzyme A dehydrogenase
CYP2R1	Encoding 25-hydroxylase
CYP24A1	Encoding 24-hydroxylase
CYP27B1	Encoding 1α-hydroxylase
C10orf88	Region harbouring the open-reading frame 88 on chromosome 10q26.13.
DBP	Vitamin D Binding Protein
DEQAS	Vitamin D External Quality Assessment Scheme
EFSA	European Food Safety Authority
FFQ	Food Frequency Questionnaire
GC	Encoding the vitamin D binding protein or GC, group-specific component
GRS	Genetic Risk Score
GWAS	Genome-Wide Association Studies
IOM	Institute of Medicine
LC-MS/MS	Isotope dilution liquid chromatography tandem mass spectrometry
LD	Linkage Disequilibrium
mRNA	Messenger RNA
MS	Multiple Sclerosis
NADSYN1/DHCR7	Nicotinamide adenine dinucleotide synthetase-1/7-dehydrocholesterol reductase
NIST	National Institute of Standards and Technology
NNRs	Nordic Nutrition Recommendations
PPF	Pigment Protection Factor
PTH	Parathyroid Hormone
RDA	Recommended Dietary Allowance
RI	Recommended Intakes
RXR	Retinoid-X Receptor
SED	Standard Erythema Doses
SNPs	Single Nucleotide Polymorphisms
SZA	Solar Zenith Angle
T1DM	Type 1 Diabetes Mellitus
VitDgen	Vitamin D in genes
VitmaD	Food with vitamin D
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Elements
UL	Tolerable Upper Intake Level
UV	Ultra-Violet
25(OH)D	25-Hydroxyvitamin D

Definition of genetic terms

Allele	An individual inherits two copies (alleles) for each gene, one from each parent, that control the same trait.
Genotype	The genetic constitution of a particular individual that determinates a specific trait (SNP), a set of traits (several SNPs), or all traits (the DNA).
Genetic risk score	The joint effect of X SNPs, calculated as the sum of number of X risk alleles.
Haplotype	A combination of closely linked DNA sequences on one chromosome that are often inherited together.
	States that genotype distribution remains constant in a randomly mating population.
Heterozygote	Individual carrying two different alleles.
Homozygote	Individual carrying two identical alleles.
Linkage disequilibrium	The alleles of a few SNPs on a haplotype predict the alleles of other SNPs, which provide redundant information.
MM	Homozygous major allele carriers or wild-type carriers.
Mm	Heterozygous carrier of one major and one minor allele.
mm	Homozygous minor allele carrier or variant.
rs	Reference sequence and a unique number for every known SNP e.g. rs4588.
SNP	Single nucleotide polymorphism; change in the DNA caused by a change in a single nucleotide (A, C, G or T).



SNP illustration, adapted from (1).

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1. Introduction

In the 21st century, vitamin D deficiency has become a worldwide problem affecting 1 billion people (2). Severe vitamin D deficiency causes osteomalacia or childhood rickets, osteoporosis and bone fractures because of reduced calcium absorption (3). Besides its established role in skeletal health, low vitamin D status is discussed as a risk factor in relation to several non-skeletal health outcomes such as cardiovascular diseases (4), obesity (5), diabetes (6), asthma (7), multiple sclerosis (MS) (8), occurrence of a large range of cancer diseases (9) and overall mortality (10,11).

Vitamin D status is modified by several external factors such as lifestyle, anthropometric factors, sun exposure and habits, latitude, diet, supplementation and fortification but also genetic variation in vitamin D modulating genes. A wide variability in heritability of 25-hydroxyvitamin D (25(OH)D, calcidiol) concentrations, ranging from 29 to 80 %, has been reported in twin and family-based studies (12–14) indicating that genetic factors may have an appreciable influence on vitamin D status, yet the genetic epidemiology of vitamin D or its metabolites has not been well studied. A better understanding of how genetic variation in the vitamin D modulating genes influences vitamin D status all year round and after fortification is needed and is the main objective of this thesis. Genetically determined variation in the vitamin D modulating enzymes may accelerate, or protect against, low vitamin D status and may help to identify who is most at risk of developing low vitamin D status. It may be used to prevent development of vitamin D deficiency in at-risk individuals and moreover preventing the development of vitamin D related diseases.

A growing number of studies have uncovered single nucleotide polymorphisms (SNPs) related to vitamin D modulating genes that affect vitamin D status independently of latitude and diet. By candidate gene analysis, five vitamin D modulating genes have identified, including *GC, CYP24A1, CYP2R1, CYP27B1* and *VDR* (15). Recently, two genome-wide association studies (GWAS) of vitamin D (16,17) confirmed the associations of common variants in *GC* and *CYP2R1* genes but also that the nicotinamide adenine dinucleotide synthetase-1/7-dehydrocholesterol reductase (*NADSYN1/DHCR7*) (17) and the region harbouring the open-reading frame 88 (*C10orf88*) (16) were associated with vitamin D status. This thesis investigates 25 genetic variations located in the aforementioned 7 vitamin D modulating genes and their association with vitamin D status in a healthy Caucasian population in late summer, end of winter, after 6-months intake of vitamin D_3-fortified bread and milk and after artificial whole body UVB irradiation during winter.

2. Background – Vitamin D

This section describes essential background information on vitamin D metabolism and biological functions, dietary recommendations, UVB exposure and genetic variations associated with vitamin D status.

2.1 Sources, metabolism and functions of vitamin D

In humans, vitamin D_3 is primarily obtained through endogen synthesis in the skin initiated by exposure to UVB irradiation (280-315 nm), contributing up to 80-90% of acquired vitamin D_3 in European populations (18) and characteristically smaller amounts are obtained through diet and supplements. Dietary vitamin D exists in two major native forms, vitamin D_2 (ergocalciferol) derived from eating invertebrates such as plants, mushrooms and yeast and vitamin D_3 (cholecalciferol) derived from animal-based sources such as fish, meat, milk and eggs (19). Vitamin D_2 differs structurally from vitamin D_3 in that it has an additional double bond and methyl group (20).

In the skin, UVB radiation converts 7-dehydrocholesterol (7-DHC) to pre-vitamin D_3, which immediately undergoes a thermal isomerization to vitamin D_3, and is completed within 2-3 days after initial sun exposure (21–23) (**Figure 1**).

Dermally synthesized vitamin D_3 diffuses via the blood to the liver tightly bound to vitamin D binding protein (DBP, also known as GC, group-specific component) whereas ingested vitamin D_2 and D_3 are absorbed in the small intestine and transported by chylomicrons and lipoproteins to the liver (20) and thus are presented to the liver in a different way. Hereafter, the metabolism of dermally or dietary synthesized vitamin D_2 or D_3 is considered to be similar even though the bioavailability of vitamin D_3 is considered to be better compared to that of vitamin D_2 (24). A distinction between these two forms is not made in the general literature and in the following vitamin "D" refers to both D_2 and D_3. Vitamin D undergoes a series of enzymatic conversions in the liver and kidneys in order to become biologically active.

In the liver, the hepatic enzyme 25-hydroxylase (encode by the *CYP2R1* gene) converts vitamin D to 25(OH)D. The conversion is loosely regulated and seems to be primarily dependent on the vitamin D concentration (20).

To become biologically active, 25(OH)D is converted into 1,25-dihydroxyvitamin D ($1,25(OH)_2D$, calcitriol) mainly in the kidneys, but also in other tissues expressing the enzyme 1α-hydroxylase

(encode by the *CYP27B1* gene). The conversion of 25(OH)D to 1,25$(OH)_2$D is tightly regulated by calcium and phosphate concentrations through a negative feedback mechanism mediated by parathyroid hormone (PTH) (25).

In the circulation, most of vitamin D, 25(OH)D and 1,25$(OH)_2$D are transported and bound to DBP but a small fraction is bound to albumin or exists in free form (26,27). DBP-bound 25(OH)D is the major circulation metabolite and with a relative long half-life of 2-3 weeks, DBP-bound 25(OH)D is the preferred biomarker of vitamin D status compared to 1,25$(OH)_2$D which have a short half-life of 10-20 h (20). Recently, it has been questioned whether 25(OH)D is the best biomarker of vitamin D status. It has been suggested that free and bioavailable 25(OH)D, measured as albumin-bound and free 25(OH)D, may be a better and more informative marker for vitamin D status (26,27).

DBP-bound 1,25$(OH)_2$D enters the circulation and travels to target tissues where is can mediate both transcriptional and rapid non-transcriptional effects. The transcriptional effects of vitamin D are mediated by 1,25$(OH)_2$D binding to nuclear vitamin D receptors (*VDR*), which then forms a heterodimer with the retinoid-X receptor (*RXR*) which binds to vitamin D response elements (VDRE) in the regulatory element region of vitamin D target genes (28). The rapid non-transcriptional (rapid response) effect of 1,25$(OH)_2$D is when 1,25$(OH)_2$D acts like a steroid hormone through activation of signal transduction pathways at or near cell surface receptors (29). The non-transcriptional response is rapid i.e. acting within seconds to minutes, whereas the transcriptional response takes a few hours to days to elicit the response (30).

To prevent excessive vitamin D signalling in target organs, both 25(OH)D and 1,25$(OH)_2$D induce 24-hydroxylase (encode by *CYP24A1*) leading to formation of biologically inactive water-soluble metabolites which are eventually excreted in the bile (20,31,32). Additionally, as vitamin D is hydrophobic it can be stored in human adipose tissues as a “non-specific” store, but the extent of accumulation or mobilization during periods of shortages of vitamin D is unknown (33).

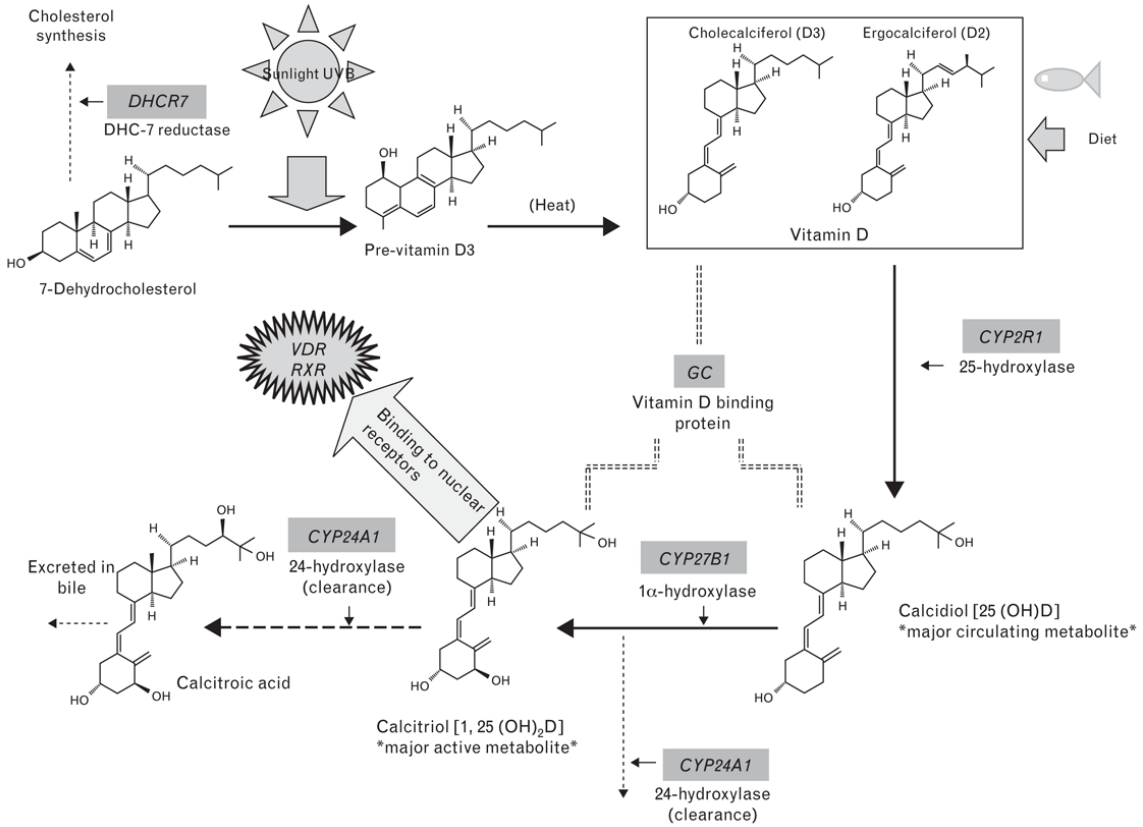

Figure 1. Genetic variations related to the vitamin D metabolism. *CYP24A1*, 24-hydroxylase; *CYP27B1*, 1α-hydroxylase; CYP2R1, 25-hydroxylase; *DHCR7*, 7-dehydrocholesterol reductase; *GC*, vitamin D binding protein; *RXR*, retinoid-X receptor; *UVB*, ultraviolet B; *VDR*, vitamin D receptor (34).

The main biological function of vitamin D is facilitation of intestinal calcium absorption and maintenance of calcium homeostasis. Calcium is essential for development and maintenance of bone, cellular processes and neuromuscular functions (35). Low blood calcium concentrations induce the release of PTH from the parathyroid gland, which stimulates 1α-hydroxylase in the kidneys to produce $1,25(OH)_2D$, which then increases calcium concentrations through three separate targets: 1) by enhancing intestinal absorption, 2) interacting with PTH to stimulate reabsorption in the kidneys and 3) mobilization from bones (36). Under normal conditions, dietary calcium if favoured over bone-mobilization, but it has been suggested that bone cells can convert $25(OH)D$ to $1,25(OH)_2D$ when calcium supply is inadequate (37).

High blood calcium concentrations and $1,25(OH)_2D$ it self suppress PTH secretion and induce 24-hydroxylase activity in the kidney converting $1,25(OH)_2D$ to 24,25-dihydroxyvitamin D which is less biologically active than $1,25(OH)_2D$ and is considered the first step of inactivation (29).

Existence of non-classical functions of vitamin D, not related to calcium homeostasis, has been suggested (30). In the human genome over 2700 VDR-binding sites have been identified in over 30 cell types, including bone, intestine, immune, kidney, pancreas, lung, heart, muscle, brain and skin, supporting the wide-ranging influence of vitamin D in human metabolism (34,35). Non-classical function of $1,25(OH)_2D$ may play a role in the innate immune system, insulin secretion, cell proliferation and differentiation (35).

2.2 Seasonal and individual variation of 25(OH)D concentrations

The efficiency of the conversion of 7-DHC to vitamin D_3 follows the seasonal variation in the solar zenith angle (SZA) inversely related to the amount of UVB photons in the solar spectrum. A small SZA results in an increased intensity of UVB photons reaching the earth and is prominently found in summer, at noon and near equator (38). In contrast, a large SZA results in less UVB photons with less intensity reaching the earth because more UVB photons are absorbed, redirected or attenuated in the atmosphere and is prominently found in winter, at early mornings, at late afternoons and at high latitudes (38). Therefore, cutaneous vitamin D_3 synthesis is influenced by the time of the day, season of the year and latitude (39). Around equator (0°) a high amount of solar UVB radiation is present all year round, contrarily, at the poles (90°) solar UVB radiation is only present a few months of the year (40). Consequently, during winter months in latitudes above 40°N, cutaneous vitamin D_3 synthesis is negligible from October to March and often referred to as the "vitamin D winter" (41). During the "vitamin D winter" period vitamin D must be acquired from dietary sources, supplementation or use of summer vitamin D storage. Vitamin D status is therefore associated with season, and hence solar UVB radiation, with the highest 25(OH)D concentrations observed during summer and the lowest 25(OH)D concentrations observed during winter (42).

Humans respond differently to UVB radiation and a number of factors affect the cutaneous synthesis of vitamin D_3. Cutaneous vitamin D synthesis is affected by geographic factors, sun-seeking behavioural factors such as duration and time spent outside, area of exposed skin, use of sunscreen, sunny holidays and age (38,43). The skins ability to synthesize vitamin D_3 decreases with age (44). It is controversial whether cutaneous vitamin D_3 synthesis is more efficient in individuals with pale skin compared to individuals with dark skin (45,46). It is believed to be an evolutionary adaptation resulting from migration to more northern and less sunny climates (47,48).

Ambient ultraviolet radiation (UV)-R may cause erythema (temporary reddening) and can be measured as the standard erythema dose (SED). SED is a standardized measure of erythemal effective radiant exposures from natural or artificial sources of UVR (49). One SED is equivalent to an erythemal effective radiant exposure of 100 Jm^{-2} at 298 nm using the International Committee of Illumination (CIE) erythema action spectrum and corresponds to a UV dose that causes perceptible erythema in the most sun-sensitive individuals (49,50). The SED is independent of skin type and a particular exposure dose in SED may cause erythema in fair skin but not in darker skin.

2.3 Health benefits and risks of solar UV radiation

Sunlight is the most prominent source of UVR, consisting of UVB and UVA radiation. Solar UVA and UVB radiation have different effects on skin. Solar UVB radiation contributes with 80% of the harmful effect of sun-exposure and solar UVA radiation with the remaining 20% (51). UVR has detrimental effects on human health and the dangers of overexposure to sunlight have been well established. Acute signs of solar UVR exposure are pigmentation (tanning) and erythema (sunburn). Chronic signs of solar UVR exposure are premature skin aging and increased risk of skin cancer due to DNA damage (52). Solar UVR is considered as a complete carcinogen and excessive solar UVR exposure causes 99% of non-melanoma skin cancers by initiating and promoting the carcinogenesis of squamous cell carcinoma and basal cell carcinoma (52). Furthermore, it is estimated that at least 20% of malign melanoma are causes due to excessive solar UVR exposure (40).

Public health guidelines have the last 40 years warned against excessive solar UVR as it causes sunburn and increased risk of skin cancer (43). Less attention has been given to acknowledge the beneficial role of UVB radiation, that being the cutaneous synthesis of vitamin D_3 (45). Adequate sun exposure is essential for human health and for most of the world's population requirement of vitamin D_3 is satisfied by photosynthesized vitamin D_3 (40). A balance is required between avoiding the increase in skin cancer risk and achieving enough UVB radiation exposure to maintain adequate vitamin D concentrations. How this is balanced remains to be clearly defined and validated (53). Limited data exist for weighting risk against benefit when considering inadequate vitamin D status vs. overexposure to sunlight (54). There have been concerns that sun avoidance may lead to inadequate vitamin D status. The overall health benefit of an improved vitamin D status may be more important than the possible increased skin cancer risk resulting from carefully increasing UVR exposure (55). Besides vitamin D_3 production, solar UVR has several beneficial effects. Heliotherapy (solar radiation) or phototherapy (artificial UVR) can treat several human skin diseases, like psoriasis, vitiligo, atopic dermatitis and localized scleroderma (56). Solar UVR may increase nitric oxide concentrations in the blood which may reduce blood pressure and improve cardiovascular health (56). Moreover, delayed tanning induced by UVB can act as a sunscreen and it has been hypothesized that vitamin D_3 produced in the skin has a protective mechanism against UVR induced carcinogenesis (57). However, due to the well-known carcinogenicity and high frequency of acute side effects sunbed use as vitamin D source is generally not recommendable (58).

Safe sun practices, intake of vitamin D-rich foods and vitamin D supplements have emerged as an alternative strategy for optimizing one's vitamin D status and may help to decrease skin cancer risk (59). A short daily sun exposure is recommended over a single long exposure regarding cutaneous vitamin D_3 synthesis (38). After about 15 minutes of sun exposure the synthesis of previtamin D_3 reaches a plateau [57] and prolonged sun exposure leads to formation of biologically inactive water-soluble metabolites to prevent reaching toxic levels of vitamin D_3 (20,31,32). It have been suggested that in summer months in Denmark, 56°N, adequate vitamin D concentrations can be obtained from 20-30 minutes of sun exposure of hands, arms and face 2-3 times a week in the middle of the day (61).

2.4 Dietary vitamin D recommendations, measurement and cut-off limits

Evaluation of vitamin D status is complex because it is modified by several external and lifestyle factors such as UVB and sun exposure habits, latitude, season, anthropometric factors, ethnicity, variation in vitamin D modulating genes, dietary vitamin D sources, vitamin D supplementation and vitamin D-fortified foods and drinks. Moreover, the optimal level of vitamin D is uncertain, which is further complicated by the lack of standardization in and between different methods for quantification of 25(OH)D concentrations (62). To compensate for method-related variability, an international standardization reference material was in 2010 introduced by the National Institute of Standards and Technology (NIST) (62). At present, 25(OH)D concentrations is generally accepted as the best biomarker of vitamin D status reflecting the sum of vitamin D from intake and cutaneous synthesis (63).

Dietary vitamin D intake has become an increasingly important source during the winter season at higher latitudes when solar exposures are negligible and low vitamin D status is frequently observed. Relatively few foods naturally contain vitamin D and the actual vitamin D content may vary considerably due to breeding circumstances, feed, species, season and cooking method (39). The vitamin D content in wild caught salmon from Alaska was approximately 25% higher compared to farmed salmon. Furthermore, the vitamin D content between species varied from 2.5 to 25 μg/100g and moreover the vitamin D content in fish decreased with 50% when fried in vegetable oil, but not when baked or microwaved (39).

In Denmark, food fortification is not a significant source of vitamin D, as it is not mandatory or common as in Norway, Finland, Sweeden, Ireland, the United Kingdom, Spain, USA and Canada (64,65). In the Danish population, the primary dietary sources of vitamin D comes from intakes of fish (57%), meat (16%), eggs (10%), milk (7%), fats (4%), bread and cereals (2%) and cheese (2%) with a mean estimated dietary intake of 2.7 μg/day in children aged 4-9 years, 2.8 μg/day in children aged 10-17 years and 4.8 μg/day in adults aged 18-75 years (66). In Denmark, use of dietary supplements is common and 2% of children aged 4-10 years, 4.6% of children aged 10-17 years and 8.5% of adults aged 18-75 years are supplement users (67). Among dietary supplement users, the total estimated vitamin D intake were 7.6-8.4 μg/day (68). Intakes are lower than the recommended intake (RI) of 10 μg/day defined by the Nordic Nutrition Recommendation (NNR) (69) as it is in most populations (70).

In Europe, vitamin D recommendations range from intakes of 2.5 to 22.5 μg/day (71), and no general agreement of which dietary vitamin D doses are needed to achieve sufficient 25(OH)D concentrations has been reached. The Institute of Medicine (IOM) recently reported that a Recommended Dietary Allowance (RDA) of 15 μg/day for individuals aged 1-70 y will cover the requirement for 97.5% of the population in the US and Canada, corresponding to 25(OH)D concentrations of at least 50 nmol/L (33). Recently, the RI for vitamin D intake in the Nordic countries was revised and increased from 7.5 μg/day to 10 μg/day for individuals aged 2-60 y to cover the requirement for 95% of the Nordic population (69,72). Both IOM and NNR 2012 based their RDA and RI on the relationship between 25(OH)D concentrations and bone health.

The Danish National Board of Health defines vitamin D status above 50 nmol/L as vitamin D sufficiency, between 25-50 nmol/L as vitamin D insufficiency, below 25 nmol/L as vitamin D deficiency and below 12.5 nmol/L as severe vitamin D deficiency (73). These definitions will be used in the present thesis, except in paper II where the American definition of vitamin D deficiency was used. In America, vitamin D insufficiency was defined as 25(OH)D concentrations below 30 nmol/L where adverse effects on bone health may be expected and vitamin D sufficiency was defined as above 50 nmol/L, which is the requirement for optimal bone health (33). No international standard has been accepted defining deficient and sufficient vitamin D status and there is an on-going international discussion regarding which cut-off values should be used. There is a general agreement in Europe that a 25(OH)D concentrations of at least 50 nmol/L is sufficient (33). Concurrently, some experts argue that a 25(OH)D concentrations >75 nmol/L is necessary to achieve a sufficient vitamin D status and non-skeletal benefits (19,74).

2.5 Consequences of low and toxic vitamin D concentrations

Vitamin D is essential in calcium homeostasis and in the development and maintenance of the skeleton. Low vitamin D status, caused by limited exposure to sunlight, poor nutrition and/or decreased dietary intake of vitamin D, has long been associated with the development of rickets in growing children or osteomalacia and osteroporosis in adults caused by an impaired mineralization of bone (75). In addition, studies have indicated that genetic factors in vitamin D modulating genes may play an important role in the susceptibility to rickets (75). Rickets, caused by failure in calcification of the growth plates, is characterized by growth retardation, muscle weakness, fractures, pain and skeletal deformities (soft bones) (76). In osteomalacia, un-calcified bone tissue gradually replaces old bone tissue, leading to weakened bone structure. The symptoms may be less pronounced in adults causing diffuse pain in bone and muscles (3,77).

Prolonged and less severe degrees of vitamin D deficiency have been suggested to play a role in osteoporosis pathogenesis caused by elevated PTH concentrations, known as secondary hyperparathyroidism, calcium mal-absorption, increased bone turnover and bone loss. Osteoporosis is characterized by low bone mass, mineralization defects and muscle weakness causing falls and high fracture risk and in the long term leading to osteomalacia (3), (78).
In Denmark, the prevalence of rickets or osteomalacia is low and mostly frequently found among immigrants (79,80). In contrast, the prevalence of osteoporosis is high in elderly, which has large public health implications (77,81).

Importantly for public health, low vitamin D status may also be related to various non-skeletal health outcomes, including cardiovascular diseases (4), obesity (5), diabetes (6), asthma (7), multiple sclerosis (8), certain cancer types (9), autoimmune diseases (82) and overall mortality (10,11).

Vitamin D is a fat-soluble vitamin and can be stored in human adipose tissues and this raises concerns about toxicity. In the general population, excessive vitamin D intakes from fortified foods and drinks or supplementation, but not endogenous synthesis, can potentially lead to a state of vitamin D "-intoxication-" or "-hypervitaminosis-" (83). In the literature there are no known cases of vitamin D toxicity resulting from extreme or unusually prolonged sun exposure, because thermal activation of pre-vitamin D_3 in the skin gives rise to multiple non-vitamin D-forms (33). Acute vitamin D intoxication leads to hypercalcemia including pain, conjunctivitis, anorexia, fever, chills,

thirst, polyuria, vomiting and weight loss. Chronic vitamin D intoxication can lead to soft tissue calcification and resultant renal and cardiovascular damage (33). Vitamin D intoxication is rare and usually not seen with 25(OH)D concentrations <325 nmol/L or daily intake <250 μg/day (33), (83). Nevertheless, lower 25(OH)D concentrations than what caused acute vitamin D intoxication may potentially be associated with adverse health outcomes (84) and the health-consequences of prolonged/life-long intake of >25 μg/day of vitamin D are at present unknown (85). A U-shaped or reverse J-shape relationship between 25(OH)D concentrations and some adverse health outcomes such as certain cancers and all-cause mortality has been found (10,11,86,87). Based on the relationship between 25(OH)D concentrations and all-cause mortality, the US dietary committee suggested that potential adverse health outcomes may occur at 25(OH)D concentrations >125 nmol/L (33). On the basic knowledge of hypercalcemia and impaired growth in children, the Tolerable Upper Intake Level (UL) for vitamin D was set to be 50 μg/day for children aged 1-10 years and 100 μg/day for children aged 11-17 years and adults by the European Food Safety Authority (EFSA) (88).

2.6 Genetic variation influence on vitamin D status

The concept of heritability contributing to disease susceptibility has been known for centuries. The study of genetic variation has a broad applicability, in elucidating disease susceptibility and in tailoring of personalised clinical strategies based on the individual's genetic make-up.

SNPs are stably inherited DNA-sequence variations, which occur when a single nucleotide (A, G, C or T) in the genome sequence is substituted for another nucleotide and occur in more than one percent of the general population. Studies of SNP variations in different ethnic groups may be essential because genotype frequencies differ between populations and could partly explain the difference in genetically determined disease susceptibility between populations. Different SNP versions state the individual's genotype, which may lead to different phenotypes. Phenotype refers to the physical and behavioural characteristics of e.g. a protein (89).

SNPs can occur in the protein-coding region of genes or between genes (intronic regions). SNPs located inside a protein-coding region can be silent (synonymous) without any functional consequence for the protein or it can change the amino acid (non-synonymous) and thereby change protein concentration and the catalytic property of the enzyme. SNPs located in the promoter region of a gene may affect the regulation of the gene and thereby affect protein concentration. Intronic SNPs may play a significant role in the stability or the slicing of the messenger RNA (mRNA), giving lower expression levels of the encoded protein.

Recently, two GWAS (16,17) and an increasing number of candidate gene studies have identified vitamin D modulating genes that are associated with vitamin D status. The two independent GWAS, based on participants from European ancestry, both identified genetic variations in three genes: *DHCR7*, *CYP2R1* and *GC*. Furthermore, Wang et al.(17) confirmed a genetic variant in *CYP24A1* and Ahn et al.(16) confirmed a variant in *C10orf88* to be associated with vitamin D status. From candidate gene studies *CYP27B1* (90–93) and *VDR* (90,94,95) have also been associated with vitamin D status.

In the following section a general introduction to the vitamin D modulating genes that have been linked to vitamin D status is described. The function and location of the genes in the vitamin D metabolisms is shown in Figure 1 (page 4).

DHCR7, located on chromosome 11q13.4 close to the *NADSYN1* gene, encodes a reductase catalysing the conversion of 7-DHC to cholesterol, thus removing precholesterol which is the substrate for 25(OH)D synthesis. Two recent studies in healthy Chinese (96,97) confirmed the findings by GWAS (16,17). In animal studies *DHCR7* inhibitors led to increased 7-DHC and 25(OH)D concentrations (98). In human, mutations in *DHCR7* are known to lead to Smith-Lemli-Optiz syndrome, but it is unknown whether their vitamin D status is affected (99). Furthermore, evidence suggests that the *DHCR7* gene is involved in the susceptibility to ocular Behçet disease (100), severity of liver fibrosis (101,102) as well as associated with risk of autoimmune diseases including rheumatoid arthritis (103), type 1 diabetes (T1DM) (104) and MS (105).

CYP2R1, located on chromosome 11p15.2, is the primarily enzyme responsible for the hydroxylation of vitamin D to 25(OH)D. It yielded a high score in both GWAS (16,17) and was prior found in a candidate gene study (106) and subsequently replicated in several studies (96,97,107,108) to be associated with 25(OH)D concentrations. In addition, external sources of vitamin D, such as season, dietary and supplemental intake, seems to modify the genetic effects of *GC* and *CYP2R1* (107,109).
A known mutation in the *CYP2R1* gene leads to vitamin D deficiency (110). Recently, a case-control study conducted in north-eastern Han Chinese children confirmed that *CYP2R1* and *GC* variation plays an important role in the susceptibility to rickets (75). Moreover, genetic variation in the *CYP2R1* gene has been associated with a broad range of diseases including recurrence of colon cancer (111), pancreas cancer (112), testis cancer (113,114), T1DM (104,108,115), chronic liver disease (102),(116), asthma (117) and eczema (118) to mention a few.

GC, located on chromosome 4q12–13, encodes the DBP, which is an albumin-like protein produced in the liver and acts as the major carrier protein for vitamin D and its metabolites. Apart from acting as the major transport carrier protein for vitamin D and its metabolites, DBP has several other important biological functions such as extracellular actin scavenging, leukocyte C5a-mediated chemotaxis, macrophage activation, stimulation of osteoclasts and transportation of fatty acids. DBP and vitamin D may jointly or independently affect disease susceptibility or resistance unrelated to their function in bone and mineral metabolism (119–121). DBP has independently been linked to bone metabolism, autoimmune disease, obesity, pulmonary disease, liver disease and MS (121) to mention a few.

There is accumulating evidence that genetic variation in the *GC* gene is associated with 25(OH)D concentrations. SNPs in the *GC* gene reached the highest score in both GWAS (16,17) and prior candidate gene studies have found evidence for association with 25(OH)D concentrations (122–130). The human DBP protein is a highly polymorphic protein with more than 120 known variants (121). The most studied *GC*-variants are the two common missense mutations rs7041 (Asp432Glu) and rs4588 (Thr436Lys), which produce a highly polymorphic protein that give rise to three major DBP-phenotypes; *Gc1F* (rs7041-T, rs4588-C), *Gc1S* (rs7041-G, rs4588-C), and *Gc2* (rs7041-T, rs4588-A). Combinations of these three DBP-phenotypes give rise to six DBP-isotypes (*Gc1F/1F, Gc1F/1S, Gc1F/2, Gc1S/1S, Gc1S/2, Gc2/2)*. They differ by amino acid substitutions and by glycosylation (128) and have different binding affinities for vitamin D metabolites inclusive 25(OH)D (26,27). Vitamin D status differed significantly depending on rs4588 (or rs2282679, $r^2 > 0.99$) and/or rs7041 genotype, where the A-allele of rs4588 and/or the T-allele of rs7041 consistently are associated with lower 25(OH)D levels (122–129). In Caucasian, rs4588 and rs7041 are in almost complete linkage disequilibrium (LD) (Haploview software version 4.2). DBP-phenotype is an independent predictor of 25(OH)D (122) and adjustment for DBP-phenotypes may therefore influence 25(OH)D concentrations. Moreover, it has recently been suggested that free and bioavailable 25(OH)D, measured as free and albumin-bound 25(OH)D, may be a more informative measure of vitamin D status than the currently used total 25(OH)D. Genetic differences in DBP phenotypes may affect the binding of 25(OH)D and, thereby, the amount of free and bioavailable 25(OH)D (26,27,131).

There is biological support that the affinity to both 25(OH)D and $1,25(OH)_2D$ is higher for the rs4588 C-allele isoform than for the A-allele isoform (132). Based on glycosylation patterns, it is suggested that *Gc2* enzyme metabolizes faster. Kawakami et al. (133) observed that the metabolic rate indeed was higher in *Gc2/2* individuals than in *Gc1/1* individuals. In addition, the *Gc2* allele, which is associated with low 25(OH)D concentrations, is also associated with low mean DBP concentrations (122). Interestingly, the *Gc2* allele frequency is higher in Caucasians and their derivatives (living in northern climates) than in the Black population indicative of an overall population variation (134).

GC genotypes have in association with 25(OH)D concentrations been linked to PTH levels and bone mass accrual in adolescence (119), vitamin D insufficiency (135), and response to UV radiation (UVR) (136).

CYP24A1, located on chromosome 20q13.2, initiates degradation of both 25(OH)D and $1,25(OH)_2D$, and was found in the GWAS of Wang et al. (17). Previous and recent candidate gene studies have not been able to find an association of variants at this locus with 25(OH)D concentrations in healthy populations (107,137,138). It has been found that baseline DNA methylation levels of *CYP24A1* may predict variation in vitamin D response (139).

In chronic kidney disease, vitamin D status is profoundly affected. Recent evidence suggests that increased *CYP24A1* expression, which results in increased degradation of both 25-(OH)D and 1,25-$(OH)_2D$, is the main cause of the disturbed vitamin D status (140). In several human cancer diseases (141–144) an over-expression of *CYP24A1* has been found, suggesting that *CYP24A1* contributes to the diminished efficacy of 1,25-$(OH)_2D$ (145). Inhibition of *CYP24A1* may potentially not only increase 1,25-$(OH)_2D$ concentrations but also inhibit intra-tumor degradation of 1,25-$(OH)_2D$ (145).

CYP27B1, located on chromosome 12q14.1, converts 25(OH)D to the active hormone $1,25(OH)_2D$, did not reach genome-wide significance (17) and has not consistently been association with 25(OH)D concentrations in candidate gene studies (90–93,106,126,146). A rare mutation in the *CYP27B1* gene is known to lead vitamin D-dependent rickets type 1 (147,148). Vitamin D deficiency has been found as a risk factor for MS and rare variants in *CYP27B1* are strongly associated with MS risk, supporting a causal role of vitamin D deficiency as a risk factor for MS (149,150). Moreover, genetic variation in the *CYP27B1* has been associated with fracture risk in the elderly (151). Like *CYP24A1, CYP27B1* is found to be up-regulated in breast tumours as compared with normal tissue (152).

C10orf88, located on chromosome 10q26.13 in the vicinity of acyl-Coenzyme A dehydrogenase (ACADSB), is involved in cholesterol and vitamin D synthesis (16) was found in the GWAS by Ahn et al. (16), but has not since been found to contribute to variations in vitamin D status in replication studies (138,153).

VDR, located on chromosome 12q13.11, encodes the nuclear hormone receptor for $1,25(OH)_2D$, and variants at this locus have typically not been associated with 25(OH)D concentrations, although some evidence for Fok1 (rs10735810) has been found in studies on MS (90,95). A strong association between *VDR* and 25(OH)D would not be expected given the metabolic distance between them. The main focus on *VDR* has been on assessing disease associations (34).

3. Rationale and aims of project

Genetic variation in vitamin D modulating genes has been associated to vitamin D status and a better understanding of how genetic variation in vitamin D modulating genes influences vitamin D status is needed. The overall aims of this thesis are:

1. Elucidate the genetic influence of 25 SNPs, located in the *CYP2R1, CYP24A1, CYP27B1, C10orf88, DHCR7/NADSYN1, GC* and *VDR* genes on vitamin D status in a healthy Caucasian population at four different scenarios; in late summer, end of winter, after intake of vitamin D_3-fortified bread and milk and after artificial UVB irradiation
2. Identify predisposed individuals, who have substantially elevated risk of developing low vitamin D status.

Paper I:

The aims of paper I was to determine the influence of 25 common genetic variations located in 7 vitamin D modulating genes on vitamin D status in late summer in Denmark. The aim was to identify genetically predisposed individuals that may have increased risk of developing low vitamin D status.

Paper II:

The aim of paper II was to assess the effect of real-life use of vitamin D_3-fortified bread and milk on vitamin D status in relation to 25 common genetic variations in 7 vitamin D modulating genes in Danish families with dependent children during a 6-months winter period. Furthermore, to assess if vitamin D_3-fortification will maintain vitamin D status during winter in those with genetically determined low vitamin D status. A secondary aim was to evaluate the amount of vitamin D needed in different genetic profiles to maintain a sufficient vitamin D status during winter.

Paper III:

In paper III, the aim was to analyze the association between the increase in vitamin D status after a given dose of artificial UVB irradiation and 25 common genetic variations in 7 vitamin D modulating genes. The aim was furthermore, to compare if vitamin D_3 acquired by artificial UVB irradiation or from consumption of vitamin D_3-fortified bread and milk during winter have similar effect on vitamin D status in relation to genetic variations in the *CYP2R1* and *GC* genes.

Publications are enclosed in the Appendix section and will be discussed in the following chapters.

4. Overview of the experimental work

The present thesis is based on three research publications based on the Food with vitamin D (VitmaD) study conducted at The National Food Institute, Technical University of Denmark and the Vitamin D in genes (VitDgen) study conducted at the Department of Dermatology, Bisperbjerg University Hospital. Papers I and II are based on the VitmaD study, whereas Paper III is based on both the VitmaD and VitDgen studies. An overview of the VitmaD and VitDgen studies is given here.

4.1 The VitmaD study

The VitmaD study was a double-blinded, randomized placebo-controlled intervention trial with apparently healthy ethnically Danish children and adults (4-60 y) recruited as 201 families (782 participants) who were randomly allocated to either vitamin D_3-fortified bread and milk or non-fortified placebo bread and milk during a 6-months winter period (September 2010 to April 2011).

During the intervention period, the adult participants were seen three times (month 0, 3 and 6) and children (4-17 years) were seen twice (month 0 and 6). Blood samples were collected at all visits and anthropometric measures (height and weight), blood pressure (only measure in adults) and information from a detailed self-administered web-based questionnaire including a semi-quantitative food frequency questionnaire (FFQ) were recorded at month 0 and 6.

The study was conducted according to the guidelines in the Declaration of Helsinki and the protocol was approved by the Research Ethics Committee of the Capital Region of Denmark (*H-4-2010-020*) and registered at http://clinicaltrials.gov (*NCT01184716*). All adult participants and guardians on the behalf of the children participants gave written consent to participate.

4.1.1 Food fortification strategy

The aim of the study design was to investigate a realistic vitamin D_3-fortification strategy in real-life settings. The aim was to increase the vitamin D intake to 7.5 µg/day (the RI at that time) (72) in as many subjects as possible while avoiding an intake above 25 µg/day for children and 50 µg/day for adults (the UL at that time) (88) and still allowing a daily use of multivitamin supplements with 10 µg vitamin D. The vitamin D_3-concentrations in fortified bread were 5.2 ± 0.3 µg vitamin D_3/100 g in wheat bread, and 4.3 ± 0.3 µg vitamin D_3/100 g in rye bread, 0.40 ± 0.01 µg vitamin D_3/100 mL in fortified milk, and <0.004 µg vitamin D_3/100 mL in un-fortified milk.

4.1.2 Biochemical analyses in both VitmaD and VitDgen studies

The primary endpoints were serum 25(OH)D concentrations, and genotyping of 25 SNPs in seven vitamin D modulating genes; *CYP2R1*, *CYP24A1*, *CYP27B1*, C10orf88, *DHCR7/NADSYN1*, *GC* and *VDR*. Blood samples were obtained without prior fasting and serum and buffy coat was stored in aliquots at -80°C until analysis.

4.1.3 Serum 25(OH)D concentrations

Measurements of serum 25(OH)D concentrations relied on the determination of both $25(OH)D_2$ and $25(OH)D_3$ and were conducted by isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) at Clinical Biochemical Department, Holbæk Hospital, Denmark. Standard reference material, vitamin D in humans (SRM972), from the National Institute of Standards and Technology (NIST, USA) was used as primary calibrator.

The analytic quality of 25(OH)D assay was assured by Vitamin D External Quality Assessment Scheme (DEQAS, http://www.deqas.org/) certification and the mean bias was -3.2% in the VitmaD study and 5.7% in the VitDgen study.

4.1.4 Genotyping

DNA was extracted from peripheral blood leukocytes and stored in TE-buffer at -80°C until analysis. All SNPs were genotyped using a Sequenom® platform (San Diego, California) and the iPLEX Gold reaction at the Department of Biomedicine, Aarhus University.

4.1.5. SNP selection

In 2012 I made a mini review of the literature on the association between common SNPs and 25(OH)D concentrations. SNPs were selected based on previously evidence of significant association with 25(OH)D concentrations or GWAS validated SNPs. Only SNPs that were known not to be in high LD with each other were selected, resulting in 25 SNPs in 7 prominent vitamin D modulating genes. These were assessed for associations to 25(OH)D concentrations. **Table 1** provides a description of each of the 25 SNPs.

Table 1. Description of SNPs examined and their previously reported association to 25(OH)D concentrations.

Gene	Reference SNP	Location on gene	Reported significant associated with 25(OH)D concentrations in 2012
CYP2R1	rs7116978	Intronic	(17)
CYP2R1	rs10741657	5' near gene/promotor	(17,106,108)
CYP2R1	rs1562902	5' near gene/promotor	(106)
CYP2R1	rs10766197	5' near gene/promotor	(106,154)
CYP24A1	rs6013897	Intronic	None
CYP24A1	rs4809960	Intronic	None
CYP24A1	rs2296241	Exon 4	(154)
CYP24A1	rs17219315	Intronic	(154)
CYP24A1	rs2426496	5' near gene/promotor	(154)
CYP27B1	rs10877012	5' near gene/promotor	(91–93)
C10orf88	rs6599638	Intronic	(16)
DHCR7/NADSYN1	rs1790349	Intronic	(16,129)
DHCR7/NADSYN1	rs12785878	Intronic	(17)
GC	rs16846876	3' Flanking	(155)
GC	rs12512631	3' UTR	(146,155)
GC	rs17467825	3' Flanking	(17,155)
GC	rs22882679	Intronic	(16,17,92,129,146)
GC	rs842999-triallelic	Intronic	(155)
GC	rs4588	Exon 11 (non-syn)	(106,123–129,137,156)
GC	rs222020	Intronic	(106)
GC	rs2298849	Intronic	(92,106)
VDR	rs731236 (TaqI)	Exon 9	None
VDR	rs757343 (TruI)	Intronic	None
VDR	rs10783219	Intronic	(126)
VDR	rs7139166	5' near gene	(94)

Linkage disequilibrium (LD) between polymorphisms was evaluated using Pearsons' r, SNAP version 2.2 (http://www.broadinstitute.org/mpg/snap/ldsearchpw.php) and Haploview software version 4.2. Deviation from Hardy–Weinberg equilibrium (HWE) was tested using Chi-square test with Bonferroni's correction (P-value 0.05/25 SNPs = 0.002). No statistically significant deviation from HWE was observed in the adult population in the VitmaD study or in the VitDgen study.

Genotyping was successful for 762 participants (99.0%) in the VitmaD study and for 102 participants (100%) in the VitDgen study. To confirm the accuracy of genotyping 10%-duplicate samples were included yielded 100% reproducibility in both studies.

In the VitmaD study, out of the 762 participants that were successfully genotyped, baseline 25(OH)D concentrations were measured in 758 participants. At the end of the study a total of 756 participants (control group n = 384 and fortification group n = 384) had complete questionnaire data, genotypes and 25(OH)D concentrations measured.

4.2 The VitDgen study

The VitDgen study was an open and controlled clinical trial conducted at Department of Dermatology, Bispebjerg University Hospital, Denmark, 56°N, including apparently healthy ethnically Danish adults (18-60 y, men and women) who over a 10-days period received 4 times artificial UVB irradiation with a total dose of 6 or 7.5 SEDs during late-winter/early-spring (January to March 2013) to stimulate cutaneous vitamin D_3-synthesis. One hundred and two participants were included in the study and a total of 92 participants had complete genotypes and measurements of baseline and end 25(OH)D concentrations.

The study was conducted according to the guideline in the Declaration of Helsinki and the protocol was approved by the Danish ethics committee (*H-4-2012-071*) and registered in ClinicalTrials.gov (*NCT01741233*). All the participants gave written informed consent.

4.2.1 Artificial UVB irradiation:

Artificial UVB irradiation were use to mimic natural cutaneous vitamin D synthesis. During a 10-day period the participants received artificial UVB irradiation 4 times with 2 or 3 days' interval (Mon, Wed, Fri, Mon). The participants' body surfaces were equally exposed in a UV-cabin (Waldmann UV1000L, Villingen-Schwenningen, Germany) equipped with a broadband UVB source consisting of 26 UV6 tubes (Waldmann GmbH, Villingen-Schwenningen, Germany) emitting UVB radiation mainly between 290-350 nm. During the treatment period the UV-intensity was weekly controlled using a Sola-Hazard spectroradiometer (Solatell, Cornwall, UK).

A total of 23 participants received a total dose of 7.5 SED (1 x 3 SED upper body and 3 x 1.5 SEDs whole body). After the first UVB irradiation, 4 participants got erythema and withdrew from the study. The SED dose was lowered to 1.5 SED and given on whole body to minimize the risk of erythema. Seventy-nine participants received a total dose of 6 SED (4 x 1.5 SEDs on whole body). 1.5 SED is equivalent to ~15 minutes of sun exposure in the middle of a clear summer day in Denmark (56°N).

4.2.2 Skin type, pigmentation and redness

Self-reported skin-type according to Fitzpatrick's classification I-VI (157) was registered at baseline. Furthermore, to follow the skin response to UVB irradiation, a skin reflectance meter (UV-optimize, Scientific, Chromo-light, Espergaerde, Denmark) was used to measure the percentage of redness (range 0-100%) and the Pigment Protection Factor (PPF, range 1.0-24.0) on

the forehead, shoulder (facultative pigmentations) and buttock (constitutive skin pigmentation) at baseline and 2 days after last UVB irradiation. The percentage of redness reflects hemoglobin levels in the skin and PPF reflects melanin levels in the skin.

4.2.3 Biochemical analyses

Measurement of 25(OH)D concentrations and genotyping were performed as described in section 4.1.3 and 4.1.4 under the VitmaD study.

In the VitDgen study, all the included 102 participants were successfully genotyped and had baseline 25(OH)D concentrations measured. At the end of the study a total of 92 participants had complete questionnaire data, genotypes and 25(OH)D concentrations measured.

5. The influence of vitamin D modulating genes on vitamin D status in late summer -Main results and discussion of paper I

Paper I describes the genetic baseline data of the VitmaD study. The main objective was to assess 25(OH)D concentrations in late summer in relation to 25 common genetic variations in CYP2R1, CYP24A1, CYP27B1, C10orf88, DHCR7/NADSYN1, GC and *VDR genes.*

In late summer, common variants located in the *CYP2R1* and *GC* genes, but not variants located in the *CYP24A1, CYP27B1*, *C10orf88*, *DHCR7/NADSYN1*, *GC* and *VDR* genes, were statistically significantly associated with 25(OH)D concentrations in both children, adults and all combined (**Table 2**). The findings that *CYP2R1* and *GC* genes were associated with vitamin D status is in agreement with the findings of two GWAS studies of Caucasian cohorts (16,17) where variants in *CYP2R1* and *GC* genes were the two "top hits".

5.1. The importance of genetic variation in the *CYP2R1* gene and its effect on vitamin D status

All 4 analysed *CYP2R1* variants; rs7116978, rs10741657, rs1562902 and rs10766197, were significantly associated with 25(OH)D concentrations. SNPs rs10741657-rs7116978, and rs10766197-rs1562902 were in strong LD and the association appeared to be driven by rs10741657 and rs10766197 and formed 4 haplotype combinations. The findings, that rs10741657 and rs10766197 in the *CYP2R1* gene are association with 25(OH)D concentrations, are consistent with prior evidence from candidate gene studies (17,96,106,117,154,158,159) and validated in two GWAS (16,17). Ahn et al. (16) and Engelman et al. (107) found that rs2060793, which is in complete LD with rs10741657, also was associated with 25(OH)D concentrations.

Genetic variants located in the *CYP2R1* gene may effect 25(OH)D synthesis and thus the blood concentration, because the *CYP2R1* gene encodes the key liver enzyme 25-hydroxylase that converts vitamin D to 25(OH)D (20). Both rs10741657 and rs10766197 are located in the promoter region and may therefore affect the 25-hydroxylase blood concentrations.

Table 2. Basic characteristics of the individual SNP and the association with serum 25(OH)D concentrations in children, adults and all combined.

				Children (n = 344)				Adults (n = 414)			All (n = 758)		
SNP	MAF	HWE	M/m	Gt	n	25(OH)D	p_{adj}	n	25(OH)D	p_{adj}	n	25(OH)D	p_{adj}
CYP2R1													
rs7116978	38.8	0.25	C/T	CC	124	67.6 (65.0-70.2)	**<0.0001**	156	67.5 (64.2-71.0)	**0.0093**	280	67.5 (65.3-69.8)	**<0.0001**
				CT	158	73.9 (71.4-76.6)		180	72.8 (69.5-76.3)		338	73.3 (71.2-75.6)	
				TT	54	79.1 (74.5-83.9)		66	77.5 (71.8-83.8)		120	78.2 (74.4-82.3)	
rs10741657	40.8	0.31	G/A	GG	118	67.9 (65.2-70.7)	**<0.0001**	150	66.6 (63.3-70.1)	**0.0067**	268	67.2 (65.0-69.5)	**<0.0001**
				GA	175	73.9 (71.5-76.4)		190	74.0 (70.7-77.4)		365	73.9 (71.8-76.1)	
				AA	51	78.8 (74.1-83.7)		74	75.2 (69.9-80.9)		125	76.6 (73.0-80.5)	
rs1562902	45.2	0.37	T/C	TT	103	68.9 (65.9-71.9)	**0.0086**	129	67.5 (63.9-71.4)	**0.0353**	232	68.1 (65.7-70.6)	**0.0005**
				TC	172	73.7 (71.2-76.2)		196	73.3 (70.0-76.6)		368	73.5 (71.4-75.6)	
				CC	69	75.0 (71.0-79.1)		89	73.4 (68.6-78.5)		158	74.1 (70.9-77.4)	
rs10766197	46.9	0.15	G/A	GG	97	76.0 (72.7-79.5)	**0.0006**	124	73.0 (69.0-77.3)	**0.0081**	221	74.3 (71.6-77.1)	**<0.0001**
				AG	168	72.7 (70.2-75.2)		191	73.2 (69.9-76.6)		359	72.9 (70.8-75.1)	
				AA	79	67.9 (64.6-71.4)		98	66.2 (62.1-70.5)		177	66.9 (64.2-69.8)	
CYP24A1													
rs6013897	20.3	0.77	T/A	TT	219	73.5 (71.3-75.8)	0.5044	264	71.8 (69.1-74.7)	0.7058	483	72.6 (70.8-74.4)	0.5228
				AT	114	70.7 (67.8-73.8)		132	70.9 (67.1-74.9)		246	70.8 (68.4-73.4)	
				AA	11	69.5 (60.7-79.5)		18	70.0 (60.3-81.3)		29	69.8 (63.0-77.4)	
rs4809960	22.7	0.35	T/C	TT	198	72.0 (69.7-74.3)	0.5674	244	72.2 (69.3-75.1)	0.2786	442	72.1 (70.2-74.0)	0.0663
				TC	121	72.9 (70.0-76.0)		152	69.7 (66.2-73.3)		273	71.1 (68.7-73.5)	
				CC	25	73.8 (67.5-80.7)		18	77.2 (66.5-89.6)		43	75.2 (69.1-81.9)	
rs2296241	49.0	0.37	G/A	GG	90	68.9 (65.8-72.2)	0.1111	103	70.3 (66.0-74.8)	0.6078	193	69.6 (66.9-72.5)	0.0501
				AG	164	72.9 (70.4-75.4)		216	71.1 (68.1-74.3)		380	71.9 (69.9-74.0)	
				AA	90	75.4 (71.9-79.0)		95	73.5 (68.8-78.4)		185	74.4 (71.4-77.5)	
rs17219315	3.1	0.75	A/G	AA	342	72.3 (70.6-74.1)	0.1836	401	71.4 (69.1-73.7)	0.3828	743	71.8 (70.3-73.3)	0.2381
				AG	2	95.4 (69.5-130.9)		13	74.3 (62.3-88.6)		15	76.8 (66.5-88.7)	
rs2426496	27.7	0.51	G/T	GG	176	71.3 (68.9-73.8)	0.2500	214	70.5 (67.5-73.6)	0.7896	390	70.8 (68.9-72.9)	0.2500
				GT	135	73.2 (70.4-76.0)		171	72.3 (68.9-75.9)		306	72.7 (70.4-75.0)	
				TT	33	75.8 (70.1-81.9)		29	73.9 (65.7-83.1)		62	74.9 (69.8-80.4)	
CYP27B1													
rs10877012	33.5	0.02	G/T	GG	156	72.8 (70.2-75.4)	0.5758	193	71.0 (67.9-74.4)	0.9451	349	71.8 (69.7-74.0)	0.9918
				GT	142	73.4 (70.7-76.2)		163	72.4 (68.9-76.0)		305	72.9 (70.6-75.2)	
				TT	46	68.4 (64.1-73.1)		57	69.9 (64.3-76.0)		103	69.2 (65.5-73.2)	
C10oft88													
rs6599638	47.8	0.20	G/A	GG	98	72.5 (69.3-75.8)	0.3197	106	72.0 (67.7-76.6)	0.8797	204	73.3 (69.5-75.1)	0.8821
				GA	171	73.5 (71.0-76.0)		219	70.8 (67.8-73.9)		390	71.9 (69.9-74.0)	
				AA	75	70.2 (66.6-73.9)		88	72.2 (67.4-77.2)		163	71.2 (68.2-74.4)	
DHCR7/NADSYN1													
rs1790349	15.1	0.55	A/G	AA	232	71.6 (69.6-73.7)	0.0923	300	70.9 (68.4-73.6)	0.3478	532	71.2 (69.5-73.0)	0.8787
				GA	105	73.2 (70.1-76.4)		103	73.9 (69.5-78.7)		208	73.6 (70.8-76.5)	
				GG	7	91.5 (77.4-108.3)		11	63.2 (52.2-76.5)		18	73.0 (64.0-83.2)	
rs12785878	27.5	0.84	T/G	TT	171	72.8 (70.4-75.4)	0.7649	218	73.0 (69.9-76.2)	0.2169	389	72.9 (70.9-75.0)	0.0998
				GT	147	72.1 (69.5-74.9)		163	69.6 (66.2-73.1)		310	70.8 (68.6-73.1)	
				GG	26	71.7 (65.7-78.4)		32	69.9 (62.5-78.2)		58	70.7 (65.7-76.1)	
GC													
rs16846876	33.2	0.88	A/T	AA	158	76.5 (73.9-79.2)	**0.0004**	184	74.1 (70.7-77.6)	**0.0024**	342	75.2 (73.0-77.4)	**<0.0001**
				AT	153	70.3 (67.8-72.8)		185	70.9 (67.7-74.3)		338	70.6 (68.5-72.8)	
				TT	33	64.5 (59.8-69.6)		45	63.6 (57.9-69.8)		78	64.0 (60.1-68.1)	
rs12512631	36.2	0.62	T/C	TT	137	68.6 (66.1-71.2)	**0.0012**	166	66.8 (63.6-70.1)	**0.0004**	303	67.6 (65.5-69.8)	**<0.0001**
				TC	157	74.4 (71.8-77.1)		196	74.6 (71.3-78.0)		353	74.5 (72.4-76.7)	
				CC	50	77.5 (72.8-82.5)		52	75.3 (69.0-82.1)		102	76.4 (72.3-80.6)	
rs17467825	27.6	0.53	A/G	AA	181	76.3 (73.9-78.8)	**<0.0001**	219	73.8 (70.7-77.0)	**0.0015**	400	74.9 (72.9-77.0)	**<0.0001**
				GA	142	70.1 (67.6-72.7)		160	70.0 (66.6-73.6)		302	70.1 (67.9-72.3)	
				GG	21	57.7 (52.5-63.3)		34	63.6 (57.1-70.8)		55	61.2 (56.9-65.9)	

rs2282679	27.4	0.41	A/C	AA	181	76.3 (73.9-78.8)	**<0.0001**	219	73.8 (70.7-77.0)	**0.0020**	400	74.9 (72.9-77.0)	**<0.0001**
				CA	138	70.0 (76.4-72.6)		156	70.1 (66.6-73.7)		294	70.0 (67.8-72.3)	
				CC	21	57.7 (52.5-63.3)		34	63.6 (57.1-70.8)		55	61.2 (56.9-65.9)	
rs842999	4.5	0.65	G/C/A	GG	105	76.7 (73.5-80.0)	**<0.0001**	112	74.2 (70.0-78.7)	**0.0046**	217	75.4 (72.7-78.3)	**<0.0001**
				GC	153	72.6 (70.1-75.2)		188	73.7 (70.4-77.1)		341	73.2 (71.1-75.4)	
				CC	57	63.7 (60.2-67.5)		75	66.6 (61.9-71.5)		132	65.3 (62.3-68.5)	
				GA	19	74.3 (67.3-82.1)		23	64.9 (57.0-73.9)		42	69.0 (63.4-75.1)	
				CA	7	76.3 (64.6-89.6)		12	55.8 (46.6-66.9)		19	62.6 (55.2-71.0)	
				AA	0	-		1	75.5 (40.5-140.9)		1	75.5 (43.6-	
rs4588	27.7	0.57	C/A	CC	181	76.3 (73.9-78.8)	**<0.0001**	219	74.1 (71.0-77.3)	**0.0008**	400	75.1 (73.1-77.2)	**<0.0001**
				CA	142	70.1 (67.6-72.7)		161	69.7 (66.3-73.2)		303	69.9 (67.7-72.1)	
				AA	21	57.7 (52.5-63.3)		34	63.6 (57.1-70.8)		55	61.2 (56.9-65.9)	
rs222020	15.6	0.13	T/C	TT	250	70.5 (68.6-72.5)	**0.0021**	291	70.5 (67.9-73.1)	0.5338	541	70.5 (68.8-72.2)	0.0739
				TC	88	78.4 (74.8-82.1)		117	73.2 (69.1-77.6)		205	75.4 (72.5-78.4)	
				CC	6	69.7 (58.3-83.5)		6	86.4 (66.7-111.8)		12	77.6 (66.1-91.1)	
rs2298849	20.2	0.57	T/C	TT	229	71.1 (69.1-73.2)	0.2204	262	70.3 (67.6-73.1)	0.4591	491	70.7 (69.0-72.5)	0.2605
				CT	99	75.4 (72.1-78.8)		137	73.4 (69.5-77.5)		236	74.2 (71.6-77.0)	
				CC	15	71.1 (63.4-79.7)		15	73.3 (62.3-86.3)		30	72.2 (65.2-79.9)	
VDR													
rs731236	40.3	0.18	T/C	TT	113	70.0 (67.1-73.0)	0.0753	154	68.9 (65.4-72.5)	0.1306	267	69.3 (67.0-71.7)	**0.0346**
				TC	181	74.2 (71.8-76.7)		186	72.3 (69.0-75.7)		367	73.2 (71.1-75.4)	
				CC	49	72.0 (67.5-76.7)		74	74.9 (69.6-80.6)		123	73.7 (70.1-77.5)	
rs757343	11.5	0.45	G/A	GG	261	73.9 (71.9-76.0)	**0.0103**	326	72.2 (69.7-74.7)	0.0896	587	72.9 (71.3-74.6)	**0.0025**
				AG	77	68.4 (65.1-72.0)		81	69.6 (64.9-74.7)		158	69.1 (66.1-72.2)	
				AA	6	63.7 (53.1-76.3)		7	59.9 (47.1-76.0)		13	61.6 (52.8-71.9)	
rs10783219	36.4	0.10	A/T	AA	147	72.5 (69.8-75.2)	0.7067	160	70.1 (66.7-73.7)	0.3913	307	71.2 (69.0-73.5)	0.2023
				TA	152	72.6 (70.0-75.2)		207	71.8 (68.7-75.0)		359	72.1 (70.0-74.3)	
				TT	45	72.1 (67.4-77.1)		47	74.6 (68.0-81.8)		92	73.4 (69.2-77.8)	
rs7139166	43.0	0.48	C/G	CC	114	72.4 (69.5-75.5)	0.4251	131	73.2 (69.2-77.3)	0.4324	245	72.8 (70.3-75.5)	0.7845
				CG	167	71.6 (69.2-74.1)		210	71.7 (68.6-74.8)		377	71.6 (69.6-73.7)	
				GG	62	74.9 (70.8-79.3)		73	67.9 (63.0-73.1)		135	71.0 (67.7-74.5)	

Bold numbers represent significant P values (<0.005).

SNP single nucleotide polymorphism (ordered by position), *MAF* minor allele frequency for the adult population in percent, *HWE* P-values for Hardy-Weinberg equilibrium in the adult population, *M/m* major and minor alleles, *Gt* genotype, *Mean*, raw serum 25(OH)D concentrations were log-transformed to approximate a normal distribution an given as geometric mean (nmol/L), *95% CI* 95%-confident interval.

P_{adj}. Linear mixed models with family as a random factor, adjusted for age, sex, BMI, ski and sun holidays, use of solarium, dietary vitamin D intake, use of multivitamin and vitamin D supplementation.

5.2 The importance of genetic variation in the *GC* gene and its effect on vitamin D status

In late summer SNPs rs16846876, rs12512631, rs17467825, rs2282679, rs842999 and rs4588 in the *GC* gene were statistically significantly associated with 25(OH)D concentrations (**Table 2**). A dose-dependent relationship between carrier of none, one or two copies of the G-allele of the tri-allelic rs842999 and 25(OH)D concentrations was observed.

SNPs rs4588 was in strong LD with rs2282679, rs17467825 and rs16846876. Moreover, rs17467825-rs2282679, and rs2282679-rs16846876 were in strong LD with each other. The association appeared to be driven by rs4588 and not by rs2282679 as found in the two GWAS (16,17). Wang et al. (17) did not include rs4588 in the GWAS because it was not included in the HapMap dataset. In agreement with our findings, several studies have found that rs4588 is in strong LD with rs2282679, and that rs4588 was the strongest independent predictor of 25(OH)D concentrations compared to rs2282679 (129), (160), (96). Zhang et al. (96) argued that it is unlikely that rs2282679 in itself is the disease-causing variant and that the possible causal variant is the non-synonymous rs4588.

The three significant *GC*-variants rs4588, rs842999, and rs12512631 formed 5 haplotype combinations. Based on haplotype analyses rs12512631 was excluded from further analyses, since the variant allele of rs12512631 was associated with high 25(OH)D concentrations and the variant alleles of rs4588 and rs842999 were associated with low 25(OH)D concentrations. Furthermore, haplotype analyses also indicated that rs4588 is the biologically relevant polymorphism rather than rs842999.

The *GC* gene encodes the DBP that binds and transport vitamin D and its metabolites in the blood. Genetic variants located in the *GC* gene may effect the DBP binding and bioavailability of 25(OH)D, and thus there may be a relationship between DBP-phenotype and blood concentrations of 25(OH)D. The non-synonymous rs4588, located in exon 11, leads to a Thr/Lys amino acid substitution at codon 420 and may give rise to a conformation change in the DBP affecting the blood concentration and the catalytic effect. The biological effect of the intronic tri-allelic rs842999 is unknown, but if functional it could interfere with binding of a regulatory protein thereby affecting transcription or degradation of the mRNA.

5.3 Genetic risk score analysis of *CYP2R1* and *GC* haplotypes

In order to elucidate the effect of *GC*-haplotype or *CYP2R1*-haplotype combinations in relation to low vitamin D status, a genetic risk score (GRS, range 0 to 4) was calculated as the sum of risk alleles of G-alleles of rs10741657 and A-alleles of rs10766197 for *GC* **(Figure 2A)** and for *CYP2R1* as the sum of risk alleles of A-alleles of rs4588 and C/A-alleles of rs842999 (**Figure 2B**). Furthermore, in order to elucidate the combined effect of *GC*- and *CYP2R1*-haplotype combinations in relation to low vitamin D status, a combined GRS (range 0 to 8) was calculated as the sum of the number of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999 (**Figure 2C**). A generally negative correlation was observed between the number of risk alleles and 25(OH)D concentrations in all 3 GRS analysis. Non-carriers of risk alleles of *CYP2R1*, *GC* or in the combined analysis of *CYP2R1* and *GC* had significantly higher 25(OH)D concentrations compared to carriers of all 4 or 8 (for the joint analysis) risk alleles. The largest %-range in mean 25(OH)D concentrations between non-carriers and carriers of all risk alleles was found for the combined analysis (80.6, 56.1 and 67.9%) compared to the GRS of *CYP2R1* (20.9, 14.1 and 16.5 %) or *GC* (35.4, 20.0 and 23.4%) analysis in children, adults and all combined, indicating a additive effect of the combined analysis of *CYP2R1* and *GC* on 25(OH)D concentrations. Important for public health, children carrying 7 or 8 risk alleles had insufficient vitamin D status (<50 nmol/L) in late summer.

In agreement with our findings, Zang et al. (96) found that both the minor A-allele (denoted T-allele in the paper) of rs4588 and the G-allele of rs2282679 were associated with reduced DBP concentrations. Participants with 3 or 4 risk alleles of the two variants were more likely to have vitamin D concentrations lower than 50 nmol/L compared with non-carriers of the risk alleles and a 0.12-fold drop in the log-25(OH)D concentrations was showed for each additional risk allele. In a study by Engelman et al. (107) women with no risk alleles of rs4588 and rs2060793 (in strong LD with rs10741657) who consumed at least 16.75 µg/d vitamin D all had 25(OH)D >50 nmol/L. For women carrying 1, 2 or 3-4 risk alleles and consuming at least 16.75 µg/d vitamin D, only 84, 72, and 62% had 25(OH)D >50 nmol/L. These results indicate that there is an additive effect of the polymorphisms in *CYP2R1* and *GC* on 25(OH)D concentrations and the more risk alleles an individual carries in the *CYP2R1* and *GC* genes, the more prone the individual will be for having a low vitamin D status.

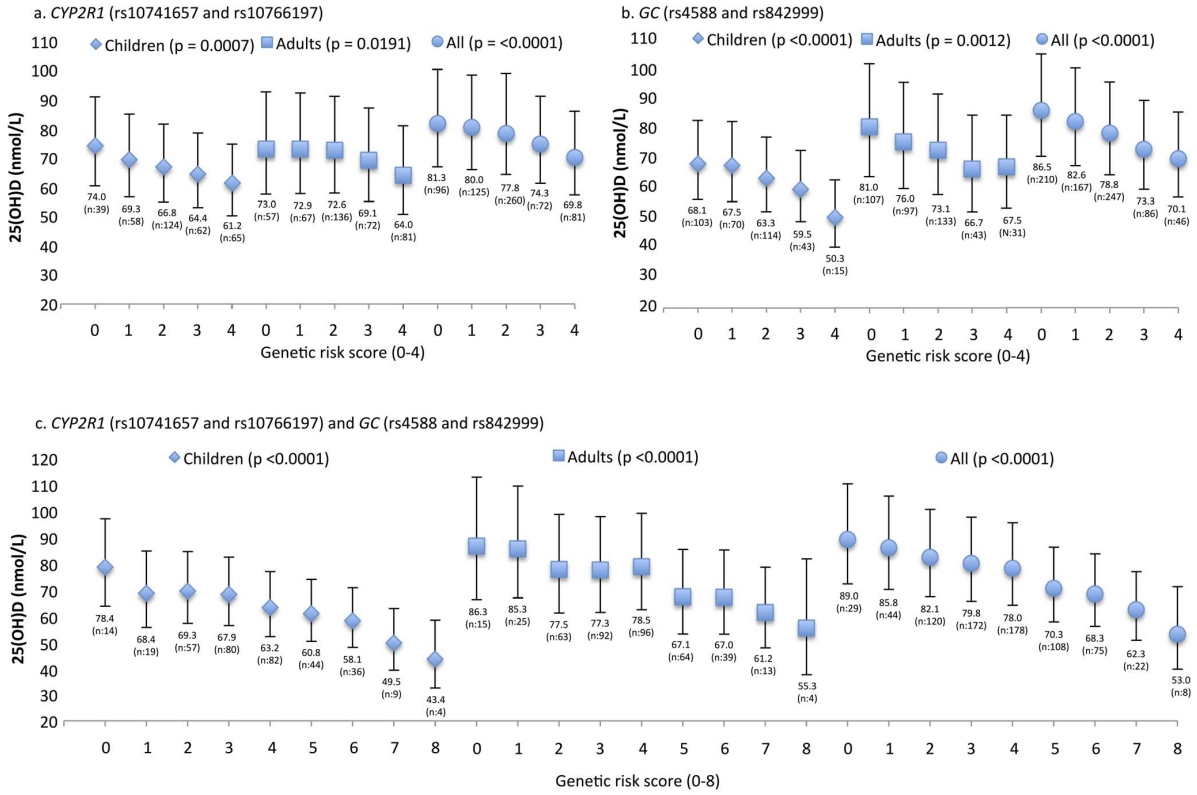

Figure 2. Genetic risk score for *CYP2R1* (rs10741657 and rs10766197) (**A**), *GC* (r4588 and rs842999) (**B**) and *CYP2R1* (rs10741657 and rs10766197) and *GC* (r4588 and rs842999) (**C**) in children, adults and all combined. X-axis stands for the sum of risk alleles. Y-axis stand for 25(OH)D (nmol/L). Errors bars stand for 95%-confidence interval and 25(OH)D concentrations are given as geometric means. Linear mixed models with family as a random factor, adjusted for age, sex, BMI, ski and sun holidays, solarium use at least once a week, dietary vitamin D intake, multivitamin and vitamin D supplement users was conducted to compare sum of risk alleles and 25(OH)D concentrations. Increasing number of risk alleles give rise to decreasing 25(OH)D concentrations.

6. The influence of vitamin D modulating genes on vitamin D status after 6 months intake of vitamin D_3-fortified bread and milk –main results and discussion of paper II

Paper II describes the VitmaD intervention study. The main objective was to assess the effect of 25 common genetic variations in CYP2R1, CYP24A1, CYP27B1, C10orf88, DHCR7/NADSYN1, GC and *VDR genes in relation to vitamin D status after real-life use of vitamin D_3-fortified bread and milk on 25(OH)D concentrations during a 6-months winter period.*

At the end of the study, there was a pronounced positive effect of real-life usage of vitamin D_3-fortified bread and milk on 25(OH)D concentrations. For the fortification group, 25(OH)D concentrations were significantly associated with rs4588 and rs842999 in *GC,* and rs10741657 in *CYP2R1*, but borderline significantly associated with rs10766197 in *CYP2R1*, resembling the results found in late summer. This indicates that when vitamin D is received primarily as vitamin D_3-fortification during winter, the association between 25(OH)D concentrations and genetic variation in *CYP2R1* and *GC* found in late summer, is maintained. On the contrary, the associations between 25(OH)D concentrations and genetic variation in *CYP2R1* and *GC* found in late summer disappeared during winter for the control group. These findings, that a genetic season effect exists, and that the genetic effect of *GC* and *CYP2R1* on 25(OH)D concentrations disappears during winter are consistent with the findings from two previous studies (107,128). A plausible explanation may be that when vitamin D synthesis is present (during summer or after vitamin D-fortification) *CYP2R1* and *GC* genes are determinants of the vitamin D status. This indicates that the *CYP2R1* and *GC* genes catalyse rate-limiting processes in vitamin D synthesis and storage. During winter months, when cutaneous vitamin D synthesis is negligible and summer vitamin D storage is being used, the *CYP2R1* and *GC* gene products are not rate limiting since the main processes are unrelated to synthesis and uptake. In our study, the control group had similar mean 25(OH)D concentrations at the end of the winter, indicating that when solar vitamin D has not been obtained during winter months, a minimum vitamin D plateau is reach, to maintain the physiological role of vitamin D.

6.2 Prevalence of 25(OH)D concentrations <30 nmol/L and <50 nmol/L

The American cut-off value was used and 25(OH)D <50 nmol/L defines the requirement for optimal bone health for the majority of the population and cut-off value <30 nmol/L defines the 25(OH)D concentrations at which adverse effects on bone health may be expected (33). In the present study, the lowest prevalence of vitamin D deficiency <30 and <50 nmol/L was observed in late summer for all the participants, with no difference in the prevalence of participants presenting

with 25(OH)D concentrations <30 nmol/L when stratified by rs10741657 (p = 0.2269) and rs10766197 (p = 0.1715) in *CYP2R1,* and rs4588 (p = 0.6953) and rs842999 (p = 0.5111) in *GC*. In contrast, there was statistically significant difference in the prevalence of participants presenting with 25(OH)D concentrations <50 nmol/L in late summer when stratified by rs10741657 (p = 0.0004) and rs10766197 (p = 0.0743) in *CYP2R1,* and rs4588 (p <0.0001) and rs842999 (p = 0.0435) in *GC*. The significant differences in prevalence <50 nmol/L disappeared during the winter for the control group, and only rs4588 (p = 0.0002) and rs842999 (p = 0.0029) in *GC* remained significant associated in the fortification group.

As anticipated, participants in the control group had a higher prevalence of vitamin D status <30 and <50 nmol/L compared to the fortification group at the end of the winter. For the fortification group the highest prevalence of 25(OH)D <50 nmol/L was observed for the rs4588-AA genotype. In contrast, rs4588-AA carriers in the control group had the lowest prevalence of 25(OH)D <50 nmol/L. This indicates that although carriers of the rs4588-AA genotype in the fortification group were more prone to be vitamin D deficient, rs4588-AA carriers in the control group were less prone to be vitamin D deficient. This may indicate that rs4588-AA carriers have a somewhat low but very stable 25(OH)D concentrations.

6.3 PTH levels

In late summer, there was no difference in PTH levels when stratified by rs10741657 and rs10766197 in *CYP2R1*and rs4588 and rs842999 in *GC* for all the participants (p = 0.2473). At the end of the study, as anticipated, PTH levels were significantly higher in the control group compared to the fortification group (p = 0.0199), because elevated levels of PTH are considered as a sensitive marker of vitamin D deficiency. A significant difference in PTH levels was observed for rs4588 in both the fortification group (p = 0.0064) and control group (p = 0.0132) and moreover a recessive effect for rs4588-AA carriers on PTH levels was observed in both groups. Participants carrying the rs4588-AA genotype have the lowest PTH levels and 25(OH)D concentrations compared to rs4588-CC or -CA carriers, indicating no physiological symptoms of vitamin D deficiency. Similar to our findings, Pekkinen et al. 2014 (119) found a dose-response effect of rs4588 on PTH concentrations with rs4588-AA carriers having the lowest PTH and 25(OH)D concentrations. Further studies are needed to investigate the underlying biological mechanism of this observation.

6.4 Genetic risk score analysis of *CYP2R1* and *GC*

As in paper I, the combined contributions of rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* on 25(OH)D concentrations were analysed with a combined GRS (range 0-8) calculated as the sum of risk alleles of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999 individually for the control and fortification group, stratified by all, adults and children (**Figure 3A, B and C)**. As anticipated, no difference in 25(OH)D concentrations and GRS was observed for the control group. For the fortification group, there was a negative linear correlation between 25(OH)D concentrations and of the number of risk alleles ranging from 0 to 7-8 risk alleles as observed in late summer. Overall, there was a mean difference in 25(OH)D concentrations of 28.2, 28.6 and 31.9 nmol/L between non-carriers and carriers of all 7-8 risk alleles in all, adults, and children, respectively. Overall, the same GRS pattern was observed for adults and children.

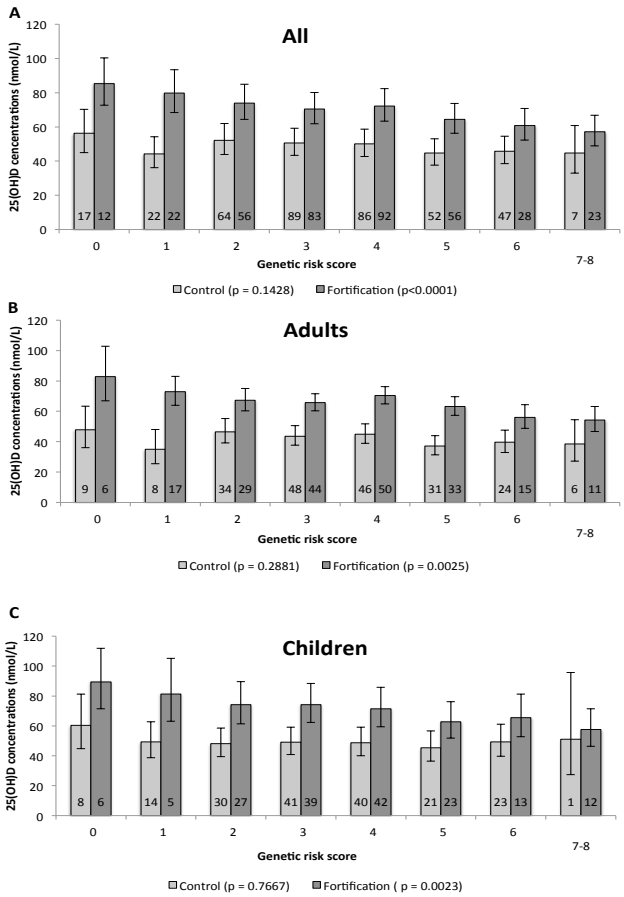

Figure 3. Estimated mean 25(OH)D concentrations at the end of the study for each genetic risk score category stratified by control and fortification group, separately for all (**A**), adults (**B**) and children (**C**).
Genetic risk score (range 0 to 7-8) was calculated as the sum of number of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999. Column numbers indicate total numbers of participants carrying the risk score. Error bars indicate 95% confidence interval.

6.5 Genetic risk score of *CYP2R1* and *GC* stratified by total vitamin D intakes

The effect of total vitamin D intake was estimated for each category GRS (range 0 to 8) of rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* for the control and fortification group (**Figure 4)**. Total vitamin D intake was estimated as the sum of dietary vitamin D intake, use of multivitamin and vitamin D supplements and furthermore for the fortification group, self-reported intake of vitamin D_3-fortified bread and milk. A total of 25.1, 22.4, 23.4, 15.6 and 13.6% of the adult participants carried 0-2, 3, 4, 5 or 6-8 risk alleles, respectively.

A statistically significant positive linear relationship between total vitamin D intake and 25(OH)D concentrations was observed among carriers of 0-2, 3, 4 or 5 risk alleles, ($p = 0.0012$, 0.0001, 0.0118 and 0.0029, respectively), but not for individuals carrying 6-8 risk alleles ($p = 0.1051$). In general, the more risk alleles an individual carries the more vitamin D supplementation is required to obtain a sufficient vitamin D status (>50 nmol/L). A total vitamin D intake of <3 µg/day was not sufficient for 95% of the study population to achieve sufficient 25(OH)D concentrations, regardless of risk alleles. For participants carrying 0-2 or 3 risk alleles, 3 to 7.4 µg/day of vitamin D seemed to be sufficient for 95% of the study population to achieve sufficient 25(OH)D concentrations. For participants carrying 4 risk alleles, a total vitamin D intake of >7.5 µg/day seemed to be sufficient for 95% of the study population to achieve sufficient 25(OH)D concentrations. For participants carrying 5 risk alleles, a total daily vitamin D intake >10 µg seemed to be sufficient for 95% of the study population to achieve sufficient 25(OH)D concentrations. For participants carrying 6-8 risk alleles, a total daily vitamin D intake >15 µg was almost enough for 95% of the study population to achieve sufficient 25(OH)D concentrations, suggesting that it is difficult to increase 25(OH)D concentrations to a sufficient level in participants carrying 6-8 risk alleles with vitamin D-fortification. For participants carrying 6-8 risk alleles, a statistically non-significant increase in 25(OH)D concentrations was found comparing the lowest and highest quintile of vitamin D intake, but with a much lower rate (+Δ17.6 nmol/L) compared to participants carrying 0-2, 3, 4 or 5 risk alleles (+Δ28.8, 36.5, 24.2 and 33.6 nmol/L), respectively. The increase in 25(OH)D concentrations are similar to the findings by Engelman et al. (107) that individuals carrying 3-4 risk alleles of rs4588 in *GC* and rs2060793 (in strong LD with rs10741657) in *CYP2R1* have the lowest increase in 25(OH)D concentrations (+Δ16.7 nmol/L) compared to individuals with fewer risk alleles (+Δ27.7 nmol/L). Furthermore, the percentage of women with sufficient 25(OH)D concentrations rose with each increasing quartile of vitamin D intake. Thus, subjects with genetic predisposition seem to benefit from dietary vitamin D supplementation, which is in agreement with our findings.

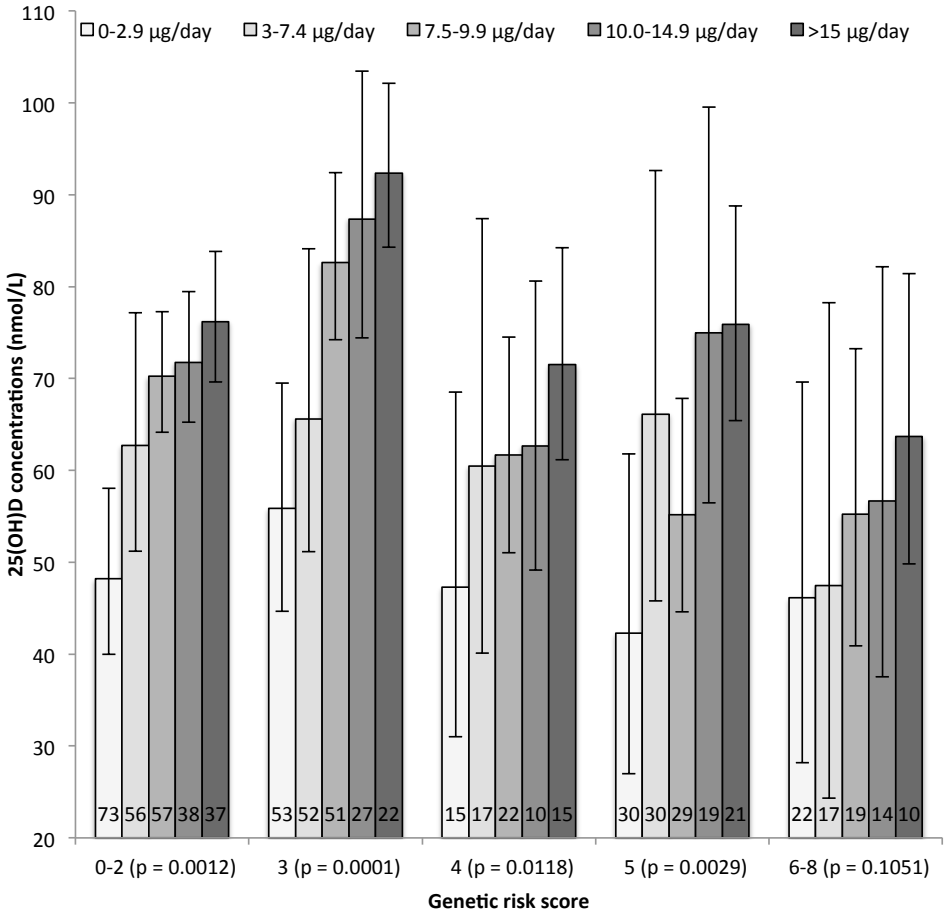

Figure 4. Mean 25(OH)D concentrations for each GRS category stratified by total vitamin D intakes .
GRS (range 0-8) calculated as the sum of number of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999. The numbers in the columns present the total numbers of participants carrying this risk score. Error bars indicate 95% confidence interval.

Low vitamin D status can be corrected by vitamin D supplementation, but individual responses to vitamin D supplementation vary, suggesting that some people might need higher doses of vitamin D to reach sufficient 25(OH)D concentration, or that there is variability in the physiologically normal concentration of 25(OH)D (109). This study provide evidence that genetic predisposition in *CYP2R1* and *GC* may have a large impact on 25(OH)D concentrations and individuals with genetically determined low 25(OH)D concentrations may need more vitamin D in order to improve their vitamin D status or there may be variability in the physiologically normal range of 25(OH)D concentrations for individuals carrying different *CYP2R1* and *GC* genotypes, demonstrating that a "one size fits all" approach may not work well for vitamin D.

Important for public health recommendations and vitamin D-fortification programs a general trend was observed. Individuals carrying a low GRS had sufficient vitamin D status and achieved an even

higher vitamin D status with increasing amount of vitamin D supplementation. Contrary, individuals carrying a high GRS often presented with a low vitamin D status and did not benefit as much from an increasing amount of vitamin D supplementation as observed for individuals carrying a low GRS. This means that individuals carrying a high GRS may have a natural lower physiologically level of 25(OH)D or have a lower uptake of vitamin D supplementation compared to individuals carrying a low GRS.

In order to raise vitamin D status to a sufficient level in 95% of individuals carrying a high GRS a vitamin D dose of >15 µg/day is needed with is above the RDA and RI. The long-term health consequences of high doses of vitamin D supplementation and the potential risk of developing vitamin D intoxication in individuals carrying a low GRS needs to be further investigated. There is evidence that a U- or J-shaped response curve exists between 25(OH)D concentrations and certain cancers and all-cause mortality (59) at 25(OH)D concentrations >125 nmol/L (33).

6.6 Genetic risk score of *CYP2R1* and *GC* stratified by total vitamin D intakes and >50 nmol/L of vitamin D status

In Europe, there is a general agreement that a 25(OH)D concentration of at least 50 nmol/L is sufficient (161). The percentage of participants with sufficient 25(OH)D (>50 nmol/L) concentrations was determined (**Figure 5**). Sufficient 25(OH)D concentrations were achieved for all participants carrying 0-2, 3 or 4 risk alleles and who consumed >15 µg/day of vitamin D. For participants carrying 5 or 6-8 risk alleles this fell to 86 and 90%, respectively. Furthermore, sufficient 25(OH)D concentrations were achieved for 87, 90, 83, 84 and 67% of the participants carrying 0-2, 3, 4, 5 or 6-8 risk alleles who consumed 10 to 14.9 µg/day. This fell to 80, 76, 86, 50 and 53% and 57, 50, 61, 52 and 41% for participants carrying 0-2, 3, 4, 5 or 6-8 risk alleles and who consumed 7.5 to 9.9 µg/day or 3.0 to 7.4 µg/day of vitamin D, respectively. In the study population, 67% of the participants carrying 6-8 risk alleles had sufficient 25(OH)D concentrations in contrast to 87, 90, 83 and 84% for participants carrying 0-2, 3, 4 or 5 risk alleles, respectively, when following IOMs RDA of 15 µg/day for individuals aged 1-70 y. Following the Nordic countries RI of 10 µg/day for individual aged 2-60 y, only 50 and 53% of the participants carrying 5 or 6-8 risk alleles, respectively, had sufficient 25(OH)D concentrations compared to 80, 76 and 86% of the participants carrying 0-2, 3 or 4 risk alleles, respectively. After stratification by total vitamin D intake and genetic predisposition in *CYP2R1* and *GC* the RDA or RI was not fulfilled by the intervention.

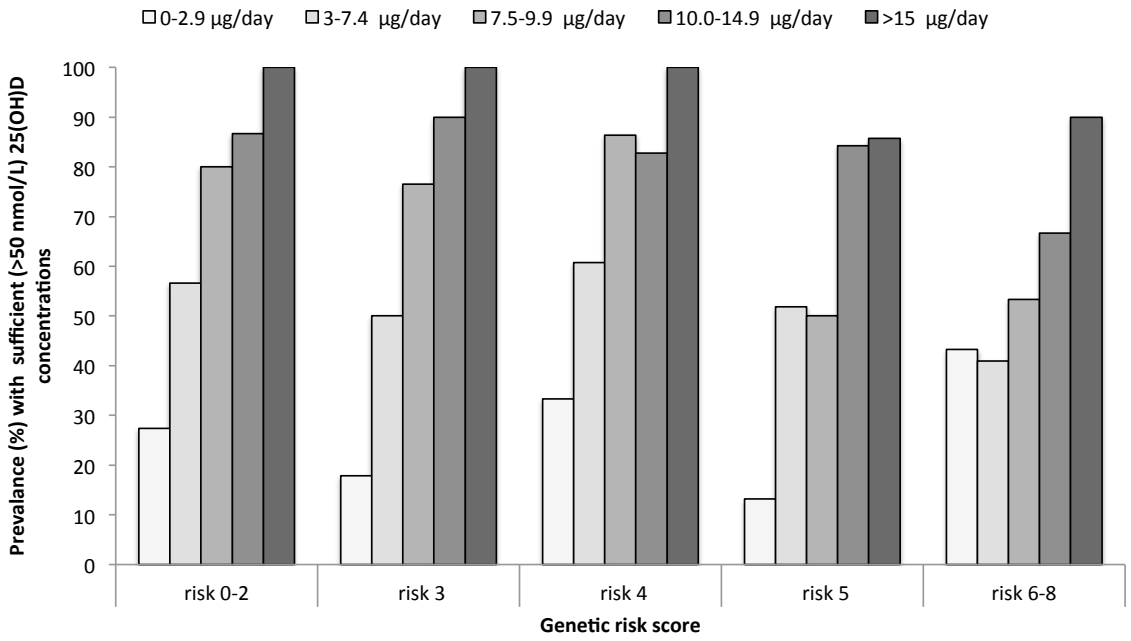

Figure 5. The %-prevalence of 25(OH)D concentrations >50 nmol/L, for each GRS category stratified by quintiles of total vitamin D intake.
GRS (range 0-8) was calculated as the sum of number of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999.

In agreement with our findings, Cranney et al. (162) concluded that vitamin D_3-doses of 10-20 µg/day may be insufficient to prevent vitamin D deficiency in at-risk-individuals. Cashman et al. (163) concluded that for a population to achieve 25(OH)D concentrations of 50 nmol/L an average intake of 9 µg/day vitamin D was needed. Nevertheless, when taking inter-individual variation into account 23.5 µg/day of vitamin D_3 was needed for 95% of the population to reach a 25(OH)D concentrations of 50 nmol/L. In the study of Engelman et al. (107), all the women with no risk alleles of rs4588 in *GC* and rs2060793 (in strong LD with rs10741657) in *CYP2R1* who consumed at least 16.75 µg/d vitamin D had 25(OH)D > 50 nmol/L. For woman who had 1, 2 or 3-4 risk alleles, who consumed at least 16.75 µg/d vitamin D, this fell to 84, 72, and 62%. Furthermore, the percentage with adequate 25(OH)D concentrations rose with increasing vitamin D intake. Furthermore, the rs4588 genotype predicts changes in 25(OH)D concentrations after long-term vitamin D supplementation. Fu et al. (127) showed that after one year supplementation with 40 µg/d or 15 µg/d, the mean percentage increase of 25(OH)D was significantly allele-specific for rs4588: 97% for CC, 151% for CA and 307% for AA genotypes. Thus, subjects with genetic predisposition seemed to benefit the least from dietary vitamin D supplementation.

These findings demonstrate that *CYP2R1* and *GC* genotypes are determinants of reduced 25(OH)D concentrations and associated with the risk of developing low vitamin D status which may have clinical importance for human health. Epidemiological studies have found association between low 25(OH)D concentrations, cancer risk and all-cause mortality, but the significance of genetically determined low 25(OH)D concentrations is not clear. Jorde et al. (164) showed that individuals carrying the DBP phenotype *GC-1f/1f* had 23-26% reduced risk of incident cancer compared to the *GC-1S/1S* and *GC-2/2* phenotypes ($p < 0.02$).

7. The influence of vitamin D modulating genes on vitamin D status after artificial UVB irradiation or after intake of vitamin D_3-fortified bread and milk –main results and discussion of paper III

Paper III focuses primarily on the VitDgen study but also data from the VitmaD study are included. The main objective was to assess the effect of 25 SNPs located in CYP2R1, CYP24A1, CYP27B1, C10orf88, DHCR7/NADSYN1, GC and *VDR genes on artificial UVB irradiation-mediated increase in 25(OH)D concentrations. Secondary, the study aimed to determine whether common genetic variations in CYP2R1 and GC have similar effects on 25(OH)D concentrations after artificial UVB irradiation and after intake of vitamin D_3-fortified bread and milk.*

After 4 whole body UVB irradiations during a 10-day period and with a total dose of 6 or 7.5 SEDs, rs10741657 in *CYP2R1* and rs4588 in *GC* predicted UVB-induced 25(OH)D concentration as previously found for the VitmaD study in late summer and after 6 months intake of vitamin D_3-fortified bread and milk. There was a gene-dose dependent relationship between GRS and the UVB-dependent increase in 25(OH)D concentrations or after intake of vitamin D_3-fortified bread and milk. Carriers of all 4 risk alleles of rs10741657 in *CYP2R1* and rs4588 in *GC* had the lowest mean 25(OH)D concentrations during winter, the smallest increase in UVB-induced 25(OH)D concentrations and after intake of vitamin D_3-fortified bread and milk during winter the largest decrease in 25(OH)D concentrations compared to non-carriers. These findings indicate that genetically predisposed individuals carrying all 4 risk alleles of rs10741657 in *CYP2R1* and rs4588 in *GC* benefit the least from UVB irradiation or intake of vitamin D_3 supplements during winter. Regardless of the method used to increase or maintain 25(OH)D concentrations during winter, the effects of UVB irradiation or intake of vitamin D_3 on 25(OH)D concentrations seem notably similar in a healthy Caucasian population. Common genetic variation in the *CYP2R1* and *GC* genes are determinants of 25(OH)D concentrations after UVB irradiation and after intake of vitamin D_3-fortified bread and milk in a Caucasian population.

7.1. The UVB-induced 25(OH)D concentrations, the VitDgen study

In the VitDgen study, 92 participants (out of 102 recruited) completed the study. In winter, 51% of the participants were vitamin D sufficient (>50 nmol/L), 43% of the participants were vitamin D insufficient (25-50 nmol/L) and 5% of the participants were vitamin D deficient (<25 nmol/L). After receiving 4 whole-body UVB irradiations with a total dose of 6 or 7 SEDs, 97% of the participants were vitamin D sufficient, 3% of the participants were vitamin D insufficient and none of the participants were vitamin D deficient. Using an artificial UVB source during winter over a short time period the increase in 25(OH)D concentrations were well controlled and an average increase of 28 nmol/L (24.1-31.1 nmol/L) was observed.

As anticipated and found for the control group in paper II, there was no statistically significant difference between 25(OH)D concentrations and the 25 analyzed SNPs, except for rs12512631 in *GC* in winter. This effect disappeared after UVB irradiation. False-positive results (type 1 errors) are common when studying associations between genetic markers and outcomes, and the relatively small sample size, resulting in statistically reduced power might explain this finding. Otherwise, our findings are in agreement with previous studies showing no genetic effects on 25(OH)D concentrations during winter months (107,128,165).

After having received UVB irradiation, there was a statistically significant association between UVB-induced 25(OH)D concentrations and rs10741657 in *CYP2R1*, and rs16846876, rs17467825, rs2282679 and rs4588 in *GC* (**Table 5**) as found in late summer (**paper I**) and after vitamin D_3 fortification (**paper II**). As in paper I and II, rs4588 was in strong LD with rs2282679 and rs17467825 and moreover rs17467825-rs2282679 and rs2282679-rs16846876 were in LD. The strongest association with 25(OH)D concentrations was observed for rs4588.

SNP rs10766197 in *CYP2R1* and rs842999 in *GC* did not predict UVB-induced 25(OH)D concentrations as found in paper I and II, and the lack of replication may be due to the small sample size. None of the analyzed SNPs in *CYP24A1, CYP27B1*, *C10orf88*, *DHCR7/NADSYN1* and *VDR* genes were statistically significantly associated with the UVB-induced 25(OH)D concentrations as found in paper I and II.

Table 5. Basic characteristics of the individual SNP and the association with 25(OH)D concentrations

SNP	MAF	HWE	M/m	Genotype	n	Winter 25(OH)D	P_{adj}[1]	UVB-induced 25(OH)D	UVB-increased 25(OH)D	P_{adj}[2]
CYP2R1										
rs7116978	39.5	0.11	C/T	CC	37	50.8 (43.8-58.9)	0.32	78.4 (72.3-85.0)	22.6 (18.3-27.8)	0.10
				CT	35	50.5 (43.4-58.7)		80.5 (74.0-87.5)	27.2 (21.8-34.1)	
				TT	18	58.1 (47.0-71.7)		93.4 (83.1-104.9)	29.8 (21.7-40.9)	
rs10741657	41.4	0.07	G/A	GG	36	50.2 (43.1-58.4)	0.28	77.0 (70.9-83.5)	21.7 (17.7-26.8)	**0.024**
				GA	36	50.2 (43.2-58.5)		81.9 (75.5-88.9)	28.6 (23.0-35.5)	
				AA	20	57.9 (47.3-71.0)		93.7 (84.0-104.6)	30.7 (22.9-41.2)	
rs1562902	43.8	0.35	T/C	TT	32	49.8 (42.4-58.4)	0.84	79.0 (72.3-86.3)	25.6 (20.4-32.1)	0.32
				TC	40	49.8 (43.1-57.4)		81.2 (75.0-87.9)	26.8 (21.7-33.2)	
				CC	20	59.9 (48.9-73.3)		90.4 (80.1-101.1)	24.6 (18.4-33.0)	
rs10766197	48.9	0.39	G/A	GG	22	56.0 (46.1-67.9)	0.40	87.3 (78.5-97.1)	25.2 (19.0-33.3)	0.13
				AG	49	52.5 (46.1-59.7)		83.7 (77.9-89.9)	26.5 (21.9-32.1)	
				AA	21	46.4 (38.0-56.5)		74.5 (66.8-83.0)	25.3 (19.1-33.7)	
CYP24A1										
rs6013897	20.5	0.83	T/A	TT	60	50.4 (44.8-56.7)	0.07	81.9 (76.7-87.5)	27.3 (23.1-32.2)	0.70
				AT	28	53.6 (45.1-63.7)		83.0 (75.4-91.5)	25.2 (19.8-32.0)	
				AA	4	61.1 (38.7-96.5)		83.1 (83.1-107.3)	12.3 (6.0-25.2)	
rs4809960	23.7	0.11	T/C	TT	58	51.8 (46.0-58.4)	0.45	82.5 (77.1-88.2)	25.8 (21.7-30.5)	0.26
				TC	31	53.1 (45.1-62.6)		81.2 (74.1-89.0)	24.4 (19.4-30.9)	
				CC	8	39.9 (23.6-67.6)		91.0 (67.8-122.2)	49.6 (24.0-102.5)	
rs2296241	46.0	0.26	G/A	GG	24	44.5 (37.1-53.5)	0.15	77.5 (70.0-80.1)	25.2 (19.5-32.7)	0.39
				AG	52	55.7 (49.1-63.0)		82.8 (77.2-88.8)	24.5 (20.3-29.4)	
				AA	16	51.5 (41.2-64.5)		88.1 (77.6-99.9)	31.8 (23.2-43.6)	
rs17219315	2.8	0.78	A/G	AA	87	51.7 (46.8-57.0)	0.53	82.0 (77.6-86.6)	25.7 (22.3-29.6)	0.29
				AG	5	54.4 (36.1-82.0)		87.5 (69.6-110.0)	29.2 (16.5-51.7)	
rs2426496	23.3	0.29	G/T	GG	54	48.9 (43.2-55.3)	0.44	78.8 (73.6-84.3)	24.3 (20.4-28.9)	0.25
				GT	35	56.7 (48.6-66.1)		86.9 (79.9-94.6)	27.8 (22.2-34.8)	
				TT	3	51.8 (30.7-87.4)		95.9 (71.9-128.1)	37.4 (18.0-77.7)	
CYP27B1										
rs10877012	35.2	0.97	G/T	GG	41	50.4 (43.7-58.1)	0.38	81.3 (75.1-88.1)	23.8 (19.4-29.2)	0.91
				GT	40	50.7 (43.9-58.6)		82.0 (75.7-88.9)	28.3 (23.0-34.7)	
				TT	11	61.9 (47.1-81.4)		87.1 (74.7-101.6)	25.8 (17.2-38.5)	
C10orf88										
rs6599638	49.4	0.29	G/A	GG	20	52.5 (42.8-64.5)	0.48	80.3 (71.7-90.0)	23.3 (17.4-31.2)	0.31
				GA	51	52.5 (46.2-59.7)		84.2 (78.4-90.4)	28.4 (23.6-34.0)	
				AA	21	49.6 (40.6-60.5)		79.7 (71.3-89.0)	23.0 (17.3-30.5)	
DHCR7/NADSYN1										
rs1790349	15.3	0.02	A/G	AA	69	50.6 (45.4-56.5)	0.35	82.2 (77.3-87.4)	26.5 (22.7-31.1)	0.70
				GA	18	56.8 (45.8-70.5)		84.7 (75.1-95.5)	23.8 (17.3-32.8)	
				GG	5	51.0 (33.9-76.7)		76.0 (60.5-95.5)	24.2 (13.7-42.9)	
rs12785878	28.4	0.32	T/G	TT	49	51.2 (44.9-58.3)	0.77	81.6 (75.9-87.8)	27.4 (22.7-33.1)	0.97
				GT	34	52.4 (44.7-61.3)		83.3 (76.3-91.0)	24.2 (19.3-30.3)	
				GG	9	53.2 (39.2-72.2)		82.2 (69.3-97.5)	24.6 (16.1-37.7)	
GC										
rs16846876	38.6	0.16	A/T	AA	32	58.8 (50.2-68.8)	0.41	92.2 (84.6-100.4)	26.6 (21.1-33.4)	**0.026**
				AT	50	50.0 (44.1-56.8)		78.8 (73.6-84.3)	25.5 (21.1-30.8)	
				TT	10	41.2 (31.3-54.6)		71.2 (61.2-83.0)	25.7 (17.2-38.6)	
rs12512631	31.6	0.07	T/C	TT	38	43.4 (37.7-49.9)	**0.025**	74.6 (69.1-80.6)	26.3 (21.2-32.5)	0.13
				TC	49	57.3 (50.6-64.8)		86.1 (80.5-92.1)	25.2 (20.9-30.4)	
				CC	5	79.8 (50.9-110.0)		111.3 (90.1-137.5)	30.1 (17.0-53.4)	
rs17467825	28.4	0.96	A/G	AA	49	53.0 (46.5-60.4)	0.50	83.9 (78.2-90.1)	24.4 (20.3-29.4)	**0.020**
				GA	36	51.3 (44.1-59.8)		83.7 (77.1-90.9)	29.1 (23.5-36.2)	
				GG	7	46.2 (32.7-65.3)		65.7 (54.5-79.3)	20.9 (12.4-35.0)	
rs2282679	28.4	0.96	A/C	AA	49	53.0 (46.5-60.4)	0.50	83.9 (78.2-90.1)	24.4 (20.3-29.4)	**0.020**
				CA	36	51.3 (44.1-59.8)		83.7 (77.1-90.9)	29.1 (23.5-36.2)	
				CC	7	46.2 (32.7-65.3)		65.7 (54.5-79.3)	20.9 (12.4-35.0)	
rs842999	44.1	0.14	G/C/A	GG	25	54.3 (45.4-65.1)	0.42	82.5 (74.4-91.4)	24.0 (18.6-30.9)	0.17
				GX[3]	50	53.1 (46.7-60.3)		84.2 (78.3-90.5)	25.8 (21.4-31.1)	
				XX[4]	13	49.7 (38.7-63.9)		75.7 (65.7-87.3)	25.9 (17.9-37.4)	
rs4588	29.0	0.84	C/A	CC	48	53.3 (46.7-60.8)	0.57	84.1 (78.3-90.4)	24.3 (20.1-29.2)	**0.020**
				CA	37	51.0 (43.9-59.3)		83.5 (77.0-90.6)	29.3 (23.6-36.2)	
				AA	7	46.2 (32.7-65.3)		65.7 (54.5-79.3)	20.9 (12.5-34.9)	
rs222020	22.2	0.84	T/C	TT	55	54.7 (48.5-61.6)	0.068	86.2 (80.7-92.0)	27.2 (22.8-32.5)	0.31
				TC	33	45.1 (38.7-52.6)		74.4 (68.4-81.0)	24.2 (19.3-30.4)	
				CC	4	77.7 (50.0-120.9)		100.6 (78.9-128.2)	22.4 (11.8-42.3)	
rs2298849	25.3	0.80	T/C	TT	51	53.4 (47.0-60.6)	0.31	85.5 (79.8-91.7)	29.0 (24.2-34.8)	0.33
				CT	35	47.7 (40.9-55.5)		76.5 (70.3-83.2)	22.2 (17.8-27.6)	
				CC	6	65.4 (45.2-94.6)		91.2 (74.4-11.8)	24.3 (14.6-40.6)	

VDR										
rs731236	42.6	0.08	T/C	TT	34	52.2 (44.6-61.0)	0.35	83.4 (76.5-91.0)	24.9 (19.9-31.2)	0.66
				TC	38	49.3 (42.5-57.1)		79.5 (73.2-86.4)	27.3 (22.0-33.9)	
				CC	20	56.4 (46.0-69.1)		86.8 (76.6-96.1)	25.0 (18.8-33.3)	
rs757343	10.8	0.98	G/A	GG	74	52.8 (47.5-58.8)	0.76	83.2 (78.4-88.2)	26.8 (23.0-31.3)	0.56
				AG	17	47.8 (38.3-59.7)		79.1 (69.9-89.6)	22.3 (16.4-30.4)	
				AA	1	47.6 (19.1-118.7)		74.9 (45.0-124.7)	27.3 (17.7-97.5)	
rs10783219	36.9	1.00	A/T	AA	36	53.0 (45.5-61.8)	0.82	82.1 (75.5-89.4)	25.4 (20.4-31.6)	0.69
				TA	43	50.5 (43.9-58.1)		81.2 (75.2-87.8)	25.6 (20.9-31.2)	
				TT	13	52.8 (41.0-68.1)		86.4 (75.0-99.5)	28.5 (19.7-41.2)	
rs7139166	40.3	0.24	C/G	CC	37	53.6 (46.1-62.3)	0.53	84.4 (77.7-91.8)	26.1 (21.0-32.4)	0.81
				CG	37	51.6 (44.4-59.9)		82.1 (75.5-89.3)	25.6 (20.6-31.7)	
				GG	18	48.7 (39.3-60.5)		78.5 (69.6-88.5)	26.2 (19.2-35.7)	

Bold numbers represent significant P values (<0.05).
SNP, single nucleotide polymorphism (ordered by position); *MAF,* minor allele frequency for the unrelated population in percentage; *HWE,* P-values for Hardy-Weinberg equilibrium in the unrelated population; *M/m,* major and minor alleles; *Mean*, raw serum 25(OH)D concentrations were log-transformed to approximate a normal distribution an given as geometric mean (nmol/L); *95%, CI* 95%-confident interval.
[1]P_{adj} Linear mixed models with family as a random factor, adjusted for age, sex, BMI, use of multivitamin and vitamin D supplementation, outdoor stay in light clothes, outdoor transport to work and sun bathing.
[2]P_{adj} Linear mixed models with family as a random factor, adjusted for age, sex, BMI and baseline serum 25(OH)D concentrations.
[3]GX, GC/GA
[4]XX, CC/CA/AA

7.2 Genetic risk score analysis of *CYP2R1* and *GC* in the VitDgen study

To determine the combined effect of rs10741657 in *CYP2R1* and rs4588 in *GC* on 25(OH)D concentrations in winter and after UVB irradiation, a GRS was calculated as the sum of the number of G-alleles of rs10741657 and A-alleles of rs4588 (range 0 to 4).

As expected and observed for the control group in paper II, there were no associations between GRS and 25(OH)D concentrations (p = 0.16) in the winter (**Figure 6**). However, after whole body UVB irradiation with a total of 6 or 7.5 SEDs, a gene-dose dependent relationship between the UVB-dependent increase in 25(OH)D concentrations and GRS was observed, in agreement with our findings in late summer (**paper I**) and after vitamin D_3-fortification (**paper II**). Overall, after UVB irradiation there was a mean difference in 25(OH)D concentrations of 20.9 nmol/L between non-carriers and carriers of all 4 risk alleles. In agreement with our findings, Engelman et al. (107) performed a GRS encompassing rs4588 in *GC* and rs2060793 (in strong LD with rs10741657) in *CYP2R1* and found that the lowest mean 25(OH)D concentrations were found in the group with 3 risk alleles and low external sources of vitamin D (<10 μg/day) or 4 risk alleles, regardless of the external sources of vitamin D.

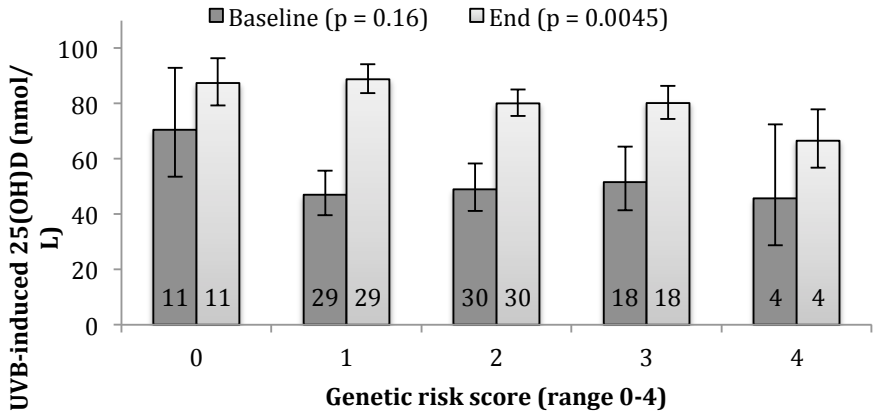

Figure 6. 25(OH)D concentrations in winter and after UVB irradiation for each genetic risk score category of rs10742657 and rs4588.
Genetic risk score (GRS) was calculated as the sum of number of G-alleles of rs10741657 and A-alleles of rs4588. Column numbers, total numbers of participants carrying the GRS; error bars, 95% confidence interval.

A statistically significant linear negative trend between the %-increase in 25(OH)D concentrations and GRS (p = 0.042) was found (**Figure 7**). Moreover, the smallest %-increase in UVB-induced 25(OH)D concentrations was also observed for carriers of all 4 risk alleles (23.05%) compared to non-carriers (54.02%).

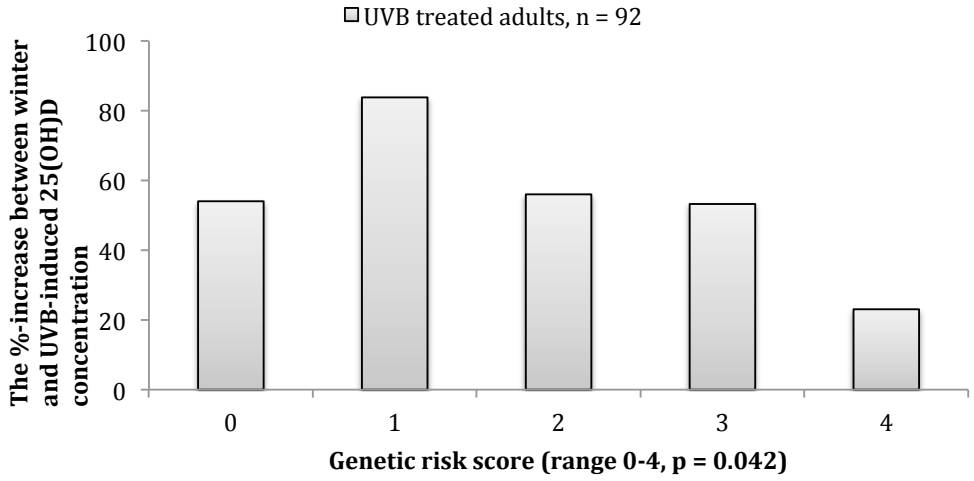

Figure 7. The %-increase in 25(OH)D concentrations after UVB irradiation for each genetic risk score category of rs10742657 and rs4588.
Genetic risk score was calculated as the sum of number of G-alleles of rs10741657 and A-alleles of rs4588.

7.3 Genetic risk score analysis of *CYP2R1* and *GC* in the VitmaD study

To evaluate and determine the genetic contribution of rs10741657 in *CYP2R1* and rs4588 in *GC* on 25(OH)D concentrations following vitamin D_3-intake, data from the adult population from the VitmaD study were used in late summer (all adults, n = 414) and after receiving vitamin D_3-fortified bread and milk for a 6-months period during winter (adults in the fortification group n = 208) (138,165,166). GRS was calculated as the sum of the number of G-alleles of rs10741657 and A-alleles of rs4588 (range 0 to 4) in late summer and after intake of vitamin D_3-fortified bread and milk. It was not necessary to weight the risk alleles by the correlation coefficient, because the coefficients of rs10741657 and rs4588 were very similar in a mixed regression model including both SNPs (data not shown). In late summer, there was a linear negative trend between 25(OH)D concentrations and carriers of 0 to 4 risk alleles ($p < 0.0001$) (**Figure 8**). After intake of vitamin D_3-fortified bread and milk for 6 months during winter, there was still a linear negative trend between 25(OH)D concentrations and being carrier of 0 to 4 risk alleles ($p = 0.027$). Nimitphong et al. 2013 (167) observed a significantly smaller increase in 25(OH)D_3 and total 25(OH)D concentrations after oral intake of 400 IU/day (10 µg/day) of vitamin D_3 for 3 months in individuals carrying the CA or AA genotypes of rs4588.

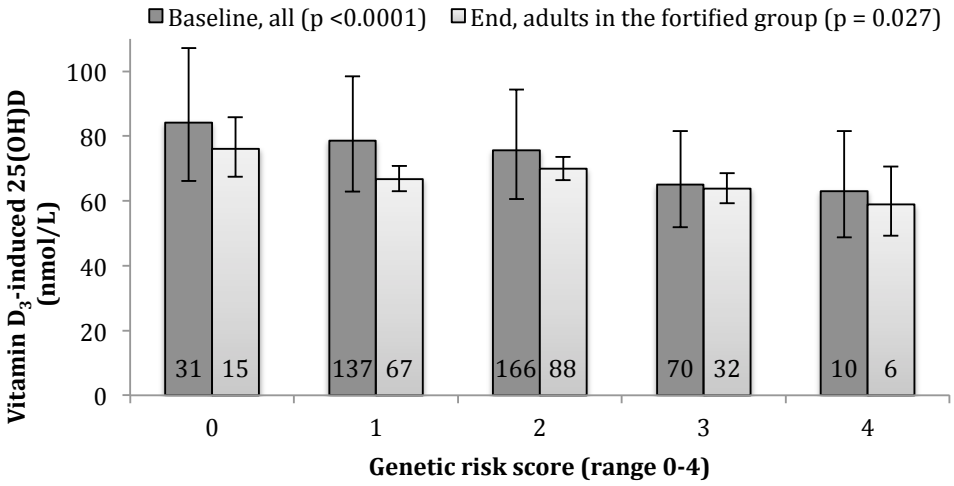

Figure 8. 25(OH)D concentrations at baseline (late summer) and after 6 months intake of vitamin D_3-fortified bread and milk (end) for each genetic risk score category of rs10742657 and rs4588
Genetic risk score (range 0 to 4) was calculated as the sum of number of G-alleles of rs10741657 and A-alleles of rs4588. The numbers in the columns present the total numbers of participants carrying the risk score. Error bars indicate 95% confidence interval.

Using a realistic vitamin D_3-fortification model, a decrease in 25(OH)D concentrations was observed during winter and the largest %-decrease in 25(OH)D concentrations were observed for carriers of all 4 risk alleles (-19.10%) compared to non-carriers (4.44%) (**Figure 9**).

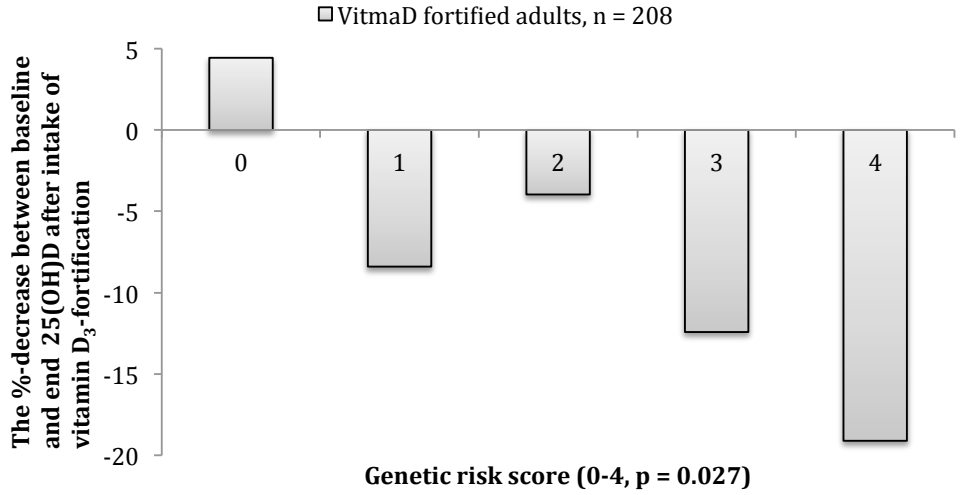

Figure 9. The %-decrease in 25(OH)D concentrations after 6 months intake of vitamin D_3-fortified bread and milk for each genetic risk score category of rs10742657 and rs4588.
Genetic risk score (range 0 to 4) was calculated as the sum of number of G-alleles of rs10741657 and A-alleles of rs4588.

Overall, these findings indicate that genetic predisposition in *CYP2R1* and *GC* genes may have a large impact on 25(OH)D concentrations. Predisposed individuals carrying all 4 risk alleles of rs10741657 in *CYP2R1* and rs4588 in *GC* benefitted the least from either whole body UVB irradiation or intake of vitamin D_3-fortified bread and milk during winter compared to individuals carrying fewer or no risk alleles of rs10741657 in *CYP2R1* and rs4588 in *GC*. Regardless of the method used to increase or maintain 25(OH)D concentrations during winter, the effects of UVB irradiation or vitamin D_3-fortification on 25(OH)D concentrations seemed remarkably similar.

Important for public health recommendations, this study emphasizes that individuals carrying a high GRS, predisposed to genetically determined low 25(OH)D concentrations, may need a longer UVB-exposure time or a higher amount of vitamin D supplement to achieve a given 25(OH)D concentration than individuals carrying a lower GRS. One the other hand, these results may indicate that there is a physiological variation in the normal range of 25(OH)D concentration, demonstrating that a "one size fits all" approach may not work well for vitamin D.

7.4 The clinical importance of variation in rs10741657 in *CYP2R1* and rs4588 in *GC*

Vitamin D has emerged as a promising target in relation to disease susceptibility. The fact that SNPs in vitamin D modulating genes have shown to predict vitamin D status has given rise to an increasing number of epidemiological studies investigating the risk of developing a large range of different adverse health outcomes in relation to genetic biomarkers. It is not known whether carriers of all 4 risk alleles of rs10741657 in *CYP2R1* and rs4588 in *GC* are at-risk individuals, who may have substantially elevated risk of developing vitamin D deficiency and subsequent adverse health outcomes.

Genetic variation in rs10741657 has been found to be associated with colon cancer recurrence (111) and inversely associated with pancreas cancer risk (AA versus GG, OR=0.70; 95% CI: 0.51-0.95) (112). Furthermore, rs10741657 has been associated with risk of T1DM in a German population. The G-allele of rs10741657 was more often transmitted to affected offspring (61% vs. 39%, p = 0.004) and was also more frequent in cases than in controls (46.1% vs. 35.7%, p = 0.03) and carriers of this allele had on average lower 25(OH)D concentrations (159). Contrary, Thorsen et al. 2013 (168) found no association between T1DM and rs10741657 in 1467 affected offspring of Danish origin, but in agreement with our and previous studies an association with 25(OH)D concentrations were found. Blanton et al. 2011 (169) did not find any association between genotype

frequencies of rs4588 and DBP concentrations and risk of T1DM. Nimitphong et al. (170) found that rs2282679 (in strong LD with rs4588) in *GC* modified the association between 25(OH)D concentrations and bone mineral density and bone markers.

There is increasing evidence indicating that *GC* genotypes (rs7041 and rs4588), giving rise to different DBP phenotypes, are associated with adverse health outcomes including premenopausal bone fracture, diabetes, severity of obstructive pulmonary disease and rheumatic fever (120,121). Abbas et al. 2008 (124) found that carriers of the Gc2/2 genotype had significantly lowered risk of postmenopausal breast cancer with an odds ratio (95% confidence interval) of 0.72 (0.54-0.96), compared with homozygous Gc1s allele carriers. Sayegh et al. 2014 (171) found that the Gc2 phenotype is prevalent among women with endometriosis and may be implicated in its pathogenesis. Li et al. 2011 (172) provide supporting evidence that the Gc2 genotype was significantly associated with asthma susceptibility in a Chinese Han population (OR = 1.35, 95% CI = 1.01-1.78 p = 0.006) compared to Gc1 carriers. In the Tromsø Study, a reduced incidence of cancer risk between 23-26% was found in Gc1f/1f carriers compared to Gc1s/1s and Gc-2/2 carriers (164). The cancer protective effect of Gc1f/1f could not be explained by differences in 25(OH)D concentrations. In a Danish study, Afzal et al. (173) found that for each increase in allele score of rs7944926 and rs11234027 in *DHCR7* and rs10741657 and rs12794714 in *CYP2R1* were associated with a 1.9 nmol/L lower 25(OH)D concentrations. Furtermore, genetically low 25(OH)D concentrations were associated with increased all cause mortality, cancer mortality, and other causes of mortality but not with cardiovascular mortality.

8. Conclusion and future perspectives

Several candidate gene studies including two GWAS have demonstrated the importance of genetic variation in vitamin D modulation genes on 25(OH)D concentrations. In this study, common genetic variations in the *CYP2R1* and *GC* genes were shown to be determinants of 25(OH)D concentrations in a healthy Caucasian population in late summer (**paper I),** after intake of vitamin D_3-fortified bread and milk (**paper II**) and after UVB irradiation (**paper III).** No association was observed between vitamin D status and genetic variation in the *CYP2R1* and *GC* genes during winter when no supplemental vitamin D sources (fortification or UVB irradiation) were given (**paper II** and **III**). In general, no differences between gender in children and adults were observed, and there were no differences between children and adults for all analysed parameters (**paper I** and **II**).

Overall, a general negative gene-dose dependent relationship was observed between increasing numbers of risk alleles of *CYP2R1* and *GC* and lower 25(OH)D concentrations, and moreover an additive effect of *CYP2R1* and *GC* on 25(OH)D concentrations was observed (**paper I, II** and **III**). The present study has shown that individuals with a high GRS stratified by rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* were more prone to have a low vitamin D status compared to carriers of a lower GRS, independently of the vitamin D source (**paper I, II** and **III)**. Predisposed individuals, with a genetic profile in *CYP2R1* and *GC* leading to low vitamin D status, were also the ones responding the least to increased exposure of the vitamin D sources, vitamin D_3-fortification and UVB irradiation (**paper II and III**). Individuals with genetically determined low 25(OH)D concentrations may need different health recommendations in order to improve their vitamin D status or, alternatively, there may be variability in the physiologically normal range of 25(OH)D concentrations, demonstrating that a "one size fits all" approach may not work well for vitamin D (**paper II**). These findings provide fundamental data for establish what is sufficient vitamin D status in different genetic profiles of *CYP2R1* and *GC*.

Genetic predisposition in rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* is linked to low vitamin D status and may be used as genetic biomarker to identify individuals at highest risk of low vitamin D status. Importantly for the use of the SNPs as a biomarkers for vitamin D status, rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* were found to be significantly associated with 25(OH)D concentrations both before and after adjustment for vitamin D confounders in late summer (**paper I**).

Identifying at-risk individuals and avoiding low vitamin D status is essential in relation to adverse health outcomes. It is crucial to implement easy-to-apply phenotypic strategies for screening at-risk individuals, which can help to improve clinical practice by better targeting individuals at need for vitamin D supplementation and/or blood testing. Today, official nutrition recommendations do not take genetic differentiation into account due to lack of scientific substantiation. The challenge of providing individualized targeted recommendations on vitamin D may be taken to a new level by including individual genetic profiling in *CYP2R1* and *GC*, which may improve nutritional recommendations and public preventive strategies. In future studies, including DBP phenotypes (rs7041 and rs4588), measuring DBP concentrations and analysing possible effects of rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in GC on free and bioavailable 25(OH)D concentrations may further improve individualized targeted recommendations on vitamin D.

However, detailed information about disease susceptibility in individuals with high GRS stratified by polymorphisms rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* (and thus low vitamin D status and low response to increase exposure to vitamin D sources) has not been elucidated. It is not known whether they have an increased risk of vitamin D related diseases or whether they are 'protected' by their genetic *CYP2R1* and *GC* profile. This needs to be addressed in future studies before recommending higher vitamin D doses.

In conclusion, this PhD thesis gives a comprehensive overview of genetic variation in vitamin D modulating genes and elucidates the genetic variability, linkage disequilibrium, haplotype structure of *CYP2R1* and *GC* in a healthy Caucasian population in late summer, in winter, after vitamin D_3-fortification and after UVB irradiation. These findings provide fundamental data for further analysis in the clarification of the relevance of genetic variation in the *CYP2R1* and *GC* genes in relation to vitamin D-fortification strategies, health recommendations, disease susceptibility and use as a biomarker for low vitamin D status.

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Common Variants in *CYP2R1* and *GC* Genes Predict Vitamin D Concentrations in Healthy Danish Children and Adults

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Abstract

Environmental factors such as diet, intake of vitamin D supplements and exposure to sunlight are known to influence serum vitamin D concentrations. Genetic epidemiology of vitamin D is in its infancy and a better understanding on how genetic variation influences vitamin D concentration is needed. We aimed to analyse previously reported vitamin D-related polymorphisms in relation to serum 25(OH)D concentrations in 201 healthy Danish families with dependent children in late summer in Denmark. Serum 25(OH)D concentrations and a total of 25 SNPs in *GC*, *VDR*, *CYP2R1*, *CYP24A1*, *CYP27B1*, *C10or88* and *DHCR7/NADSYN1* genes were analysed in 758 participants. Genotype distributions were in Hardy–Weinberg equilibrium for the adult population for all the studied polymorphisms. Four SNPs in *CYP2R1* (rs1562902, rs7116978, rs10741657 and rs10766197) and six SNPs in *GC* (rs4588, rs842999, rs2282679, rs12512631, rs16846876 and rs17467825) were statistically significantly associated with serum 25(OH)D concentrations in children, adults and all combined. Several of the SNPs were in strong linkage disequilibrium, and the associations were driven by *CYP2R1*-rs10741657 and rs10766197, and by *GC*-rs4588 and rs842999. Genetic risk score analysis showed that carriers with no risk alleles of *CYP2R1*-rs10741657 and rs10766197, and/or *GC* rs4588 and rs842999 had significantly higher serum 25(OH)D concentrations compared to carriers of all risk alleles. To conclude, our results provide supporting evidence that common polymorphisms in *GC* and *CYP2R1* are associated with serum 25(OH)D concentrations in the Caucasian population and that certain haplotypes may predispose to lower 25(OH)D concentrations in late summer in Denmark.

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Introduction

Vitamin D deficiency is a widespread problem in developed countries [1]. Severe vitamin D deficiency causes osteomalacia, or childhood rickets, osteoporosis and fractures because of reduced calcium absorption [2]. Low vitamin D concentrations may also be related to various non-skeletal health outcomes, including cardiovascular diseases [3], obesity [4], diabetes [5], asthma [6], multiple sclerosis [7], occurrence of a large range of cancer diseases [8] and overall mortality [9,10].

In humans, vitamin D is produced mainly in the skin during exposure to solar ultraviolet blue (UVB) radiation (270–300 nm) [11]. UVB radiation converts 7-dehydrocholesterol (7-DHC) in the skin to pre-vitamin D_3, which immediately undergoes a thermal isomerization to vitamin D_3. Dietary sources provide two forms of vitamin D: Vitamin D_2 (ergocalciferol) derived from invertebrates (plants and fungi) and vitamin D_3 (cholecalciferol) derived from animal sources. Ingested vitamins D_2 and D_3 are absorbed in the small intestine and transported with chylomicrons and lipoproteins to the liver, whereas dermally synthesized vitamin D_3 diffuses via the blood to the liver tightly bound to group-specific complement (GC) [12].

Dietary or dermally synthesized vitamin D (hereafter "D" refers to D_2 and D_3) undergoes a series of enzymatic conversions in the liver and kidneys to become biologically active. The hepatic enzyme 25-hydroxylase (CYP2R1) converts vitamin D to 25-hydroxyvitamin D (25(OH)D). This is the major circulating form of vitamin D in the blood. To become biologically active, 25(OH)D is converted to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). This occurs mainly in the kidneys, but also in other tissues expressing the enzyme 25(OH)D-1α-hydroxylase (CYP27B1). The biological effect of vitamin D is mediated when $1,25(OH)_2D$ binds to the vitamin D receptor (VDR). To prevent excessive vitamin D signalling in the target organs, $1,25(OH)_2D$ limits its own activity by inducing 24-hydroxylase (CYP24A1) converting $1,25(OH)_2D$ to the biologically inactive water-soluble calcitroic acid which is excreted in the bile [1,12,13].

The best biomarker of vitamin D concentration is the serum 25(OH)D concentration. Approximately 25% of the inter-individual variability in plasma 25(OH)D concentrations can be

explained by external factors such as diet, regular use of vitamin D supplements and exposure to sunlight (dependent on season and latitude) [14,15]. Genetic factors may contribute to vitamin D concentrations. Results from twin and family-based studies indicate that blood vitamin D concentrations to some extent are under genetic control. The results have been inconsistent with a wide variability in heritability estimates ranging from 23 to 80% [15–21]. Furthermore, ethnic differences in vitamin D concentrations have also been described [22].

Genetic epidemiology of vitamin D is in its infancy and a better understanding of how genetic variation influences vitamin D concentrations is needed. A growing number of studies have uncovered polymorphisms associated with vitamin D concentrations. By candidate gene analysis, five genes have been found, including *GC, CYP24A1, CYP2R1, CYP27B1* and *VDR* [23]. Recently, two genome-wide association studies (GWAS) of vitamin D [24,25] confirmed the associations of common variants in *GC* and *CYP2R1* genes. Furthermore, nicotinamide adenine dinucleotide synthetase-1/7-dehydrocholesterol reductase (NADSYN1/DHCR7), and the region harbouring the open-reading frame 88 (C10orf88) on chromosome 10q26.13 were also found to be associated with vitamin D concentrations in blood.

In Denmark, low vitamin D status is common during the winter due to inadequate dietary intakes and lack of solar radiation from September to April [26]. We assessed vitamin D status in late summer (September to October), where the Danes vitamin D concentration peaks but are not saturated [27], in families with a broad span in age in both children and adults. In children, the role of genetic variation in determining serum 25(OH)D concentrations is an understudied area.

In this study, we analysed previously reported vitamin D-related polymorphisms in relation to serum 25(OH)D concentrations in 201 healthy Danish families with dependent children to confirm previous findings and thus help identifying individuals that may have increased risk of developing vitamin D insufficiency.

Subjects and Methods

Study population

The present cross-sectional study used baseline data from the VitmaD intervention study described in detail elsewhere [28]. Briefly, 201 Danish families with dependent children (n = 782) were enrolled. The participants were 4- to 60-years old. Baseline blood samples were collected in September and October 2010 and were obtained from 770 participants. The study was conducted according to the guidelines in the Declaration of Helsinki and the protocol was approved by the Research Ethics Committee of the Capital Region of Denmark (H-4-2010-020) and registered at http://clinicaltrials.gov (NCT01184716). All adult participants and guardians on the behalf of the children participants gave written consent to participate.

DNA extraction and genotyping

DNA was extracted from peripheral blood leukocytes as described by Miller *et al.* [29] and stored in TE-buffer at -80°C. The DNA was diluted to 10 ng/µl using a Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington). Single nucleotide polymorphisms (SNPs) were genotyped using the Sequenom MassARRAY iPLEX Gold platform (Sequenom, San Diego, California) at the Department of Biomedicine, Aarhus University, Denmark. Genotyping was successful for 762 participants (99.0%). To confirm the accuracy of genotyping duplicate samples (10%) yielded 100% reproducibility.

All SNPs were located in or near genes involved in vitamin D synthesis, activation or degradation. The following SNPs were selected on the basis of evidence of significant association in previous studies: ***CYP2R1*** (rs1562902; rs7116978; rs10741657; rs10766197) ***CYP24A1*** (rs229624; rs2426496; rs4809960; rs6013897; rs17219315) ***CYP27B1*** (rs10877012) ***C10orf88*** (rs6599638) ***DHCR7/NADSYN1*** (rs1790349; rs12785878) ***GC*** (rs4588; rs222020; rs842999-triallelic; rs2882679; rs2298849; rs12512631; rs16846876; rs17467825) ***VDR*** (rs731236 (TaqI), rs757343 (TruI); rs7139166; rs10783219).

Deviation from Hardy–Weinberg equilibrium (HWE) was tested for the adult population using Chi-square test with Bonferroni's correction (P-value 0.05/25 SNPs = 0.002). No significant deviation from HWE was observed. Linkage disequilibrium (LD) between polymorphisms was evaluated using Pearsons' r, SNAP version 2.2 (http://www.broadinstitute.org/mpg/snap/ldsearchpw.php) and Haploview software version 4.2 for the adult population.

Measurement of serum 25(OH)D concentrations

Measurements of serum 25(OH)D concentrations are described in detail elsewhere [28]. Briefly, blood samples were obtained without prior fasting and serum was stored in aliquots at –80°C until analysis. Measurements of serum 25(OH)D concentrations relied on the determination of both $25(OH)D_2$ and $25(OH)D_3$ and were conducted by isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) at Clinical Biochemical Department, Holbæk Hospital, Denmark. As primary calibrator the standard reference material, vitamin D in humans (SRM 972) from the National Institute of Standards and Technology was used. The analytic quality of 25(OH)D assay was assured by Vitamin D External Quality Assessment Scheme certification and the mean bias was –3.2%. The Inter-assay CVs for $25(OH)D_2$ were 7.6% and 4.6% at 43 and 150 nmol/L, respectively, and for $25(OH)D_3$ 2.2% and 2.8% at 30 and 180 nmol/L, respectively [28]. Of the 762 participants that were successfully genotyped, baseline serum 25(OH)D concentrations were measured for 758 participants.

Statistical analysis

Statistical analyses were performed using SAS Enterprise Guide 4.3 (SAS Institute, Inc., Cary. USA). Serum 25(OH)D concentrations were log transformed to approximate a normal distribution and all means are presented as geometric means. A nominal P-value of 0.05 was considered statistically significant. Linear mixed models with family as a random factor were applied to account for the possible dependence between the participants. Furthermore, in the linear mixed models the following categorical variables were included: age (4–11, 12–17, 18–40, 41–60 years), sex (male, female), BMI (underweight, normal weight, overweight, obese) according to standards for children [30] and the WHO International standards for adults [31], ski or sun holidays (yes, no), solarium use at least once a week (yes, no), dietary vitamin D (quartiles: <1.7, 1.7–2.4, 2.5–3.3 and >3.3 µg/d), multivitamin and vitamin D supplement users (yes, no). The data were obtained from a self-administered web-based questionnaire and a semi-quantitative food frequency questionnaire based on the last six months. Pearson's r were calculated on the adult population and were used to assess the degree of linkage between linked SNPs. Haplotypes were inferred manually among the adults, only since the children were not population-based. The inferred haplotype combinations described 100% and 97% of the observed genotypes among the adults for *CYP2R1* and *GC* genes, respectively. Among the children the inferred haplotype combinations described 100%

and 96% of the observed genotypes for *CYP2R1* and *GC* genes, respectively. Each derived haplotype was assigned a number. Homozygote haplotype combinations were numbered with two identical numbers e.g. 11. The combinations of heterozygote haplotypes were given by the combination of the number of each haplotype e.g. 1+ 2 = 12.

Genetic risk scores were calculated as the sum of risk alleles and included as risk factors in linear mixed models adjusted for family and confounding variables. The correlation coefficient for rs10741657, rs10766197, rs4588 and rs842999 were very similar and therefore it was not necessary to weight the score by effect size. All the analyses were performed separately for children, adults and for all combined.

Results

Genotyping and serum 25(OH)D concentrations were available for 758 participants. Table 1 summarizes the basic characteristics of the study population, previously described in detail elsewhere [28]. The median age among children was 10 years (range: 4 to17) among adults 41 years (range: 18 to 60) and for all combined 30 years.

Associations between genotypes and serum 25(OH)D concentrations are shown for children, adults and all combined in Table 2. After adjustment for family and confounding factors, all four analysed SNPs in *CYP2R1* were statistically significantly associated with serum 25(OH)D concentrations in all three groups. Furthermore, for all three groups none of the analysed SNPs in *CYP24A1*, *CYP27B1*, *C10orf88* and *DHCR7/NADSYN1* were statistically significantly associated with serum 25(OH)D concentration. For all three groups all analysed SNPs in *GC*, except rs2298849 (in all three groups) and rs222020 (in adults and all), were statistically significantly associated with serum 25(OH)D concentration. The *VDR* rs731236 was only statistically significantly associated with 25(OH)D concentration in all combined and rs757343 was statistically significant in children and all combined. Only SNPs that were statistically significantly associated with 25(OH)D concentrations in children, adults and all combined were included in further analyses.

Haplotype and genetic risk score analysis of *CYP2R1*

In the adult population, rs10741657-rs7116978 (Pearson's r = 0.90), and rs1076697-rs1562902 (Pearson's r = –0.86, data not shown) were in strong LD. To establish which of the SNPs had the strongest association to serum 25(OH)D concentrations, we assess the association between one SNP and serum 25(OH)D concentrations while adjusting for the other SNPs, family and confounding factors in a linear mixed model. After adjustment, rs10766197 (p = 0.0846) had the strongest association compared to rs1562902 (p = 0.8211), and rs10741657 (p = 0.2545) had the strongest association compared to rs7116978 (p = 0.3087, data not shown). In further analysis only rs1076697 and rs10741657 were included.

The two *CYP2R1* variants rs10741657 and rs7116978 formed four haplotypes, where haplotype 1 and 2 were most frequent (Table 3). The possible combinations of the four homozygote haplotype are shown in table 3. One genotype combination could be assigned to both haplotype combinations 12 or 34, but based on the observed haplotype frequencies, the most likely combination was 12. After adjustment for family and confounding factors, carriers of 2 copies of the AG-haplotype (haplotype combination 33) had the highest mean serum 25(OH)D concentration (73.8 (60.1–90.6), 72.9 (57.3–92.5) and 81.3 (66.4–99.6) nmol/L) in children, adults and all combined, respectively. In a linear mixed model, only the homozygous haplotype combinations were included and haplotype combination 44 was excluded because only two participants carried this haplotype combination. The homozygous haplotype combinations were significantly associated with serum 25(OH)D concentrations (p = 0.0059, 0.0450 and 0.0007) in children, adults and all combined, respectively.

We calculated a genetic risk score (range 0–4) as the sum of the number of G-alleles of rs10741657 and A-alleles of rs10766197 (Figure 1, A). After adjustment for family and confounding factors, carriers of no risk alleles had significantly higher serum 25(OH)D concentrations (74.0 (60.3–90.0), 73.0 (57.5–92.6) and 81.3 (66.4–99.5) nmol/L) compared to carriers of all four risk alleles (61.2 (57.5–92.6), 64.0 (50.6–80.9) and 69.8 (57.0–85.4) nmol/L) in children, adults and all combined, respectively. Overall, there was 20.9, 14.1 and 16.5% difference in serum 25(OH)D concentrations between carrying no risk alleles and carrying all four risk alleles in children, adults or all combined, respectively.

Haplotype and genetic risk score analysis of *GC*

In the adult population, rs4588 was in strong LD with rs2282679 (Pearson's r = 0.997), rs17467825 (Pearson's r = 0.997) and rs16846876 (Pearson's r = 0.805). Furthermore, rs17467825-rs2282679 (Pearson's r = 1.00), and rs2282679-rs16846876 (Pearson's r = 0.8021, data not shown) were also in strong LD. To establish which of the 4 SNPs had the strongest association to serum 25(OH)D concentrations, we assess the association between one SNP and serum 25(OH)D concentrations while adjusting for

Table 1. Basic characteristics of the study population and determinants of serum 25(OH)D concentrations

Characteristics	Children	Adults	All
Number	348	414	762
Female/Male (n/n)	181/167	209/205	390/372
Age, median (range)	10 (4–17)	41 (18–60)	30 (4–60)
BMI (kg/m^2)*	17.44±2.89	25.47±4.30	21.79±5.45
Serum 25(OH)D (nmol/L)*	74.38±17.31	74.87±21.70	74.65±19.82
Dietary Vitamin D (μg/d)*	2.69±1.35	2.96±2.04	2.84±1.77
Multivitamin or vitamin D supplement users (yes/no)	141/203	113/297	254/500
Solarium use (yes/no)	2/342	10/401	12/743
Ski or sun holidays (yes/no)	195/149	220/191	415/340

*Mean ± SD.

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Table 2. Basic characteristics of the individual SNP and the association with serum 25(OH)D concentrations in children, adults and all combined.

					Children (n = 344)				Adults (n = 414)				All (n = 758)			
SNP	**MMAF**	**HWE**	**M/m**	**Gt**	**N**	**25(OH)D, nmol/L (95% CI)**	**p[1]**	**p_{adj}[2]**	**N**	**25(OH)D, nmol/L (95% CI)**	**p[1]**	**p_{adj}[2]**	**N**	**25(OH)D, nmol/L (95% CI)**	**p[1]**	**p_{adj}[2]**
CYP2R1																
rs7116978	38.8	0.25	C/T	CC	124	67.6 (65.0–70.2)	**<0.0001**	**<0.0001**	156	67.5 (64.2–71.0)	**0.0218**	**0.0093**	280	67.5 (65.3–69.8)	**<0.0001**	**<0.0001**
				CT	158	73.9 (71.4–76.6)			180	72.8 (69.5–76.3)			338	73.3 (71.2–75.6)		
				TT	54	79.1 (74.5–83.9)			66	77.5 (71.8–83.8)			120	78.2 (74.4–82.3)		
rs10741657	40.8	0.31	G/A	GG	118	67.9 (65.2–70.7)	**<0.0001**	**<0.0001**	150	66.6 (63.3–70.1)	**0.0039**	**0.0067**	268	67.2 (65.0–69.5)	**<0.0001**	**<0.0001**
				GA	175	73.9 (71.5–76.4)			190	74.0 (70.7–77.4)			365	73.9 (71.8–76.1)		
				AA	51	78.8 (74.1–83.7)			74	75.2 (69.9–80.9)			125	76.6 (73.0–80.5)		
rs1562902	45.2	0.37	T/C	TT	103	68.9 (65.9–71.9)	**0.0233**	**0.0086**	129	67.5 (63.9–71.4)	**0.0574**	**0.0353**	232	68.1 (65.7–70.6)	**0.0022**	**0.0005**
				TC	172	73.7 (71.2–76.2)			196	73.3 (70.0–76.6)			368	73.5 (71.4–75.6)		
				CC	69	75.0 (71.0–79.1) 79.1			89	73.4 (68.6–78.5)			158	74.1 (70.9–77.4)		
rs10766197	46.9	0.15	G/A	GG	97	76.0 (72.7–79.5)	**0.0048**	**0.0006**	124	73.0 (69.0–77.3)	0.0557	**0.0081**	221	74.3 (71.6–77.1)	**0.0013**	**<0.0001**
				AG	168	72.7 (70.2–75.2)			191	73.2 (69.9–76.6)			359	72.9 (70.8–75.1)		
				AA	79	67.9 (64.6–71.4)			98	66.2 (62.1–70.5)			177	66.9 (64.2–69.8)		
CYP24A1																
rs6013897	20.3	0.77	T/A	TT	219	73.5 (71.3–75.8)	0.2887	0.5044	264	71.8 (69.1–74.7)	0.9033	0.7058	483	72.6 (70.8–74.4)	0.4702	0.5228
				AT	114	70.7 (67.8–73.8)			132	70.9 (67.1–74.9)			246	70.8 (68.4–73.4)		
				AA	11	69.5 (60.7–79.5)			18	70.0 (60.3–81.3)			29	69.8 (63.0–77.4)		
rs4809960	22.7	0.35	T/C	TT	198	72.0 (69.7–74.3)	0.8163	0.5674	244	72.2 (69.3–75.1)	0.3402	0.2786	442	72.1 (70.2–74.0)	0.4658	0.0663
				TC	121	72.9 (70.0–76.0)			152	69.7 (66.2–73.3)			273	71.1 (68.7–73.5)		
				CC	25	73.8 (67.5–80.7)			18	77.2 (66.5–89.6)			43	75.2 (69.1–81.9)		
rs2296241	49.0	0.37	G/A	GG	90	68.9 (65.8–72.2)	**0.0301**	0.1111	103	70.3 (66.0–74.8)	0.6048	0.6078	193	69.6 (66.9–72.5)	0.0801	0.0501
				AG	164	72.9 (70.4–75.4)			216	71.1 (68.1–74.3)			380	71.9 (69.9–74.0)		
				AA	90	75.4 (71.9–79.0)			95	73.5 (68.8–78.4)			185	74.4 (71.4–77.5)		
rs17219315	3.1	0.75	A/G	AA	342	72.3 (70.6–74.1)	0.0895	0.1836	401	71.4 (69.1–73.7)	0.6621	0.3828	743	71.8 (70.3–73.3)	0.3674	0.2381
				AG	2	95.4 (69.5–130.9)			13	74.3 (62.3–88.6)			15	76.8 (66.5–88.7)		
rs2426496	27.7	0.51	G/T	GG	176	71.3 (68.9–73.8)	0.3094	0.2500	214	70.5 (67.5–73.6)	0.6377	0.7896	390	70.8 (68.9–72.9)	0.2573	0.2500
				GT	135	73.2 (70.4–76.0)			171	72.3 (68.9–75.9)			306	72.7 (70.4–75.0)		
				TT	33	75.8 (70.1–81.9)			29	73.9 (65.7–83.1)			62	74.9 (69.8–80.4)		
CYP27B1																
rs10877012	33.5	0.02	G/T	GG	156	72.8 (70.2–75.4)	0.1846	0.5758	193	71.0 (67.9–74.4)	0.7822	0.9451	349	71.8 (69.7–74.0)	0.3792	0.9918
				GT	142	73.4 (70.7–76.2)			163	72.4 (68.9–76.0)			305	72.9 (70.6–75.2)		
				TT	46	68.4 (64.1–73.1)			57	69.9 (64.3–76.0)			103	69.2 (65.5–73.2)		

Table 2. Cont.

					Children (n = 344)				Adults (n = 414)				All (n = 758)			
SNP	**MMAF**	**HWE**	**M/m**	**Gt**	**N**	**25(OH)D, nmol/L (95% CI)**	**p[1]**	**p_{adj}[2]**	**N**	**25(OH)D, nmol/L (95% CI)**	**p[1]**	**p_{adj}[2]**	**N**	**25(OH)D, nmol/L (95% CI)**	**p[1]**	**p_{adj}[2]**
C10orf88																
rs6599638	47.8	0.20	G/A	GG	98	72.5 (69.3–75.8)	0.3569	0.3197	106	72.0 (67.7–76.6)	0.8394	0.8797	204	73.3 (69.5–75.1)	0.8349	0.8821
				GA	171	73.5 (71.0–76.0)			219	70.8 (67.8–73.9)			390	71.9 (69.9–74.0)		
				AA	75	70.2 (66.6–73.9)			88	72.2 (67.4–77.2)			163	71.2 (68.2–74.4)		
DHCR7/NADSYN1																
rs1790349	15.1	0.55	A/G	AA	232	71.6 (69.6–73.7)	**0.0174**	0.0923	300	70.9 (68.4–73.6)	0.2381	0.3478	532	71.2 (69.5–73.0)	0.3767	0.8787
				GA	105	73.2 (70.1–76.4)			103	73.9 (69.5–78.7)			208	73.6 (70.8–76.5)		
				GG	7	91.5 (77.4–108.3)			11	63.2 (52.2–76.5)			18	73.0 (64.0–83.2)		
rs12785878	27.5	0.84	T/G	TT	171	72.8 (70.4–75.4)	0.9087	0.7649	218	73.0 (69.9–76.2)	0.4356	0.2169	389	72.9 (70.9–75.0)	0.4273	0.0998
				GT	147	72.1 (69.5–74.9)			163	69.6 (66.2–73.1)			310	70.8 (68.6–73.1)		
				GG	26	71.7 (65.7–78.4)			32	69.9 (62.5–78.2)			58	70.7 (65.7–76.1)		
GC																
rs16846876	33.2	0.88	A/T	AA	158	76.5 (73.9–79.2)	**<0.0001**	**0.0004**	184	74.1 (70.7–77.6)	**0.0161**	**0.0024**	342	75.2 (73.0–77.4)	**<0.0001**	**<0.0001**
				AT	153	70.3 (67.8–72.8)			185	70.9 (67.7–74.3)			338	70.6 (68.5–72.8)		
				TT	33	64.5 (59.8–69.6)			45	63.6 (57.9–69.8)			78	64.0 (60.1–68.1)		
rs12512631	36.2	0.62	T/C	TT	137	68.6 (66.1–71.2)	**0.0007**	**0.0012**	166	66.8 (63.6–70.1)	**0.0022**	**0.0004**	303	67.6 (65.5–69.8)	**<0.0001**	**<0.0001**
				TC	157	74.4 (71.8–77.1)			196	74.6 (71.3–78.0)			353	74.5 (72.4–76.7)		
				CC	50	77.5 (72.8–82.5)			52	75.3 (69.0–82.1)			102	76.4 (72.3–80.6)		
rs17467825	27.6	0.53	A/G	AA	181	76.3 (73.9–78.8)	**<0.0001**	**<0.0001**	219	73.8 (70.7–77.0)	0.0519	**0.0015**	400	74.9 (72.9–77.0)	**<0.0001**	**<0.0001**
				GA	142	70.1 (67.6–72.7)			160	70.0 (66.6–73.6)			302	70.1 (67.9–72.3)		
				GG	21	57.7 (52.5–63.3)			34	63.6 (57.1–70.8)			55	61.2 (56.9–65.9)		
rs2282679	27.4	0.41	A/C	AA	181	76.3 (73.9–78.8)	**<0.0001**	**<0.0001**	219	73.8 (70.7–77.0)	0.0672	**0.0020**	400	74.9 (72.9–77.0)	**<0.0001**	**<0.0001**
				CA	138	70.0 (76.4–72.6)			156	70.1 (66.6–73.7)			294	70.0 (67.8–72.3)		
				CC	21	57.7 (52.5–63.3)			34	63.6 (57.1–70.8)			55	61.2 (56.9–65.9)		
rs842999	4.5	0.65	G/C/A	GG	105	76.7 (73.5–80.0)	**<0.0001**	**<0.0001**	112	74.2 (70.0–78.7)	**0.0114**	**0.0046**	217	75.4 (72.7–78.3)	**<0.0001**	**<0.0001**
				GC	153	72.6 (70.1–75.2)			188	73.7 (70.4–77.1)			341	73.2 (71.1–75.4)		
				CC	57	63.7 (60.2–67.5)			75	66.6 (61.9–71.5)			132	65.3 (62.3–68.5)		
				GA	19	74.3 (67.3–82.1)			23	64.9 (57.0–73.9)			42	69.0 (63.4–75.1)		
				CA	7	76.3 (64.6–89.6)			12	55.8 (46.6–66.9)			19	62.6 (55.2–71.0)		
				AA	0	-			1	75.5 (40.5–140.9)			1	75.5 (43.6–130.8)		
rs4588	27.7	0.57	C/A	CC	181	76.3 (73.9–78.8)	**<0.0001**	**<0.0001**	219	74.1 (71.0–77.3)	**0.0167**	**0.0008**	400	75.1 (73.1–77.2)	**<0.0001**	**<0.0001**
				CA	142	70.1 (67.6–72.7)			161	69.7 (66.3–73.2)			303	69.9 (67.7–72.1)		

Table 2. Cont.

					Children (n = 344)				Adults (n = 414)				All (n = 758)			
SNP	**MMAF**	**HWE**	**M/m**	**Gt**	**N**	**25(OH)D, nmol/L (95% CI)**	**p[1]**	**p_{adj}[2]**	**N**	**25(OH)D, nmol/L (95% CI)**	**p[1]**	**p_{adj}[2]**	**N**	**25(OH)D, nmol/L (95% CI)**	**p[1]**	**p_{adj}[2]**
				AA	21	57.7 (52.5–63.3)			34	63.6 (57.1–70.8)			55	61.2 (56.9–65.9)		
rs222020	15.6	0.13	T/C	TT	250	70.5 (68.6–72.5)	**0.0009**	**0.0021**	291	70.5 (67.9–73.1)	**0.1954**	**0.5338**	541	70.5 (68.8–72.2)	**0.0103**	0.0739
				TC	88	78.4 (74.8–82.1)			117	73.2 (69.1–77.6)			205	75.4 (72.5–78.4)		
				CC	6	69.7 (58.3–83.5)			6	86.4 (66.7–111.8)			12	77.6 (66.1–91.1)		
rs2298849	20.2	0.57	T/C	TT	229	71.1 (69.1–73.2)	**0.0170**	**0.2204**	262	70.3 (67.6–73.1)	0.4399	0.4591	491	70.7 (69.0–72.5)	**0.0390**	0.2605
				CT	99	75.4 (72.1–78.8)			137	73.4 (69.5–77.5)			236	74.2 (71.6–77.0)		
				CC	15	71.1 (63.4–79.7)			15	73.3 (62.3–86.3)			30	72.2 (65.2–79.9)		
VDR																
rs731236	40.3	0.18	T/C	TT	113	70.0 (67.1–73.0)	0.1929	0.0753	154	68.9 (65.4–72.5)	0.1499	0.1306	267	69.3 (67.0–71.7)	0.0753	**0.0346**
				TC	181	74.2 (71.8–76.7)			186	72.3 (69.0–75.7)			367	73.2 (71.1–75.4)		
				CC	49	72.0 (67.5–76.7)			74	74.9 (69.6–80.6)			123	73.7 (70.1–77.5)		
rs757343	11.5	0.45	G/A	GG	261	73.9 (71.9–76.0)	**0.0134**	**0.0103**	326	72.2 (69.7–74.7)	0.2350	0.0896	587	72.9 (71.3–74.6)	**0.0144**	**0.0025**
				AG	77	68.4 (65.1–72.0)			81	69.6 (64.9–74.7)			158	69.1 (66.1–72.2)		
				AA	6	63.7 (53.1–76.3)			7	59.9 (47.1–76.0)			13	61.6 (52.8–71.9)		
rs10783219	36.4	0.10	A/T	AA	147	72.5 (69.8–75.2)	0.9862	0.7067	160	70.1 (66.7–73.7)	0.4908	0.3913	307	71.2 (69.0–73.5)	0.6600	0.2023
				TA	152	72.6 (70.0–75.2)			207	71.8 (68.7–75.0)			359	72.1 (70.0–74.3)		
				TT	45	72.1 (67.4–77.1)			47	74.6 (68.0–81.8)			92	73.4 (69.2–77.8)		
rs7139166	43.0	0.48	C/G	CC	114	72.4 (69.5–75.5)	0.6063	0.4251	131	73.2 (69.2–77.3)	0.2755	0.4324	245	72.8 (70.3–75.5)	0.8342	0.7845
				CG	167	71.6 (69.2–74.1)			210	71.7 (68.6–74.8)			377	71.6 (69.6–73.7)		
				GG	62	74.9 (70.8–79.3)			73	67.9 (63.0–73.1)			135	71.0 (67.7–74.5)		

Bold numbers represent significant P values.

SNP single nucleotide polymorphism (ordered by position), *MAF* minor allele frequency for the adult population in procent, *HWE* P-values for Hardy-Weinberg equilibrium in the adult population, *M/m* major and minor alleles, *Gt* genotype, *Mean*, raw serum 25(OH)D concentrations were log-transformed to approximate a normal distribution an given as geometric mean (nmol/L), *95% CI* 95%-confident interval.

[1]Unadjusted P values.

[2]Adjusted P values. Linear mixed models with family as a random factor, adjusted for age, sex, BMI, ski and sun holidays, use of solarium, dietary vitamin D intake, use of multivitamin and vitamin D supplementation.

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Table 3. Distribution of *CYP2R1* haplotype combinations and serum 25(OH)D concentrations in children, adults and all combined.

				Children (n = 348)				Adults (n = 413)				All (n = 761)			
Haplotype-combination	rs10741657	rs10766197	Alleles[1]	N	Raw mean 25(OH)D[2] (95% CI)	Adj. Mean 25(OH)D[3] (95% CI)	P_{adj}	N	Raw mean 25(OH)D[2] (95% CI)	Adj. Mean 25(OH)D[3] (95% CI)	P_{adj}	N	Raw mean 25(OH)D[2] (95% CI)	Adj. Mean 25(OH)D[3] (95% CI)	P_{adj}
11	GG	AA	Mm	65	67.3 (63.8–71.1)	64.9 (46.8–89.9)	**0.0059**	81	65.7 (61.3–70.4)	65.2 (52.5–81.1)	**0.0450**	146	66.4 (63.4–69.5)	67.9 (56.0–82.2)	**0.0007**
22	AA	GG	mM	39	80.6 (75.2–86.4)	78.7 (56.9–108.9)		57	74.3 (68.4–80.8)	74.6 (59.2–94.0)		96	76.8 (72.7–81.3)	78.2 (64.5–94.8)	
33	GG	GG	MM	8	68.6 (58.9–80.0)	63.7 (43.7–92.8)		13	70.5 (59.3–83.8)	66.4 (50.4–87.6)		21	69.8 (61.9–78.6)	68.0 (54.5–85.0)	
44	AA	AA	mm	1	50.9 (33.0–78.6)	-		1	79.2 (42.4–147.9)	-		2	63.5 (43.1–93.5)	-	
12*	GA	AG		112	74.2 (71.2–77.3)			119	75.5 (71.3–80.0)			231	74.8 (72.2–77.6)		
13	GG	AG		47	68.5 (64.3–73.1)			56	67.2 (61.8–73.0)			103	67.8 (64.2–71.6)		
23	GA	GG		50	73.8 (69.4–78.5)			54	72.3 (66.4–78.7)			104	73.0 (69.2–77.0)		
14	GA	AA		15	72.1 (64.2–81.0)			16	68.0 (58.2–79.5)			31	69.9 (63.3–77.3)		
24	AA	AG		11	75.5 (66.2–86.1)			16	78.2 (66.9–91.4)			27	77.1 (69.4–85.6)		

Bold numbers represent significant P values.

Haplotype combinations were manually inferred and numbered. Homozygote haplotype combinations were numbered 11, 22, 33 and 44. The combinations of the heterozygote haplotypes (12 to 24) were given by one number of each homozygote haplotype e.g. 11+ 22 = 12.

* Also haplotype combination 34, but the most likely haplotype combination is 12.

[1]*M* major allele, *m* minor allele.

[2]Raw geometric mean of serum 25(OH)D concentrations (nmol/L) and corresponding 95%-confidence interval.

[3]Adjusted geometric mean of 25(OH)D concentrations (nmol/L)) and corresponding 95%-confidence interval. Linear mixed models with family as a random factor, adjusted for age, sex, BMI, ski and sun holidays, use of solarium, dietary vitamin D intake, use of multivitamin and vitamin D supplements.

[adj]Adjusted P values. Haplotype combination 44 was excluded in the linear mixed model due to inadequate participants carrying this haplotype combination.

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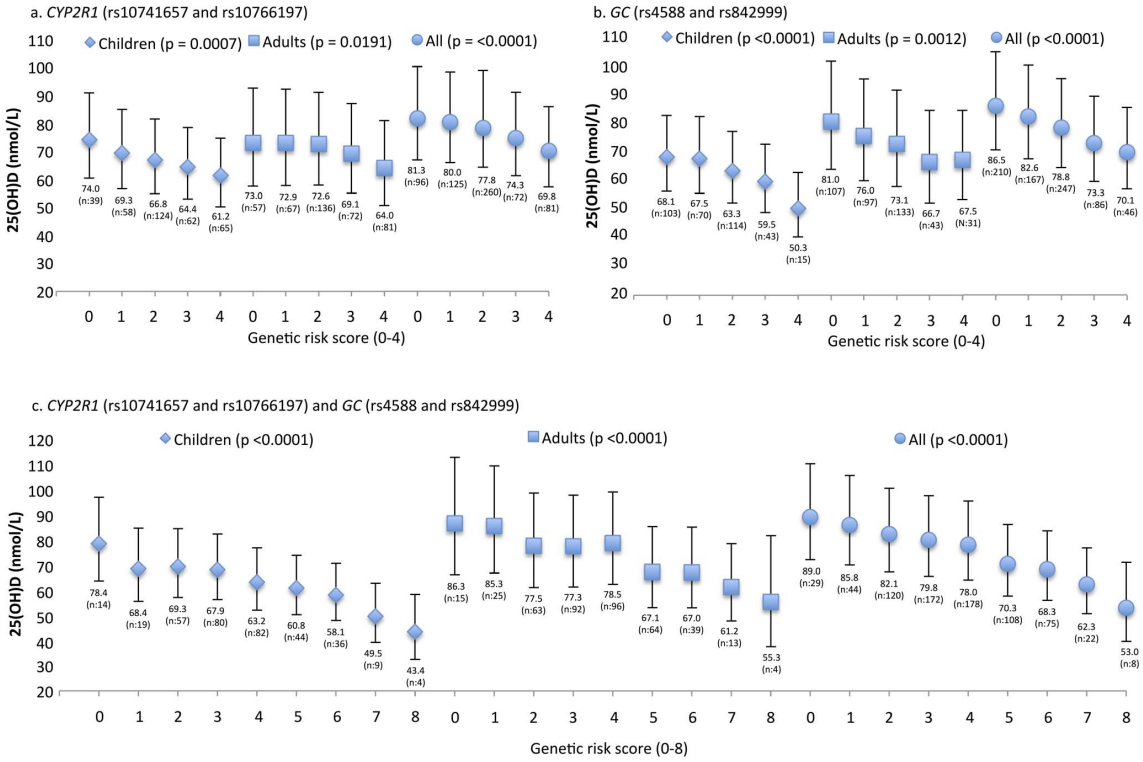

Figure 1. Genetic risk score for *CYP2R1* (rs10741657 and rs10766197) (Figure A), *GC* (r4588 and rs842999) (figure B) and *CYP2R1* (rs10741657 and rs10766197) and *GC* (r4588 and rs842999) (figure C) in children, adults and all combined. X-axis stands for the sum of risk alleles. Y-axis stand for serum 25(OH)D (nmol/L). Errors bars stand for 95%-confidence interval and serum 25(OH)D concentrations are given as geometric means. Linear mixed models with family as a random factor, adjusted for age, sex, BMI, ski and sun holidays, solarium use at least once a week, dietary vitamin D intake, multivitamin and vitamin D supplement users was conducted to compare sum of risk alleles and serum 25(OH)D concentrations. Increasing number of risk alleles give rise to decreasing 25(OH)D concentrations.
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the other SNPs, family and confounding factors in a linear mixed model. The strongest association was observed for rs4588 ($p = 0.0099$) compared to rs2282679 ($p = 0.0230$), rs17467825 ($p = 0.0230$) and rs16846876 ($p = 0.5669$, data not shown). Further analyses only included rs4588. None of the other *GC*-variants were in LD.

The three significant *GC*-variants (rs4588, rs842999, and rs12512631) formed five haplotypes, where haplotype 1 and 2 were the most frequent (Table 4). The combinations of the five haplotypes are shown in table 4. The five haplotypes could explain 723 of the 762 (95%) observed genotype combinations in *GC* (data not shown). The association between haplotype combinations and serum 25(OH)D concentrations was statistically significant in children ($p = 0.0344$), and all combined ($p = 0.0018$) but not in adults ($p = 0.1541$).

Carriers of haplotype combination 22 encompassing the variant alleles of rs4588 and rs842999 had low serum 25(OH)D concentrations. Conversely, carriers of haplotype combination 11 encompassing the variant allele of rs12512631 had high serum 25(OH)D concentration. Thus, the variant allele of rs12512631 was associated with high low serum 25(OH)D concentrations and the variant alleles of rs4588 and rs842999 were associated with low serum 25(OH)D concentrations. Since the lowest serum 25(OH)D concentrations were observed for haplotype combination 22 carriers, this could indicate that rs4588 is the biologically relevant polymorphism rather than rs842999 since haplotype combination 44 encompassing the C-allele of rs842999 is associated with higher serum 25(OH)D concentrations.

The genetic risk score (range 0–4) was calculated as the sum of the number of A-alleles of rs4588 and C/A-alleles of rs842999 (Figure 1, B). After adjustment for family and confounding factors, we found that an increasing number of risk alleles was associated with lower serum 25(OH)D concentrations. Carriers of no risk alleles had significantly higher serum 25(OH)D concentrations (68.1 (56.2–82.6), 81.0 (64.2–102.2) and 86.5 (70.9–105.5) nmol/L) compared to carriers of all four risk alleles (50.3 (40.3–62.7), 67.5 (53.6–84.9) and 70.1 (57.2–84.8) nmol/L) in both children, adults and all combined, respectively. Overall, there was a mean difference in 25(OH)D concentrations of 35.4, 20.0 and 23.4% between carrying no risk alleles and carrying all four risk alleles in children, adults and all combined, respectively.

For the tri-allelic variant rs842999, there was a dose-dependent relationship between serum 25(OH)D concentrations and carriage of none, one or two copies of the G-allele (Figure 2). Thus, carriers of two copies of the G-allele, had statistically significantly higher serum 25(OH)D concentrations (69.2 (56.8–84.3), 79.0 (62.8–99.4) and 84.8 (69.6–103.4) nmol/L) compared to carriers of only one G–allele (65.6 (53.9–79.9), 73.7 (58.8–92.4) and 79.0 (64.9–96.1)

Table 4. Distribution of *GC* haplotype combinations and serum 25(OH)D concentrations in children, adults and all combined.

					Children (n = 215)				Adults (n = 262)				All (n = 488)			
Haplotype-combination	**rs1251 2631**	**rs84 2999**	**rs4588**	**Alleles[1]**		**Raw mean**	**Adj. Mean**			**Raw mean**	**Adj. Mean**			**Raw mean**	**Adj. Mean**	
					N	**25(OH)D[2]**	**25(OH)D[3]**	**P_{adj}**	**N**	**25(OH)D[2]**	**25(OH)D[3]**	**P_{adj}**	**N**	**25(OH)D[2]**	**25(OH)D[3]**	**P_{adj}**
						(95% CI)	**(95% CI)**			**(95% CI)**	**(95% CI)**			**(95% CI)**	**(95% CI)**	
11	CC	GG	CC	mMM	48	78.0 (73.3–82.9)	86.3 (65.7–106.3)	**0.0344**	49	75.6 (69.4–82.5)	71.8 (48.3–106.8)	0.1541	97	76.8 (72.7–81.1)	88.3 (63.3–123.1)	**0.0018**
22	TT	CC	AA	Mmm	15	56.1 (50.3–62.5)	61.6 (47.1–80.7)		31	65.9 (59.2–73.5)	58.2 (40.8–82.9)		46	62.5 (57.8–67.7)	69.3 (50.3–95.4)	
33	TT	GG	CC	MMM	7	69.2 (59.0–81.2)	74.4 (56.9–97.2)		14	69.7 (59.3–82.0)	64.6 (41.8–99.7)		21	69.6 (61.9–78.2)	79.8 (56.0–113.9)	
44	TT	CC	CC	MmM	8	68.9 (59.4–80.0)	69.7 (53.0–91.8)		9	74.9 (61.2–91.7)	66.3 (40.6–108.3)		17	72.0 (63.2–82.1)	78.6 (54.7–113.1)	
55	TT	AA	CC	MmM	0	–	–		1	75.5 (41.2–138.3)	–		1	75.5 (44.1–129.3)	–	
12	TC	GC	CA		65	71.9 (68.2–75.7)			77	74.2 (69.3–79.5)			142	73.1 (69.9–76.5)		
13	TC	GG	CC		48	76.7 (72.1–81.5)			44	77.5 (70.7–84.9)			92	77.1 (72.8–81.5)		
14	TC	GC	CC		30	78.0 (72.3–84.3)			51	79.3 (72.9–86.3)			81	78.8 (74.3–83.7)		
23	TT	GC	CA		34	70.3 (65.4–75.6)			39	69.6 (63.2–76.7)			73	69.9 (65.7–74.5)		
42	TT	CC	CA		33	66.4 (61.6–71.5)			32	66.6 (59.8–74.1)			65	66.5 (62.2–71.1)		
15	TC	GA	CC		11	70.0 (61.6–79.4)			16	67.0 (57.6–77.9)			27	68.2 (61.5–75.6)		
34	TT	GC	CC		15	76.1 (68.3–84.8)			16	72.9 (62.6–84.8)			31	74.4 (67.6–82.0)		
35	TT	GA	CC		8	80.7 (69.5–93.7)			7	60.4 (48.0–75.9)			15	70.5 (61.3–81.0)		
45	TT	CA	CC		5	77.9 (64.6–94.1)			6	52.9 (41.4–67.8)			11	63.1 (53.7–74.2)		
25	TT	CA	CA		2	71.7 (53.2–96.6)			6	58.9 (46.0–75.4)			8	61.9 (51.1–74.8)		

Bold numbers represent significant P values.

Haplotype combinations were manually inferred and numbered. Homozygote haplotype combinations were numbered 11, 22, 33, 44 and 55. The combinations of the heterozygote haplotypes (12 to 45) were given by one number of each homozygote haplotype e.g. 1 + 2 = 12.

[1]*M* major allele, *m* minor allele.

[2]Raw geometric mean of serum 25(OH)D concentrations (nmol/L) and corresponding 95%-confidence interval.

[3]Adjusted geometric mean of 25(OH)D concentrations (nmol/L) and corresponding 95%-confidence interval. Linear mixed models with family as a random factor, adjusted for age, sex, BMI, holiday, use of solarium, dietary vitamin D intake, use of multivitamin and vitamin D supplements.

[adj]Adjusted P values. Haplotype combination 44 was excluded in the linear mixed model due to inadequate participants carrying this haplotype combination.

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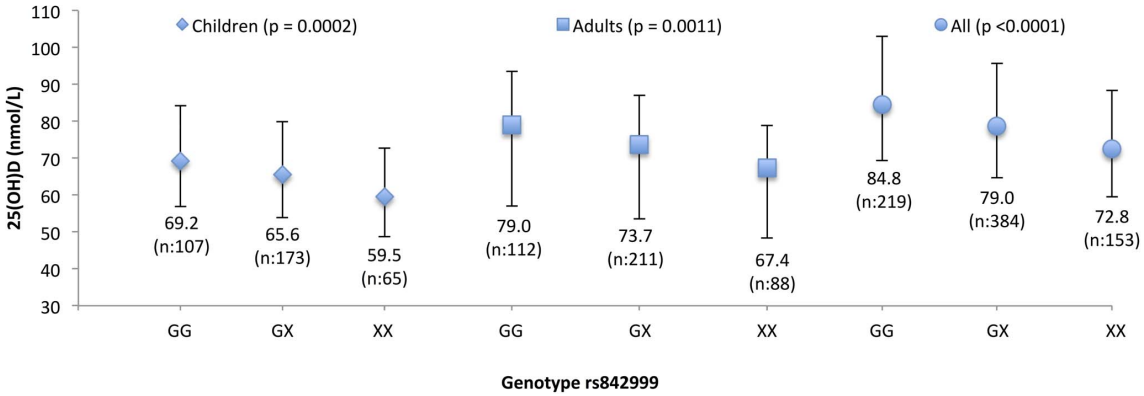

Figure 2. Dose-dependent relationship between genotype GG, GX and XX of rs842999 and serum 25(OH)D concentrations. X-axis stands for genotype GG (GG), GX (GC or GA) and XX (CC, CA or AA) of rs842999. Y-axis stand for serum 25(OH)D (nmol/L). Errors bars stand for 95%-confidence interval and serum 25(OH)D concentrations are given as geometric means. Linear mixed models with family as a random factor, adjusted for age, sex, BMI, ski and sun holidays, solarium use at least once a week, dietary vitamin D intake, multivitamin and vitamin D supplement users was conducted to compare rs842999 genotypes with serum 25(OH)D concentrations. There was a dose-dependent relationship between serum 25(OH)D concentrations and carriers of none, one or two copies of the G-allele. Carriers of two copies of the G-allele, had higher serum 25(OH)D concentrations compared to carriers with only one G-allele or non-carriers in children, adults and all combined, respectively.
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nmol/L) in children, adults and all combined, respectively. The lowest serum 25(OH)D concentrations were observed in non-carriers of the G-allele (59.5 (48.7–72.6), 67.4 (53.8–84.4) and 72.8 (59.7–88.8) nmol/L) in both children, adults and all combined, respectively.

Finally, we made a joint genetic risk score analysis including *CYP2R1* (rs10741657 and rs10766197) and *GC* (rs4588 and rs842999) (Figure 1, C). The genetic risk score (range 0–8) was calculated as the sum of the number of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999 (Figure 1, C). After adjustment for family and confounding factors, carriers of no risk alleles had statistically significantly higher 25(OH)D concentrations (78.4 (63.6–96.7), 86.3 (66.1–112.7) and 89.0 (72.0–110.0) nmol/L) compared to carriers of all eight risk alleles 43.4 (32.4–58.2), 55.3 (37.5–81.4) and 53.0 (39.6–70.9) nmol/L) in children, adults and all combined, respectively. Overall there was a mean difference in 25(OH)D concentrations of 80.6, 56.1 and 67.9% between carriage of no risk alleles and carriage of all four risk alleles in children, adults and all combined, respectively.

Discussion

In this present study, we studied the association of 7 prominent vitamin D-related genes with serum 25(OH)D concentrations in 201 Danish families with dependent children in late summer in Denmark, and found that common variants in *CYP2R1* and *GC* genes were statistically significantly associated with serum 25(OH)D concentrations.

The *CYP2R1* gene encodes the key enzyme that converts vitamin D to 25(OH)D in the liver [12] and thus genetic variation in this gene might affect 25(OH)D synthesis. We found that *CYP2R1* variants rs1562902, rs7116978, rs10741657 and rs10766197, were significantly associated with serum 25(OH)D concentrations in both children, adults and all combined. Furthermore, rs10741657-rs7116978, and rs10766197-rs1562902 were in strong LD. The association appeared to be driven by rs10741657 and rs10766197, which are located in the promoter region of the *CYP2R1* gene. We found that non-carriers of rs10741657 and rs10766197 risk alleles had the highest mean serum 25(OH)D concentrations.

Our results are consistent with previous findings. In the study of Wjst et al. [21], rs10766197 was significantly associated with 25(OH)D concentrations in 872 subjects from the German Asthma Family Study. Ramos-Lopez et al. [32] found a statistically significant association between rs10741657 and serum 25(OH)D concentrations in 203 German diabetes families. Two genome-wide association studies (GWAS) of vitamin D concentrations were published in 2010 [24,25]. Ahn et al. [24] performed a combined meta-analyses in 4,501 subjects from five adult Caucasian cohorts and found that rs2060793, which is in LD with rs10741657 ($D' = 1$, $r^2 = 1$, HapMap Data Rel 24/phase II Nov 08), was associated with serum 25(OH)D concentrations. Furthermore, these findings were successfully replicated in 2,221 subjects. Wang et al. [25] found that rs10741657 was significantly associated with 25(OH)D concentrations in 30,000 subjects of European descent from 15 cohorts. In the study of Bu et al. [33], rs10741657 and rs10766197 were found to be significantly associated with serum 25(OH)D concentrations in 496 unrelated healthy Caucasian subjects. Lasky-Su et al. [34] conducted a combined analysis in 1,164 subjects from two cohorts of Caucasian and Costa Rica asthmatic children and found that rs10741657 was significantly associated with 25(OH)D concentrations. Zhang et al. [35] found that rs10766197 was significantly associated with 25(OH)D concentrations in 2,897 unrelated healthy Chinese subjects from the Shanghai Osteoporosis Study. In the study of Engelman et al. [36], rs2060793 (in LD with rs10741657 as mentioned previously) was significantly associated with 25(OH)D concentrations in 1,204 women of European descent from the Women's Health Initiative Observational Study. All the aforementioned studies demonstrate that variants in the *CYP2R1* gene predicts 25(OH)D concentrations.

The *GC* gene encodes the vitamin D binding protein (DBP) that binds and transports blood 25(OH)D and other vitamin D metabolites to their target organs. Less than 0.04% of blood 25(OH)D circulates in free form (bioavailable). Most is bound with high affinity to DBP (83–85%) and with lower affinity to albumin

(12–15%) [37]. Variants in the *GC* gene may affect the DBP binding and bioavailability of 25(OH)D and other vitamin D metabolites. Thus, there may be a relationship between phenotype and blood 25(OH)D concentrations.

There is accumulating evidence that variants in the *GC* gene are associated with 25(OH)D concentrations. The most studied *GC*-variants are rs4588 and rs7041, giving three common *GC*-isoforms, *GC1F* (rs7041-T, rs4588-C), *GC1S* (rs7041-G, rs4588-C), and *GC2* (rs7041-T, rs4588-A), which differ by amino acid substitutions and/or by glycosylation (Gozdzik et al. 2011). Several studies have shown that vitamin D status differs significantly depending on rs4588 and/or rs7041 genotype, where the A-allele of rs4588 and the T-allele of rs7041 are consistently associated with lower 25(OH)D concentrations [17,38–45]. In agreement, we found that the A-allele of rs4588 is associated with lower 25(OH)D concentrations. There is biological support that the affinity of both 25(OH)D and $1,25(OH)_2D$ is higher for the C-allele of rs4588 than for the A-allele [46]. Based on glycosylation patterns, it is suggested that *GC2* phenotypes that is associated with low vitamin D concentrations should be metabolized faster. Kawakami et al. observed that the metabolic rate was indeed higher in *GC2-2* individuals than in *GC1-1* individuals [47]. In addition, the *GC2* genotype, which is associated with low 25(OH)D concentrations, is also associated with low mean DBP [43]. Strangely, the *GC2* genotype is more frequent in populations living in northern climates [48].

Since the two GWAS studies [24,25] found a strong association between rs2282679 and 25(OH)D concentrations, there has been increased focus on this polymorphism. Several studies have been published supporting the finding [22,34,35,49–51]. The GWAS *GC* variant rs2282679 is in high LD with rs4588. Wang et al. [25] did not include rs4588 because it is not in the HapMap dataset. In one study sample the authors found that rs4588 was in LD with several associated variants from the GWAS study. In the study of Lu et al. [45], rs4588 and rs2282679 ($r^2 = 0.97$) were significantly associated with 25(OH)D concentrations in 3,210 Han Chinese. In the study by Berry et al. [52], rs4588 was in strong LD with rs228697 ($r^2 = 0.98$), and rs4588 was significantly associated with 25(OH)D concentrations in 6,551 subjects from the British birth cohort. Zhang et al. [35] found that 2282679 and rs4588 were in strong LD in 2,897 unrelated healthy Chinese subjects and the strongest association was observed for rs4588, which accounted for 0.7% of the variation in serum 25(OH)D concentrations. Our results support that rs228697 is in strong LD with rs4588 (Pearson's $r = 0.997$, SNAP proxy D' $= 1$ $r^2 = 0.98$) and that the association with serum 25(OH)D concentrations is most likely driven by rs4588. Zhang et al. [35] argued that it is unlikely that rs2282679 in itself is the disease-causing variant. The possible causal variant is the non-synonymous rs4588, where the C/A base pair change in codon 436 (previously known as 420 [36]) causes a Thr to Lys amino acid substitution. In agreement with Zhang et al. [35] we found that rs4588 was the strongest independent predictor of 25(OH)D concentrations compared to rs2282679. Furthermore, Zang et al [35] found that both the minor T-allele of rs4588 and G- allele of rs2282679 were associated with reduced DBP concentrations. Participants with 3 or 4 risk alleles of the two variants were more likely to have vitamin D concentrations lower than 50 nmol/L (20 ng/mL) compared with non-carriers of the risk alleles.

In our study, several of the significant *GC* variants were in strong LD and the strongest associations with serum 25(OH)D concentrations were observed for rs4588 and rs842999. We observed a dose-dependent relationship between carrying none, one or two copies of the G-allele of the tri-allelic rs842999 and 25(OH)D concentrations. Furthermore, genetic risk score analysis for rs4588 and rs842999 showed that non-carriers of the risk alleles of rs4588 and rs842999 had the highest serum 25(OH)D concentrations.

We made a joint genetic risk score analysis for all four risk variants (*CYP2R1*-rs10741657 and rs10766197, and *GC*-rs4588 and rs842999), and found the largest%-range in mean serum 25(OH)D concentrations (80.6, 56.1 and 67.9%) compared to genetic risk score analysis of *CYP2R1* (rs10741657 and rs10766197; 20.9, 14.1 and 16.5%) or *GC* (rs4588 and rs842999; 35.4, 20.0 and 23.4%) indicating an additive effect. In general, there was a better association between genetic risk score and serum 25(OH)D concentrations in children than in adults. We speculate that the more risk alleles in *CYP2R1* and *GC* genes a subject carries, the more prone the subject will be for having a low serum 25(OH)D concentration. In Denmark, sufficient serum 25(OH)D concentrations are defined as >50 nmol/L [53]. Notably, in late summer in Denmark, where vitamin D status peaks in Danes, children carrying 7 or 8 risk alleles had insufficient serum 25(OH)D concentrations (49.4 and 43.4 nmol/L).

In our study population, none of the investigated SNPs in *CYP24A1*, *CYP27B1*, *C10orf88* or *DHCR7/NADSYN1* were associated with serum 25(OH)D concentrations. Furthermore, *VDR*-rs731236 was only statistically significant in all combined and rs757343 was statistically significant in children and all combined. False-positive (type 1 errors) results, which are common in studies of the association between genetic markers and outcomes, and the relative small sample size, resulting in statistical reduced power might explain these findings. We consider children and adults as two natural subpopulations due to biological differences, difference in lifestyle, eating patterns and use of multivitamins [28]. We did not use Bonferroni-corrected P-values because a statistically significant association both in children and in adults by itself may be considered a confirmation of an association. A limitation of the study is that the participants' general vitamin D status relies on a single measurement of serum 25(OH)D concentration. We were not able to calculate the genetic contribution due to the familiar design used in the linear mixed model. A strength of this study is that it is conducted in a healthy Caucasian population and thus the potential impact of diseases is minimized. Furthermore, the blood samples were collected in a relatively small geographical area in Denmark in September to October 2010 and analysed in a single batch with LC-MS/MS with low variation. Furthermore, many known predictors of serum 25(OH)D concentrations were assessed by questionnaire data.

Genetic variants may accelerate or protect against vitamin D deficiency and the genetic effect is life-long. We speculate that individuals with genetically determined low vitamin D concentrations may need different health recommendations in order to improve their serum 25(OH)D concentrations thereby avoiding adverse health outcomes. A study by Engelman et al. [36] found that in women with no risk alleles of rs4588 and rs2060793 (in strong LD with rs10741657 as mentioned previously) who consumed at least 670 IU/d vitamin D all (100%) had 25(OH)D > 50 nmol/L. For women carrying 1, 2 or 3–4 risk alleles and consuming at least 670 IU/d vitamin D, only 84, 72, and 62% had 25(OH)D > 50 nmol/L. Furthermore, the percentage of women with adequate 25(OH)D concentrations rose with each increasing quartile of vitamin D intake. Thus, subjects with genetic predisposition seem to benefit from dietary vitamin D supplementation. In the study by Madsen et al. [28], vitamin D_3-fortification of bread and milk reduced the decrease in serum 25(OH)D concentrations seen during winter and ensured 25(OH)D >50 nmol/L in healthy Danish families. Whether such a dietary intervention program could ensure adequate serum 25(OH)D

concentrations in subjects with genetic predisposition for vitamin D deficiency warrants further study.

Conclusions

In conclusion, our results support the current evidence that common genetic variation in *GC* and *CYP2R1* may contribute to the variation of serum 25(OH)D concentrations in a healthy population. Notably, genetic risk score analysis revealed that non-carriers of risk alleles of *CYP2R1* rs10741657 and rs10766197, and/or *GC* rs4588 and rs842999 had statistically significantly higher serum 25(OH)D concentrations compared to carriers of all risk alleles.

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Author Contributions

Conceived and designed the experiments: LBR GRH RA HM KHM UV JN. Performed the experiments: JN KHM BH. Analyzed the data: JN BH UV. Contributed reagents/materials/analysis tools: JN EWA GRH. Wrote the paper: JN UV.

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RESEARCH PAPER

Real-life use of vitamin D_3-fortified bread and milk during a winter season: the effects of *CYP2R1* and *GC* genes on 25-hydroxyvitamin D concentrations in Danish families, the VitmaD study

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Abstract Common genetic variants rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* and a combined genetic risk score (GRS) of these four variants influence late summer 25-hydroxyvitamin D (25(OH)D) concentrations. The objectives were to identify those who are most at risk of developing low vitamin D status during winter and to assess whether vitamin D_3-fortified bread and milk will increase 25(OH)D concentrations in those with genetically determined low 25(OH)D concentrations at late summer. We used data from the VitmaD study. Participants were allocated to either vitamin D_3-fortified bread and milk or non-fortified bread and milk during winter. In the fortification group, *CYP2R1* (rs10741657) and *GC* (rs4588 and rs842999) were statistically significantly associated with winter 25(OH)D concentrations and *CYP2R1* (rs10766197) was borderline significant. There was a negative linear trend between 25(OH)D concentrations and carriage of 0–8 risk alleles ($p < 0.0001$). No association was found for the control group ($p = 0.1428$). There was a significant positive linear relationship between different quintiles of total vitamin D intake and the increase in 25(OH)D concentrations among carriers of 0–2 ($p = 0.0012$), 3 ($p = 0.0001$), 4 ($p = 0.0118$) or 5 ($p = 0.0029$) risk alleles, but not among carriers of 6–8 risk alleles ($p = 0.1051$). Carriers of a high GRS were more prone to be vitamin D deficient compared to carriers of a low GRS. Furthermore, rs4588-AA carriers have a low but very stable 25(OH)D concentration, and interestingly, also low PTH level.

Keywords SNPs · Vitamin D · 25-Hydroxyvitamin D · D_3-fortification · Genetic risk score · Vitamin D insufficiency

Abbreviations

DBP	Vitamin D-binding protein
GC	Vitamin D-binding protein gene or group-specific component
GRS	Genetic risk score
GWAS	Genome-wide association studies
IOM	Institute of Medicine
LC–MS/MS	Isotope dilution liquid chromatography tandem mass spectrometry
LD	Linkage disequilibrium
NNRs	Nordic nutrition recommendations
PTH	Parathyroid hormone

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RDA	Recommended dietary allowance
RI	Recommended intakes
25(OH)D	25-Hydroxyvitamin D
SNPs	Single-nucleotide polymorphisms
UVB	Ultraviolet B radiation

Introduction

In northern latitudes (>40°N), low vitamin D status in humans, measured as 25-hydroxyvitamin D (25(OH)D) concentrations, is common during winter months. This is because vitamin D cannot be synthesized in the skin due to the lack of solar ultraviolet B radiation (UVB) and because the average dietary intake of vitamin D is insufficient (Thuesen et al. 2012). Moreover, twin- and family-based studies indicate that genetic factors may influence 25(OH)D concentrations appreciably (Engelman et al. 2008; Shea et al. 2009; Karohl et al. 2010). Two genome-wide association studies (GWAS) and several candidate gene studies have shown single-nucleotide polymorphisms (SNPs) to influence 25(OH)D concentrations (Engelman et al. 2008; Sinotte et al. 2009; Bogh et al. 2010; Ahn et al. 2010; Bu et al. 2010; Zhang et al. 2012; Monticielo et al. 2012; Engelman et al. 2013; Zhang et al. 2013; Nissen et al. 2014). These SNPs are located in the group-specific component also known as Gc globulin (*GC*) and in or near genes involved in vitamin D synthesis, activation or degradation. These findings indicate that 25(OH)D concentrations do not only depend on vitamin D intake and sun exposure, but also on genetic factors. Thus, genetic factors may help to identify individuals at risk of low vitamin D status.

We have previously found genetic variants in *CYP2R1* (rs10741657 and rs10766197) and *GC* (rs4588 and rs842999) genes to predict late summer 25(OH)D concentrations in Danish families in a study of 25 SNPs in vitamin D metabolism (Nissen et al. 2014). The main focus of this study is therefore on the influence of rs10741657 and rs10766197 in *CYP2R1*, and rs842999 and rs4588 in *GC* on 25(OH)D concentrations in participants allocated to either vitamin D_3-fortified bread and milk or non-fortified bread and milk during winter.

CYP2R1, a member of the cytochrome P450 family of enzymes, is the primary enzyme that hydroxylates vitamin D to 25(OH)D in the liver. Genetic variants of the *CYP2R1* gene are strongly associated with 25(OH)D concentration (Wjst et al. 2006; Ramos-lopez and Brück 2007; Bu et al. 2010; Zhang et al. 2012, 2013; Nissen et al. 2014) and reached a high score in two GWAS (Ahn et al. 2010; Wang et al. 2010). Furthermore, Ahn et al. (Ahn et al. 2010) observed heterogeneity between different cohorts in the GWAS and the association of 25(OH)D concentration with *CYP2R1*. A missense mutation in *CYP2R1* in exon 2 (L99P) is known to lead to vitamin D deficiency (Cheng et al. 2004).

Genetic variants in the *GC* gene reached the highest score in two GWAS (Ahn et al. 2010; Wang et al. 2010), and several candidate gene studies have found association with 25(OH)D concentrations (Lauridsen et al. 2005; Kurylowicz et al. 2006; Abbas et al. 2008; Engelman et al. 2008; Sinotte et al. 2009; Fu et al. 2009; Gozdzik et al. 2011; Lu et al. 2012; Nissen et al. 2014).

The *GC* gene encodes the vitamin D-binding protein (DBP), which is the primary vitamin D carrier protein. DBP binds with high affinity 85–90 % of circulating 25(OH)D, albumin binds with low affinity 10–15 % of circulating 25(OH)D and less than 1 % of 25(OH)D is in the free form (Bikle et al. 1986). The main function of DBP is to stabilize and prolong the half-life of 25(OH)D and other vitamin D metabolites (Speeckaert et al. 2006). DBP has several other important biological functions including fatty acid transportation, extracellular actin scavenging, leucocyte C5a-mediated chemotaxis, macrophage activation and stimulation of osteoclasts (Pekkinen et al. 2014).

The most studied *GC* SNPs are rs4588 and rs7041 that give rise to three common DBP isoforms, *GC1F* (rs7041-T, rs4588-C), *GC1S* (rs7041-G, rs4588-C) and *GC2* (rs7041-T, rs4588-A), which differ by amino acid composition and glycosylation (Gozdzik et al. 2011). Vitamin D status differed significantly depending on rs4588 (or rs2282679, $r^2 > 0.99$) and/or rs7041 genotypes, where the A-allele of rs4588 and/or the T-allele of rs7041 were consistently associated with lower 25(OH)D concentrations (Lauridsen et al. 2005; Kurylowicz et al. 2006; Abbas et al. 2008; Engelman et al. 2008; Sinotte et al. 2009; Fu et al. 2009; Gozdzik et al. 2011; Lu et al. 2012). In Caucasian, rs4588 and rs7041 are in almost complete linkage disequilibrium (LD) (Haploview software version 4.2). There is biological support that the affinity to both 25(OH)D and $1,25(OH)_2D$ is higher for the rs4588 C-allele isoform than for the A-allele isoform (Arnaud and Constans 1993). Based on glycosylation patterns, it is suggested that the *GC2* phenotype is fast metabolizer. Kawakami et al. (1981) observed that the metabolic rate indeed was higher in *GC2-2* individuals than in *GC1-1* individuals. In addition, the *GC2* genotype, which is associated with lower 25(OH)D concentrations, is also associated with low mean DBP concentration (Lauridsen et al. 2005). The *GC2* and *GC1S* isoforms are more frequent in people with light skin whereas the GC1F isoform is more frequent in people with dark skin (Kamboh and Ferrell 1986).

Measurement of 25(OH)D concentration in blood is currently the best biological marker of vitamin D status and

reflects total vitamin D exposure—from diet, supplements and cutaneous synthesis. Severe vitamin D deficiency (<12 nmol/L) is a medical condition associated with osteomalacia in adults and rickets in children. Vitamin D deficiency can lead to osteoporosis due to increased bone resorption caused by increased serum concentrations of parathyroid hormone (PTH) (Holick 2007). Moreover, vitamin D deficiency is associated with muscle weakness, falls and osteoporotic fractures (Lips and van Schoor 2011). Maintaining a sufficient vitamin D status (>50 nmol/L) is important, not only for bone health, but also because vitamin D deficiency may be associated with various non-skeletal health outcomes (Borradale and Kimlin 2009). Thus, a sufficient vitamin D status may have a disease risk-reduction potential (Grant 2011). Moreover, a U-shaped association exists between 25(OH)D concentrations and risk of cardiovascular disease, certain cancers and overall mortality (Ross et al. 2011).

There is an on-going international discussion regarding which cut-off values should define sufficient 25(OH)D concentrations. There is a general agreement that a 25(OH)D concentration of at least 50 nmol/L is sufficient (Ross et al. 2011; Nordic Council of Ministers 2014). Concurrently, some experts argue that a 25(OH)D concentration >75 nmol/L is required to achieve sufficient vitamin D status and non-skeletal benefits (Holick and Chen 2008; Zhang and Naughton 2010).

It is not easy to determine which doses of vitamin D are required to achieve sufficient 25(OH)D concentrations. The Institute of Medicine (IOM) recently reported that a recommended dietary allowance (RDA) of 15 μg/day for individuals aged 1–70 years will cover the requirement for 97.5 % of the population in the USA and Canada, corresponding to 25(OH)D concentrations of at least 50 nmol/L (Ross et al. 2011). Recently, the recommended intakes (RI) for vitamin D in the Nordic countries were increased from 7.5 to 10 μg/day for individuals aged 2–60 years. This will cover the requirement for 95 % of the Nordic population (Nordic Council of Ministers 2004; Nordic Council of Ministers 2014). Both IOM and Nordic nutrition recommendations (NNRs) 2012 based their RDA and RI on the relationship between 25(OH)D concentrations and bone health.

It is a public health concern that vitamin D intakes in most populations are lower than the RDA or RI (Andersen et al. 2005; Madsen et al. 2013; Nordic Council of Ministers 2014). Food fortification is an effective way to increase vitamin D intake in the general population (O'Mahony et al. 2011), thus ensuring that the general vitamin D intake aligns with the recommendations. During wintertime, a dietary intake of 10 μg/day is needed to maintain 25(OH)D concentrations around 50 nmol/L for the majority of the population in the Nordic countries. For people with little or no sun-exposure, an intake of 20 μg/day of vitamin D is recommended (Nordic Council of Ministers 2014). In Denmark, the mean dietary vitamin D intake is between 2.0 and 2.9 μg/day and does not meet the recommendations for the majority of the population (Tetens et al. 2011). Thus, during wintertime in Denmark, 50–90 % of the population will develop deficient vitamin D status between 30 and 50 nmol/L (Andersen et al. 2005; Thuesen et al. 2012; Madsen et al. 2013).

The main objective of this study was to assess the effect of real-life use of vitamin D_3-fortified bread and milk on 25(OH)D concentrations in relation to common genetic variants in *CYP2R1* (rs10741657 and rs10766197) and *GC* (rs4588 and rs842999) in ethnic Danish families with dependent children during a 6-month winter period and furthermore to assess whether vitamin D supplementation will increase 25(OH)D concentration in those with genetically determined low 25(OH)D concentrations. A secondary objective was to evaluate the amount of vitamin D needed to maintain a sufficient 25(OH)D concentrations >50 nmol/L.

Participants and methods

Study design

The present study used data from the VitmaD intervention conducted in Gladsaxe Municipality in Denmark (latitude 56°N). The study design and methods are described in detail elsewhere (Madsen et al. 2013). Briefly, a double-blinded, randomized placebo-controlled intervention trial with apparently healthy ethnically Danish children and adults recruited as families was randomly allocated to either vitamin D_3-fortified bread and milk or non-fortified placebo bread and milk during a 6-month winter period (September 2010 to April 2011) without sunlight exposure. The aim of the study design was to investigate a realistic D_3-fortification strategy in real-life settings. Participants were instructed to replace their usual consumption of bread and milk with the products provided and in all other aspects, to live a normal life without changing any habits. The study was conducted according to the guideline in the Declaration of Helsinki, and the protocol was approved by the Danish ethics committee (H-4-2010-020) and registered in ClinicalTrials.gov (NCT01184716).

Study population

A total of 201 Danish families with dependent children ($n = 782$), 4–60 years of age, randomly drawn from the Danish Civil Registration System, participated in the study. Inclusion criteria were age between 4 and 60 years and a

permanent address in the Gladsaxe Municipality in Denmark. Exclusion criteria were pregnancy, disease or medication influencing vitamin D metabolism, including dietary supplements with >10 or >5 μg vitamin D/day for children or adults, respectively. All the adult participants and guardians of the children gave written informed consent.

Vitamin D intakes

The participants' vitamin D intakes were obtained from a self-administered web-based questionnaire based on a semi-quantitative food frequency questionnaire (Andersen et al. 2005) at baseline and at the end of the study. Dietary vitamin D intake was calculated based on the self-reported consumption frequencies and dietary contents of vitamin D (National Food Institute, Technical University of Denmark). Vitamin D intake from dietary supplements was calculated as self-reported frequency of use multiplied with the self-reported vitamin D content of the supplements. The contribution of vitamin D from intakes of vitamin D_3-fortified bread and milk was calculated based on the self-reported consumption frequencies, amount and the measured vitamin D contents in the fortified products (5.2 ± 0.3 μg/100 g in wheat bread, 4.3 ± 0.3 μg/100 g in rye bread and 0.38 μg/100 mL in milk) (Madsen et al. 2013). The fortification strategy was to increase vitamin D intake to 7.5 μg/day as recommended in the Nordic nutrition recommendations (NNRs) until September 2013 (Nordic Council of Ministers 2004). Total vitamin D intake was estimated as the sum of dietary vitamin D, usage of multivitamin and vitamin D supplementation and furthermore intake of vitamin D_3-fortified bread and milk for the fortification group.

Biochemical analyses

Non-fasting venous blood samples were drawn, and serum and plasma were stored at −80 °C until analysis at Clinical Biochemical Department, Holbæk Hospital, Denmark. Measurements of serum 25(OH)D concentrations relied on the determination of both $25(OH)D_2$ and $25(OH)D_3$ and were conducted by isotope dilution liquid chromatography tandem mass spectrometry (LC–MS/MS). As primary calibrator, the standard reference material, vitamin D, in humans (SRM 972) from the National Institute of Standards and Technology was used. The analytic quality of 25(OH)D assay was assured by Vitamin D External Quality Assessment Scheme certification, and the mean bias was −3.2 %. The inter-assay CVs for $25(OH)D_2$ were 7.6 and 4.6 % at 43 and 150 nmol/L, respectively, and for $25(OH)D_3$ 2.2 and 2.8 % at 30 and 180 nmol/L, respectively, (Madsen et al. 2013). In Denmark, 25(OH)D concentrations <25 nmol/L are defined as vitamin D deficient, between 25 and 50 nmol/L as vitamin D insufficient and >50 nmol/L as vitamin D sufficient for the majority of the population (National Board of Health 2010). 25(OH)D concentrations can be divided by 2.496 to convert from nmol/L to ng/ml.

Plasma PTH levels (CV: 3.4 %) was measured by using immunology analyser Cobas e601 (Roche Diagnostics), and total calcium (CV 3.4 %) was measured by using a chemistry analyser Cobas c501 (Roche Diagnostics).

SNP selection and genotyping

In a previous study (Nissen et al. 2014), we genotyped 25 SNPs in seven vitamin D-related genes (*CYP2R1*, *CYP24A1*, *CYP27B1*, *C10orf88*, *DHCR7/NADSYN1*, *GC* and *VDR*) selected based on the reports from two GWAS and several candidate gene studies. We found a strong association between common SNPs in *CYP2R1* and *GC* genes and baseline 25(OH)D concentrations in the presently studied 201 healthy Danish families with dependent children. We found that four SNPs, rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC,* predicted baseline 25(OH)D concentrations. None of the four SNPs were in LD with each other: rs10741657 and rs10766196 (Pearson's $r = 0.60$), rs10741567 and rs842999 (Pearson's $r = 0.03$), rs10741657 and rs4588 (Pearson's $r = 0.10$), rs10766197 and rs842999 (Pearson's $r = 0.09$), rs10766197 and rs4588 (Pearson's $r = 0.05$) and rs842999 and rs4588 (Pearson's $r = 0.0.31$) were in LD. For the tri-allelic rs842999, there was a dose-dependent relationship between 25(OH)D concentrations and carriers of none, one or two copies of the G-allele and genotypes are presented as GG, GX and XX, where X represents C- or A-alleles.

DNA was purified from buffy coats as described by Miller et al. (1988). SNPs were genotyped using a Sequenom® platform (San Diego, California) and the iPLEX Gold reaction. The SNPs and the primers used are listed in Supplementary Table 1. Each PCR reaction contained 10 ng genomic DNA, 0.5 U HotStart Taq (Qiagen), 1.25 × Enzyme Buffer (Qiagen), 3.5 mM $MgCl_2$, 1 mM of each deoxynucleotide. The primers were added to a final concentration of 500 nM each. The PCRs were performed at the following cycling parameters: 15 min preheat to 94 °C, 45 cycles (20 s 94 °C, 30 s 56 °C, 1 min 72 °C) followed by 3 min 72 °C and stored at −20 °C. The PCR products were treated with shrimp alkaline phosphatase, dephosphorylate unincorporated dNTPs and extension with molecular weight-modified nucleotides were performed in concordance to the manufacturer's recommendations. The PCRs were cleaned with resin and dispend on SpectroCHIP® bioarrays. The SpectroCHIP® bioarrays were

placed in a MALDI-TOF mass spectrometer, and the results were analysed by MassARRAY Type 4.0 (Sequenom) (Nissen et al. 2014).

Of the 782 recruited children and adults, DNA was obtained from 769 participants (98.3 %). A total of 762 (99.1 %) were successfully genotyped. For quality control, 344 duplicated samples (44 %) were randomly placed throughout each of the 384-well plates and the reproducibility was 100 %. No deviation from Hardy–Weinberg equilibrium was observed for the adult population (χ^2 testing, $p > 0.05$).

Statistical analysis

All statistical analyses were carried out using SAS Enterprise Guide 4.3 (SAS Institute, Inc., Cary. USA). Linear mixed models with family as a random factor were applied in all analyses to account for the non-independency of the participants. Before analysis, 25(OH)D concentrations and PTH levels were log-transformed to approximate a normal distribution and all means are presented as geometric means, unless otherwise specified. A nominal p value of 0.05 was considered statistically significant.

The following categorical variables were used: age (4–11, 12–17, 18–40, 41–60 years), sex (male, female), BMI (underweight, normal weight, overweight, obese) according to standards for children (Cole et al. 2000) and the WHO International standards for adults (World Health Organization 2000) measured at baseline, went on ski and sun vacation during the study period (yes, no), solarium use at least once a week (yes, no) and total calcium at baseline and at the end of the study. The continuous variables are log 25(OH)D concentrations and log PTH levels at baseline and at the end of the study, total vitamin D intake from diet, multivitamins and vitamin D supplements (μg/day).

A genetic risk score (GRS) was calculated as the sum of number of risk alleles. The GRS (range 0–8) was calculated as the sum of number of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999. A linear mixed model, adjusted for family and confounding variables, was fitted to the log 25(OH)D concentration with GRS as an explanatory factor. The

Table 1 Basic characteristics of the study population ($n = 762$)

	Fortification group	Control group	p value
Participants (n)	377	385	–
Female/male (n)	191/186	199/186	0.7771
Age (n)			0.5430
4–10 years	94	91	0.5893
11–17 years	75	88	0.7064
18–40 years	111	87	0.4928
41–60 years	97	119	0.4802
BMI (kg/m^2)	21.7 (21.17–22.3)	21.9 (21.3–22.4)	0.5515
25(OH)D (nmol/L)			
Baseline	72.7 (70.8–74.7)	71.1 (68.9–73.3)	0.4688
End	67.1 (65.2–69.0)	41.5 (39.6–43.5)	**<0.0001**
PTH (ng/L)			
Baseline	35.3 (34.1–36.5)	34.5 (33.3–35.7)	0.2473
End	36.8 (35.5–38.1)	40.1 (38.7–41.6)	**0.0199**
Total calcium (mmol/L)			
Baseline	2.44 (2.43–2.45)	2.45 (2.44–2.46)	**0.0438**
End	2.43 (2.42–2.44)	2.43 (2.42–2.44)	0.8165
Total vitamin D intake (μg/day)			
Baseline	2.9 (2.8–3.1)	2.7 (2.5–2.9)	0.4972
End	11.7 (11.0–12.4)	4.1 (3.8–4.5)	**<0.0001**
Supplement users (n)			
Baseline	127	127	0.7163
End	230	242	0.5991
Ski and sun vacation during the study (n)	135	100	**0.0006**
Solarium users during the study (n)	0	8	**0.0059**
Sunscreen use (n)			
Always/most times/sometimes/seldom	82/108/144/36	105/114/132/32	**0.3361**

All means are presented as geometric means with 95 % confidence interval in parentheses. Continuously variables are tested with t test, and categorical variable are tested with Chi-square

Bold numbers represent significant P values

Table 2 The association between SNPs in *CYP2R1* and *GC* gene, and 25(OH)D concentrations at baseline (all) and at the end of the study (control/fortification)

Baseline						End of study				
Geometric mean 25(OH)D nmol/L (95 % CI)						Geometric mean 25(OH)D nmol/L (95 % CI)				
CYP2R1						**Group**				
rs10741657		**AA**	**GA**	**GG**	P_{adj}		**AA**	**GA**	**GG**	P_{adj}
	All	76.6 (73.0–80.5)	73.9 (71.8–76.1)	67.2 (65.0–69.5)	**<0.0001**	Control	40.6 (36.4–45.3)	43.1 (40.3–46.1)	39.8 (36.8–43.1)	0.1240
		($n = 125$)	($n = 365$)	($n = 268$)			($n = 66$)	($n = 175$)	($n = 128$)	
						Fortification	69.1 (64.3–74.3)	69.7 (67.0–72.5)	63.1 (60.4–66.0)	**0.0130**
							($n = 51$)	($n = 171$)	($n = 133$)	
rs10766197		**GG**	**AG**	**AA**			**GG**	**AG**	**AA**	
	All	74.3 (71.6–77.1)	72.9 (70.8–75.1)	66.9 (64.2–69.8)	**<0.0001**	Control	41.5 (38.2–45.1)	42.6 (39.9–45.6)	38.7 (34.8–43.0)	0.1996
		($n = 221$)	($n = 359$)	($n = 177$)			($n = 116$)	($n = 181$)	($n = 72$)	
						Fortification	68.3 (64.6–72.1)	68.0 (65.3–70.8)	64.3 (61.0–67.7)	0.0599
							($n = 91$)	($n = 164$)	($n = 99$)	
GC										
rs4588		**CC**	**CA**	**AA**			**CC**	**CA**	**AA**	
	All	75.1 (73.1–77.2)	69.9 (67.7–72.1)	61.2 (56.9–62.9)	**<0.0001**	Control	41.2 (38.7–44.0)	41.4 (38.5–44.5)	44.6 (37.1–53.5)	0.4163
		($n = 400$)	($n = 303$)	($n = 55$)			($n = 193$)	($n = 152$)	($n = 24$)	
						Fortification	70.9 (68.3–73.5)	64.6 (61.8–67.4)	55.0 (49.9–60.6)	**<0.0001**
							($n = 191$)	($n = 137$)	($n = 27$)	
rs842999		**GG**	**GX (GA or GC)**	**XX (CC, CA, AA)**			**GG**	**GX (GA or GC)**	**XX (CC, CA, AA)**	
	All	75.4 (72.7–78.3)	72.7 (70.7–74.8)	65.0 (62.2–68.0)	**<0.0001**	Control	40.5 (37.1–44.2)	43.5 (40.8–46.5)	38.0 (34.4–42.0)	0.4099
		($n = 217$)	($n = 383$)	($n = 152$)			($n = 104$)	($n = 185$)	($n = 79$)	
						Fortification	72.4 (68.9–76.1)	66.8 (64.3–69.4)	60.2 (56.5–64.0)	**<0.0001**
							($n = 105$)	($n = 179$)	($n = 67$)	

Bold numbers represent significant *P* values. Major, major homozygotes; het, heterozygotes; Minor, minor homozygotes

P_{adj} linear mixed models with family as a random factor, adjusted for age, sex, BMI, ski and sun vacation, total vitamin D intake estimated as the sum of dietary vitamin D, usage of multivitamin and vitamin D supplementation and for the fortification group intake of vitamin D_3-fortified bread and milk

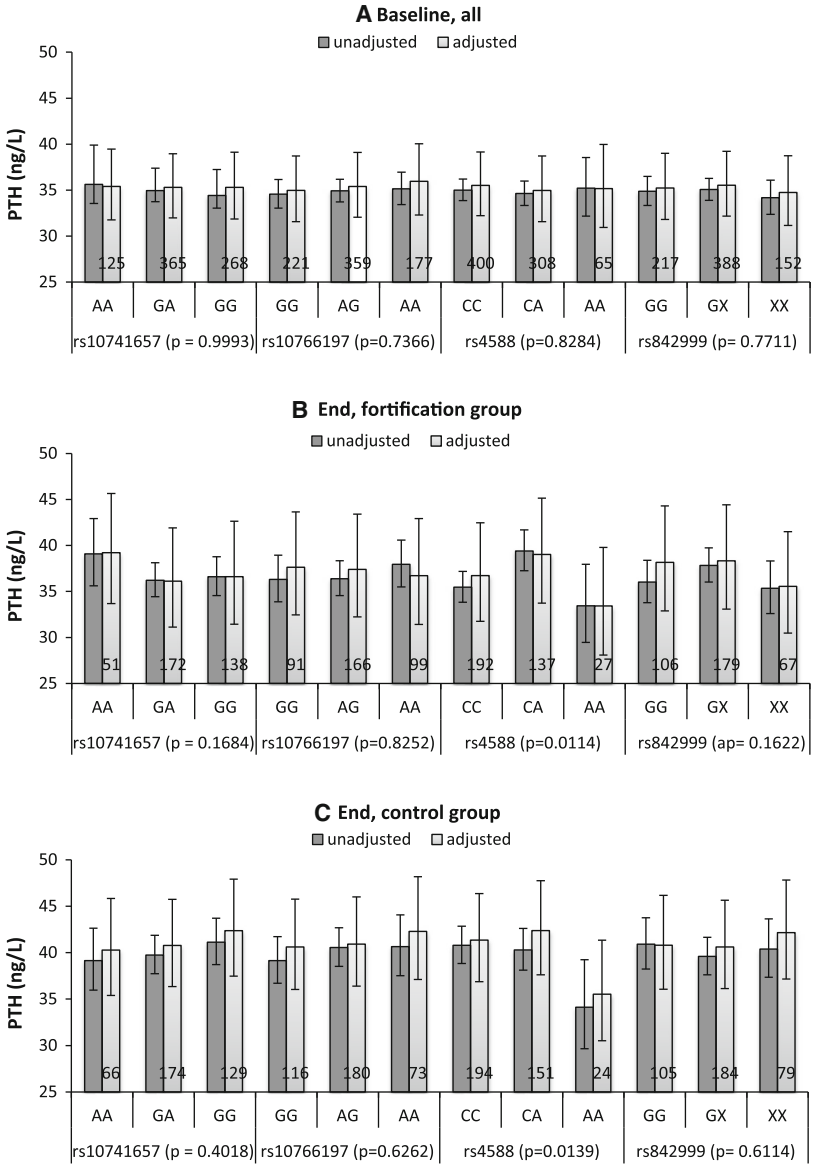

Fig. 1 Association of rs10741657, rs10766197, rs4588 and rs842999 with PTH levels at baseline for all the participants and stratified by fortification and group control at the end of the study. Results are presented as unadjusted and adjusted geometric means. At baseline, the following variables were adjusted for age, sex, BMI, vacation and baseline total calcium, and at end of the study, the following variables were adjusted for age, sex, BMI, vacation, baseline 25(OH)D concentration, baseline PTH levels and end total calcium. Adjusted *p* values are given for each genotype. The *numbers* in the *columns* present the total numbers of participants carrying this genotype. *Error bars* indicate 95 % confidence interval. A statistically significant difference in PTH levels was observed for rs4588 in both the fortification and control group at the end of the study

adjusted mean concentration of 25(OH)D was calculated for each GRS. All the analyses were performed for control and fortification group and separately for adults and children.

Furthermore, each GRS category was stratified by quintile of total vitamin D intake (Q1: 0–2.9 μg/day; Q2: 3–7.4 μg/day; Q3: 7.5–9.9 μg/day; Q4: 10.0–14.9 μg/day; and Q5: >15.0 μg/day). Total vitamin D intake was estimated as the sum of dietary vitamin D, use of multivitamin and vitamin D supplementation and, for the fortification group, intake of vitamin D_3-fortified bread and milk. The final concentration of 25(OH)D was estimated for each GRS by intake groups adjusted for family and confounding variables.

The prevalence (%) of participants with sufficient (>50 nmol/L) 25(OH)D concentrations was estimated for each GRS by intake groups adjusted for family and confounding variables.

Results

Of the 782 recruited children and adults, 762 participants had complete questionnaire data, genotypes and 25(OH)D

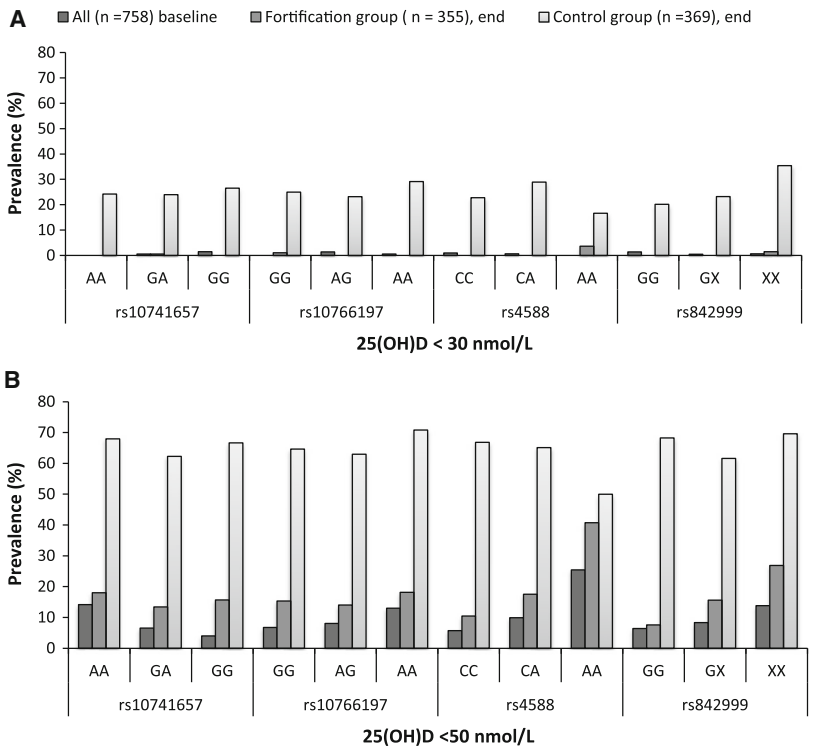

Fig. 2 The prevalence (%) of <30 nmol/L **a** and <50 nmol/L **b** 25(OH)D concentrations in carriers of different genotypes of rs10741657, rs10766197, rs4588 and rs842999 at baseline for all the participants and at the end of the study stratified by control and fortification group. Cut-off value of 25(OH)D <50 nmol/L defines the requirement for optimal bone health for the majority of the population, and cut-off value <30 nmol/L defines the 25(OH)D concentration at which adverse effects on bone health may be expected (Ross et al. 2011)

concentrations measured at baseline. At the end of the study, a total of 756 participants (control group $n = 384$ and fortification group $n = 384$) had complete questionnaire data, genotypes and 25(OH)D concentrations measured. Characteristics of the study population are listed in Table 1, as previously described in detail elsewhere (Madsen et al. 2013; Nissen et al. 2014). At baseline, participants in the control group had significantly higher total calcium levels ($p = 0.0438$) compared to participants in the fortification group, as previously reported (Madsen et al. 2013). Furthermore, there was a statistically significant difference between the control and fortification group for the use of solarium ($p = 0.0059$), and ski and sun vacation ($p = 0.0006$) during the intervention period as previously reported (Madsen et al. 2013).

In a previous study (Nissen et al. 2014), we found that at baseline, *CYP2R1* (rs10741657 and rs10766197) and *GC* (rs4588 and rs842999) were strongly associated with 25(OH)D concentrations among all participants (Table 2). At the end of the study, no associations between SNPs rs10741657 and rs10766197 in *CYP2R1* or rs4588 and rs842999 in *GC* and 25(OH)D concentrations were found for the control group. For the fortification group, rs10741657 in *CYP2R1* and rs4588 and rs842999 in *GC* were statistically significantly associated with 25(OH)D concentrations. The association with *CYP2R1* (rs10766197) was borderline significant ($p = 0.0599$). At the end of the study, total vitamin D intake ($p < 0.0001$) and 25(OH)D concentrations ($p < 0.0001$) were, as expected, significantly higher in the fortified group compared to the control group as previously reported (Madsen et al. 2013).

There was no difference in PTH levels when stratified by rs10741657, rs10766197, rs4588 and rs842999 for all the participants at baseline (Fig. 1). As anticipated, PTH levels were significantly higher in the control group compared to the fortification group ($p = 0.0199$) at the end of the study (Table 1). Furthermore, there was a significant difference in PTH levels for rs4588 in both the fortification group ($p = 0.0064$) and control group ($p = 0.0132$) at the end of the study. Carriers of the rs4588-AA genotype had significantly lower PTH levels compared to carriers of either the rs4588-CA or rs4588-CC genotype.

The prevalence of participants with 25(OH)D concentration <30 nmol/L and <50 nmol/L was estimated for each genotype of rs10741657, rs10766197, rs4588 and rs842999 for all the participants at baseline and separately for the control and fortification group at the end of the study (Fig. 2a, b).

At baseline, there was no difference in the prevalence of participants having 25(OH)D concentrations <30 nmol/L

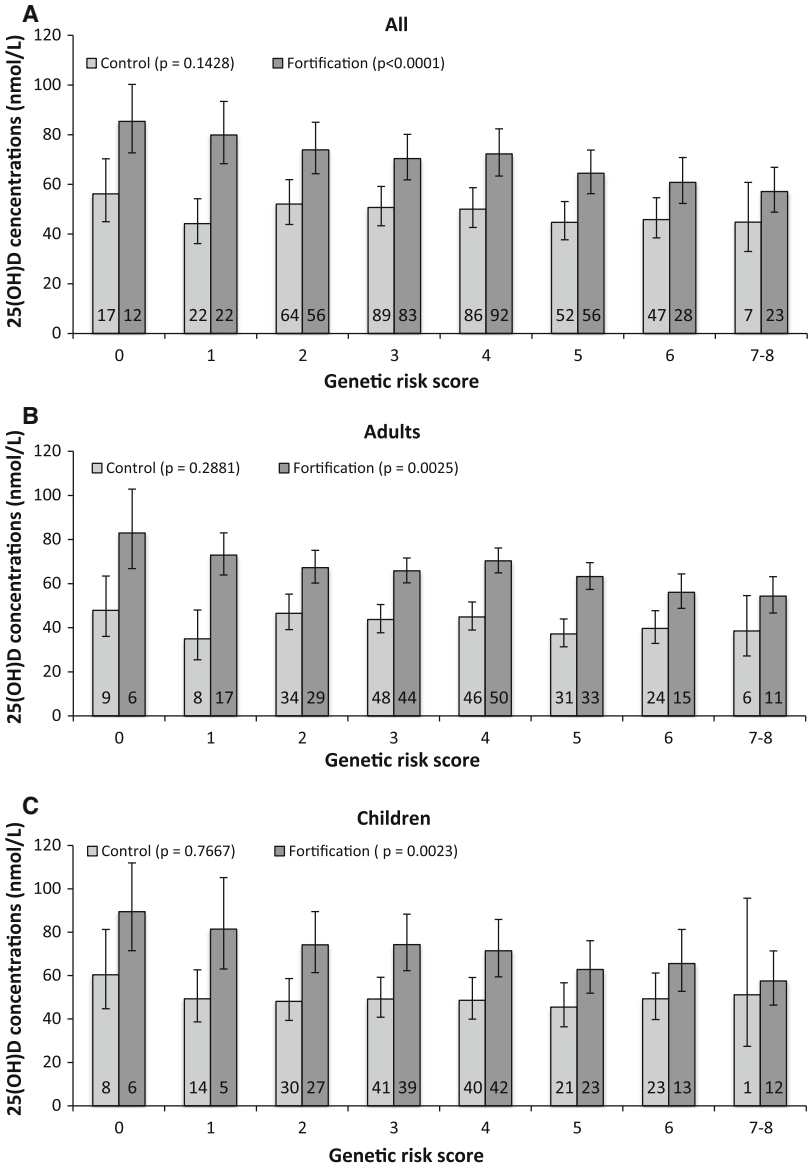

Fig. 3 Estimated mean 25(OH)D concentrations at the end of the study for each genetic risk score category stratified by control and fortification group, separately for all (**a**), adults (**b**) and children (**c**). Individuals carrying 7 or 8 (7–8) risk alleles were combined due to small sample size. Genetic risk score (range 0 to 7–8) was calculated as the sum of number of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999. The *numbers* in the *columns* present the total numbers of participants carrying the risk score. *Error bars* indicate 95 % confidence interval

stratifying by genotype rs10741657, rs10766197, rs4588 and rs842999 ($p = 0.2269$, 0.1715, 0.6953 and 0.5111), respectively. In contrast, there was significant difference in the prevalence of participants having 25(OH)D concentrations <50 nmol/L for rs10741657, rs4588 and rs842999 ($p = 0.0004$, <0.0001 and 0.0435), respectively, and rs10766197 was borderline significantly associated ($p = 0.0743$).

At the end of the study, for the fortification group, a significant difference in the prevalence of participants having 25(OH)D concentrations was found for rs4588 ($p = 0.0023 < 30$ nmol and for <50 nmol/L $p = 0.0002$) and rs842999 ($p = 0.0029 < 50$ nmol/L). No difference in prevalence was observed for rs10741657 and rs10766197 ($p = 0.5830$ and 0.2348 for <30 nmol/L and for <50 nmol/L $p = 0.5466$ and 0.6652), respectively. Furthermore, no difference in prevalence was found for rs842999 ($p = 0.1194$ for <30 nmol/L).

For the control group, only rs842999 <30 nmol/L was significant ($p = 0.0455$). No significant difference was observed for rs10741657, rs10766197 and rs4588 ($p = 0.8694$, 0.6130 and $0.2651 < 30$ nmol/L and

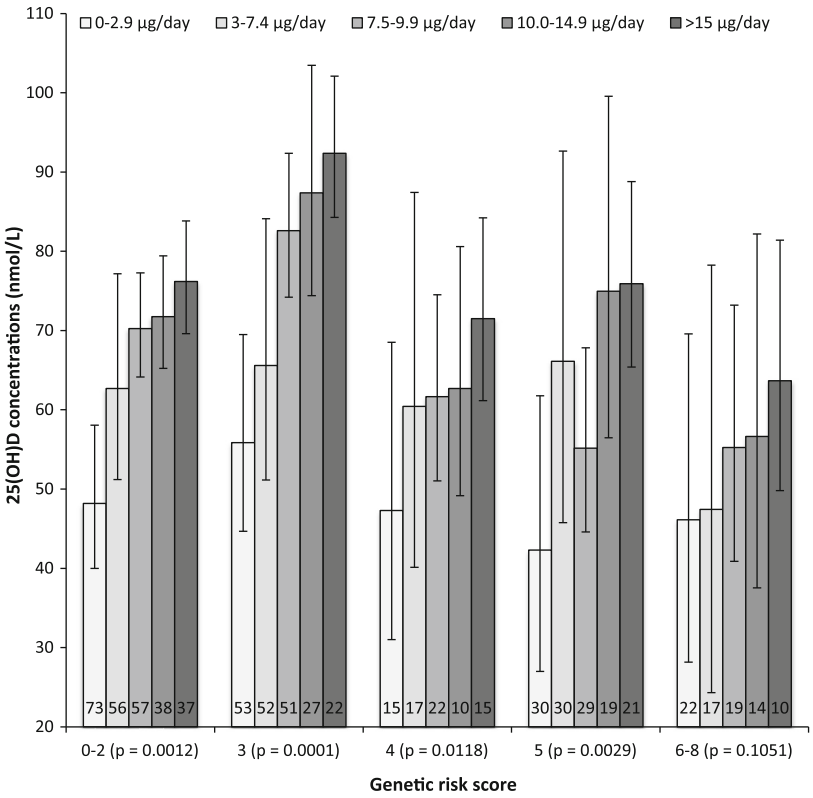

Fig. 4 Mean 25(OH)D concentrations at the end of the study for each genetic risk score category stratified by total vitamin D intakes for the study population. Total vitamin D intake was estimated as the sum of dietary vitamin D, usage of multivitamin and vitamin D supplementation and, for the fortification group, intake of vitamin D_3-fortified bread and milk. The following quintile stratification was used: quintile 1: 0–2.9 µg/day; quintile 2: 3–7.4 µg/day; quintile 3: 7.5–9.9 µg/day; quintile 4: 10.0–14.9 µg/day; and quintile 5: >15.0 µg/day. Genetic risk score (range 0–8) was calculated as the sum of number of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999. Individuals carrying 0, 1 or 2 (0–2) risk alleles and individuals carrying 6, 7 or 8 (6–8) risk alleles were combined due to small sample size after quintile stratification by total vitamin D intake. The *numbers* in the *columns* present the total numbers of participants carrying this risk score. *Error bars* indicate 95 % confidence interval

$p = 0.5645$, 0.4948 and 0.2641 for <50 nmol/L), respectively. Furthermore, rs842999 <50 nmol/L was also found to be non-significant ($p = 0.3402$). In general, the lowest prevalence of vitamin D deficiency <30 and <50 nmol/L was observed at baseline ($p = 0.0001$ and 0.0001), respectively. Participants in the control group presented more often with vitamin D deficiency <30 and <50 nmol/L compared to the fortification group ($p = 0.0001$ for <30 nmol/L and for <50 nmol/L $p = 0.0001$),

At the end of the study, to determine the combined contributions of rs10741657, rs10766197, rs4588 and rs842999, a GRS was calculated individually for the control and fortification group and separately for all, adults and children (Fig. 3a–c). Participants carrying seven or eight (7–8) risk alleles were combined due to small sample size. The coefficients for rs10741657, rs10766197, rs4588 and rs842999 were very similar in a mixed regression model including all SNPs, and therefore, it was not necessary to weight the different risk alleles by the correlation coefficient. A linear mixed model with family as a random factor, adjusted for age, sex, BMI, total vitamin D intake, and ski and sun vacation showed that for the control group, there was no difference in 25(OH)D concentrations for carriers of 0 to 7–8 risk alleles ($p = 0.1428$, 0.2881 and 0.7667) for all, adults and children, respectively. For the fortification group, there was a negative linear trend between 25(OH)D concentrations and carriers of 0 to 7–8 risk alleles for all, adults and children ($p < 0.0001$, 0.0025 and 0.0023, respectively). Overall, there was a mean difference in 25(OH)D concentrations of 28.2, 28.6 and 31.9 nmol/L between carriers of no risk alleles and

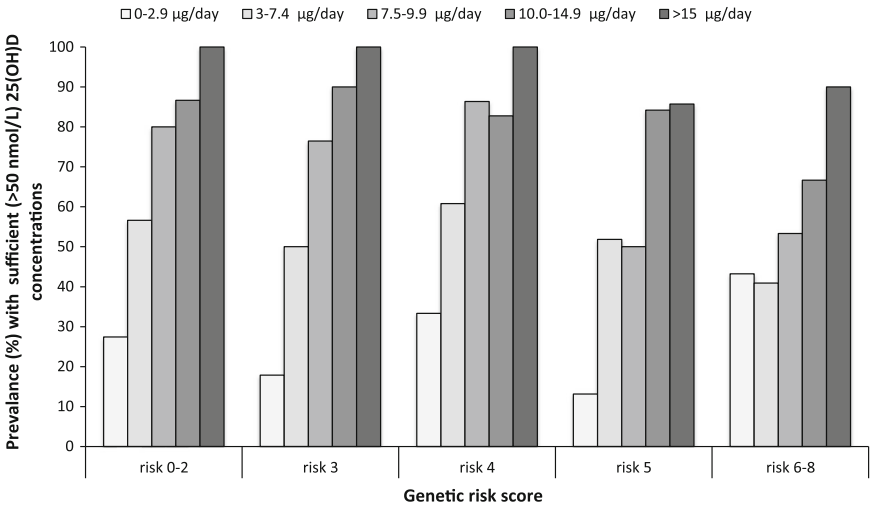

Fig. 5 The prevalence (%) of sufficient 25(OH)D concentrations, defined as >50 nmol/L, for each genetic risk score category stratified by quintile of total vitamin D intake at the end of the study. Total vitamin D intake was estimated as the sum of dietary vitamin D, use of multivitamin and vitamin D supplements and, for the fortification group, intake of vitamin D_3-fortified bread and milk. The following quintile stratification was used: quintile 1: 0–2.9 µg/day; quintile 2: 3–7.4 µg/day; quintile 3: 7.5–9.9 µg/day; quintile 4: 10.0–14.9 µg/day; and quintile 5: >15.0 µg/day. Genetic risk score (range 0–8) was calculated as the sum of number of G-alleles of rs10741657, A-alleles of rs10766197, A-alleles of rs4588 and C/A-alleles of rs842999. Individuals carrying 0, 1 or 2 (0–2) risk alleles and individuals carrying 6, 7 or 8 (6–8) risk alleles were combined due to small sample size after quintile stratification by total vitamin D intake

carriers of all 7–8 risk alleles in all, adults and children, respectively. Overall, the same GRS pattern was observed for adults and children.

We estimated the effect of total vitamin D intake for each category GRS (range 0–8), for the combined contributions of rs10741657, rs10766197, rs4588 and rs842999 (Fig. 4). Each participant was stratified by quintile of total vitamin D intake. Total vitamin D intake was estimated as the sum of dietary vitamin D, use of multivitamin and vitamin D supplements and, for the fortification group, self-reported intake of vitamin D_3-fortified bread and milk. Quintile stratification for total vitamin D intake was based on different RDA or RI: <3 µg/day (no supplementation), <7.5 µg/day (old NNRs 2004), <10 µg/day (present NNRs 2012), <15 µg/day (IOM) or >15 µg/day. The following quintile stratification cut-off values were used: quintile 1: 0–2.9 µg/day; quintile 2: 3–7.4 µg/day; quintile 3: 7.5–9.9 µg/day; quintile 4: 10.0–14.9 µg/day and quintile 5: >15.0 µg/day. The control and fortification groups were combined in the linear mixed model. Individuals carrying 0, 1 or 2 (0–2) risk alleles or individuals carrying 6, 7 or 8 (6–8) risk alleles were combined due to small sample sizes after quintile stratification by total vitamin D intake. A total of 25.1, 22.4, 23.4, 15.6 and 13.6 % of the adult participants carried 0–2, 3, 4, 5 or 6–8 risk alleles, respectively. The majority of the participants in the control group had low total vitamin D intake and were therefore primarily located in the first two quintiles. In general, there was a statistically significant, positive linear relationship between total vitamin D intake and 25(OH)D concentrations among carriers of 0–2, 3, 4 or 5 risk alleles, ($p = 0.0012$, 0.0001, 0.0118 and 0.0029, respectively). For individuals carrying 6–8 risk alleles, there was no statistically significant relationship between total vitamin D intake and 25(OH)D concentrations ($p = 0.1051$).

A the end of the winter season in Denmark, a total vitamin D intake of <3 µg/day was not sufficient for 95 % of the study population to achieve sufficient (>50 nmol/L) 25(OH)D concentrations, regardless of the number of risk alleles they carried (Fig. 4). For participants carrying 0–2 or 3 risk alleles, a total daily vitamin D intake between 3 and 7.4 µg seemed to be sufficient for 95 % of the study population to achieve sufficient 25(OH)D concentrations. For participants carrying four risk alleles, a total daily vitamin D intake >7.5 µg seemed to be sufficient for 95 % of the study population to achieve sufficient 25(OH)D concentrations. For participants carrying five risk alleles, a total daily vitamin D intake >10 µg seemed to be sufficient for 95 % of the study population to achieve sufficient 25(OH)D concentrations. For participants carrying 6–8 risk alleles, a total daily vitamin D intake >15 µg was almost enough for 95 % of the study population to achieve sufficient 25(OH)D concentrations.

In addition, we determined the percentage of participants with sufficient 25(OH)D concentrations (Fig. 5). Sufficient 25(OH)D concentrations were achieved for all

participants carrying 0–2, 3 or 4 risk alleles and who consumed >15 μg/day of vitamin D. For participants carrying 5 or 6–8 risk alleles, this fell to 86 and 90 %, respectively. Furthermore, sufficient 25(OH)D concentrations were achieved for 87, 90, 83, 84 and 67 % of the participants carrying 0–2, 3, 4, 5 or 6–8 risk alleles and who consumed 10–14.9 μg/day. This fell to 80, 76, 86, 50 and 53 % and 57, 50, 61, 52 and 41 % for participants carrying 0–2, 3, 4, 5 or 6–8 risk alleles and who consumed 7.5–9.9 μg/day or 3.0–7.4 μg/day of vitamin D, respectively.

Discussion

In the present study, we show that genetic variation influences 25(OH)D concentrations considerably. Genetically predisposed individuals carrying 6–8 risk alleles of rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* need >15 μg/day or more vitamin D to reach 25(OH)D concentrations >50 nmol/L during winter. Furthermore, there was a statistically significant dose-dependent relationship between 25(OH)D concentration and total vitamin D intake for carriers of 0–5 risk alleles of SNPs rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC*. A dose-dependent relationship was also observed for carriers of 6–8 risk alleles, but the increase in 25(OH)D concentrations was not statistically significant.

At baseline, our study showed that there was statistically significant difference in the prevalence of participants presenting with 25(OH)D concentration <50 nmol/L for rs10741657, rs10766197, rs4588 and rs842999. The significant differences in prevalence disappeared during the winter for the control group, but were maintained for rs4588 and rs842999 in the fortification group. For the fortification group, the highest prevalence of 25(OH)D <50 nmol/L was observed for the rs4588-AA genotype. In contrast, in the control group, rs4588-AA carriers had the lowest prevalence of 25(OH)D <50 nmol/L. This indicates that although carriers of the rs4588-AA genotype in the fortification group were more prone to be vitamin D deficient, rs4588-AA carriers in the control group were less prone to be vitamin D deficient. This may indicate that rs4588-AA carriers have a somewhat low but very stable 25(OH)D concentrations. Paradoxically, a recessive effect was observed for rs4588-AA carriers on PTH levels in both the fortification and control group at the end of the study. Participants with the rs4588-AA genotype have the lowest PTH levels and 25(OH)D concentrations compared to rs4588-CC or rs4588-CA carriers. Similar to our findings, Pekkinen et al. (2014) found a dose–response effect of rs4588 on PTH concentrations in 231 Finnish children and adolescents aged 7–19 years, with rs4588-AA carriers having the lowest PTH and 25(OH)D concentrations. Further studies are warranted to investigate the underlying biological mechanism of this observation.

At the end of the study, there was a pronounced positive effect of real-life usage of vitamin D_3-fortified bread and milk on 25(OH)D concentrations. For the fortification group, 25(OH)D concentrations were significantly associated with rs10741657 in *CYP2R1*, and with rs4588 and rs842999 in *GC*. Furthermore, rs10766197 in *CYP2R1* was borderline significantly associated with 25(OH)D concentrations. These winter results resemble the results found at baseline (late summer) and indicate that when vitamin D is received primarily as vitamin D_3-food fortification during the winter, the association between 25(OH)D concentrations and genetic variation observed at late summer for rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* is maintained. In contrast, the baseline association between 25(OH)D concentrations and rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC* disappeared during the winter for the control group. Our findings are consistent with the findings from two previous studies (Gozdzik et al. 2011; Engelman et al. 2013). Gozdzik et al. (2011) found that rs4588 in *GC* was associated with 25(OH)D concentrations in Canadians of European descent during the fall ($p = 0.009$), but not during the winter ($p = 0.535$). Similarly, Engelman et al. (Engelman et al. 2013) found two SNPs in *GC* (rs4588 and rs7041) and four SNPs in *CYP2R1* (rs105000804, rs11023380, 2060763, 11023374) to be strongly associated with 25(OH)D concentrations in individuals whose blood was drawn in summer but not in individuals whose blood was drawn in winter month.

Engelman et al. (2013) performed a GRS for rs4588 in *GC* and rs2060793 in *CYP2R1*. The risk scores were highly significantly associated with 25(OH)D concentrations in individuals with high external source of vitamin D (>10 μg/day) but not in individuals with low external source of vitamin D (<10 μg/day). In addition, Gozdzik et al. (2011) found that vitamin D intake was significantly predictive of 25(OH)D concentrations in individuals carrying the rs4588 (T436 K) or in *GC* diplotypes during fall and winter. Our results support these findings by Engelman et al. (2013) and Gozdzik et al. (2011). We performed a GRS including the four SNPs rs10741657 and rs10766197 in *CYP2R1* and rs4588 and rs842999 in *GC*. For the fortification group, the GRS was highly significantly associated with 25(OH)D concentrations ($p < 0.0001$) but not for the control group ($p = 0.1428$) during winter. In general, children had higher mean 25(OH)D concentrations compared to adults. For the fortification group, an explanation could be that the children consumed more vitamin D_3-fortified bread and milk compared to the adults.

Approximately 90 % of the total intake of consumed bread and milk was the products provided by the study, with no difference in compliance between children and adults (Madsen et al. 2013). In general, the children were more often multivitamin users compared to the adults (Madsen et al. 2013).

When stratifying total vitamin D intake into quintiles, our data suggest that it is difficult to raise 25(OH)D concentrations to a sufficient level in participants carrying 6–8 risk alleles with vitamin D_3-fortified bread and milk. A statistically non-significant increase in 25(OH)D concentrations was found comparing the lowest and highest quintile of vitamin D intake for participants carrying 6–8, but with a much lower rate (+Δ17.6 nmol/L) compared to participants carrying 0–2, 3, 4 or 5 risk alleles (+Δ28.8, 36.5, 24.2 and 33.6 nmol/L), respectively, (Fig. 4). Whether this also applies for vitamin D synthesized in the skin during UVB exposure remains to be further investigated. These increases are similar to the findings by Engelman et al. (2013). They found that among individuals carrying 3–4 risk alleles of *GC* (rs4588) and *CYP2R1* (rs2060793), the lowest increase in 25(OH)D concentrations was observed in individuals carrying 3–4 risk alleles (+Δ16.7 nmol/L) compared to individuals with fewer risk alleles (+Δ27.7 nmol/L).

In our study population, 67 % of the participants carrying 6–8 risk alleles had sufficient 25(OH)D concentrations in contrast to 87, 90, 83 and 84 % for participants carrying 0–2, 3, 4 or 5 risk alleles, respectively, when following IOMs RDA of 15 μg/day for individuals aged 1–70 years. Following the Nordic countries RI of 10 μg/day for individual aged 2–60 years, only 50 and 53 % of the participants carrying 5 or 6–8 risk alleles, respectively, had sufficient 25(OH)D concentrations compared to 80, 76 and 86 % of the participants carrying 0–2, 3 or 4 risk alleles, respectively. This indicates that genetic predisposition may have a large impact on 25(OH)D concentrations. Participants having a high GRS may need a higher amount of vitamin D supplementation than participants carrying a lower GRS in order to reach sufficient 25(OH)D concentrations. We provide evidence that participants with different genetic profiles need different amounts of vitamin D supplementation to achieve sufficient 25(OH)D concentrations. Epidemiological studies have found association between blood levels of vitamin D concentrations and risk of cancer, but the significance of genetically determined low vitamin D concentration is not clear.

In agreement with our findings, Cranney et al. (2007) concluded that vitamin D_3-doses of 10–20 μg/day may be insufficient to prevent vitamin D deficiency in at-risk individuals. Cashman et al. (2011) concluded that for a population to achieve 25(OH)D concentrations of 50 nmol/L, an average intake of 9 μg/day vitamin D was needed. Nevertheless, taking inter-individual variation into account 23.5 μg/day of vitamin D_3 was needed for 95 % of the population to reach a 25(OH)D concentration of 50 nmol/L. Engelman et al. (2013) found that all of the individuals with no risk alleles of rs4588 and rs2060793 who consumed at least 17 μg/day (670 IU/day) had 25(OH)D >50 nmol/L. This fell to 84, 72 and 62 %, respectively, for individuals carrying 1, 2 or 3–4 risk alleles who also consumed at least 17 μg/day.

Our study has several strengths in that we ensured a large age span (4–60 years), had both genders represented, and both children and adults were included due to the family-based design (Madsen et al. 2013). 25(OH)D concentrations were measured by a specific analytical method (LC–MS/MS). We took into account that non-genetic factors such as vitamin D intake and season are known to influence 25(OH)D concentrations. We estimated total vitamin D intake, and blood samples were drawn during the same seasons for all the participants. A disadvantage is that some of the known predictors of 25(OH)D concentration were quantified by self-reported questionnaire data.

In summary, we found that after consuming vitamin D_3-fortified bread and milk during a winter season, the effect of genetic variation in the *CYP2R1* and *GC* genes on 25(OH)D concentrations resembles the results found in late summer. The association with genetic variation observed for *CYP2R1* and *GC* genes in late summer disappeared during the winter season for the control group. We found that carriers of the rs4588-AA genotype had the highest prevalence of 25(OH)D concentration <50 nmol/L at baseline and at the end of the study for the fortification group. In contrast, rs4588-AA carriers in the control group had the lowest prevalence. It seems like rs4588-AA carriers have a low but very stable 25(OH)D concentration, and interestingly, also low PTH level.

In this study, we demonstrated that carriers of a high GRS of *CYP2R1* (rs10741657 and rs10766197) and *GC* (rs4588 and rs842999) are more prone to be vitamin D deficient compared to carriers of a low GRS. Furthermore, carriers of a high GRS may need a higher amount of vitamin D_3 supplementation to achieve sufficient 25(OH)D concentrations. Importantly, for public health recommendations, it seems that with increasing vitamin D intake, genetically determined low risk carriers with sufficient 25(OH)D concentrations achieve even higher 25(OH)D concentrations with the used real-life vitamin D_3-fortification model.

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Conflict of interest The authors declare that they have no conflict of interest.

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Common variants in CYP2R1 and GC genes are both determinants of serum 25-hydroxyvitamin D concentrations after UVB irradiation and after consumption of vitamin D$_3$-fortified bread and milk during winter in Denmark.

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Common variants in *CYP2R1* and *GC* genes are both determinants of serum 25-hydroxyvitamin D concentrations after UVB irradiation and after consumption of vitamin D_3–fortified bread and milk during winter in Denmark[1–4]

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ABSTRACT

Background: Little is known about how the genetic variation in vitamin D modulating genes influences ultraviolet (UV)B–induced 25-hydroxyvitamin D [25(OH)D] concentrations. In the Food with vitamin D (VitmaD) study, we showed that common genetic variants rs10741657 and rs10766197 in 25-hydroxylase (*CYP2R1*) and rs842999 and rs4588 in vitamin D binding protein (*GC*) predict 25(OH)D concentrations at late summer and after 6-mo consumption of cholecalciferol (vitamin D_3)–fortified bread and milk.

Objectives: In the current study, called the Vitamin D in genes (VitDgen) study, we analyzed associations between the increase in 25(OH)D concentrations after a given dose of artificial UVB irradiation and 25 single nucleotide polymorphisms located in or near genes involved in vitamin D synthesis, transport, activation, or degradation as previously described for the VitmaD study. Second, we aimed to determine whether the genetic variations in *CYP2R1* and *GC* have similar effects on 25(OH)D concentrations after artificial UVB irradiation and supplementation by vitamin D_3–fortified bread and milk.

Design: The VitDgen study includes 92 healthy Danes who received 4 whole-body UVB treatments with a total dose of 6 or 7.5 standard erythema doses during a 10-d period in winter. The VitmaD study included 201 healthy Danish families who were given vitamin D_3–fortified bread and milk or placebo for 6 mo during the winter.

Results: After UVB treatments, rs10741657 in *CYP2R1* and rs4588 in *GC* predicted UVB-induced 25(OH)D concentrations as previously shown in the VitmaD study. Compared with noncarriers, carriers of 4 risk alleles of rs10741657 and rs4588 had lowest concentrations and smallest increases in 25(OH)D concentrations after 4 UVB treatments and largest decreases in 25(OH)D concentrations after 6-mo consumption of vitamin D_3–fortified bread and milk.

Conclusion: Common genetic variants in the *CYP2R1* and *GC* genes modify 25(OH)D concentrations in the same manner after artificial UVB-induced vitamin D and consumption of vitamin D_3–fortified bread and milk. The VitDgen study was registered at clinicaltrials.gov as NCT01741233. The VitmaD study was registered at clinicaltrials.gov as NCT01184716. *Am J Clin Nutr* 2015;101:218–27.

Keywords genetic polymorphism, SNPs, UVB radiation, vitamin D status, 25-hydroxyvitamin D, vitamin D supplements

INTRODUCTION

Vitamin D deficiency is a common health problem in many countries (1). It is well recognized that vitamin D is important for maintaining bone health. Traditional clinical conditions linked to vitamin D deficiency are rickets in children and osteromalacia and osteroporosis in adults (1). A sufficient vitamin D status, which is measured as the 25-hydroxyvitamin D [25(OH)D][5] concentration in blood, may be associated with lower risk of several nonskeletal adverse health outcomes including autoimmune diseases, some cancers, risk of hypertension, and overall mortality (2, 3).

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[3] Supplemental Figures 1 and 2 and Supplemental Table 1 are available from the "Supplemental data" link in the online posting of the article and form the same link in the online table of contents at http://ajcn.nutrition.org.

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[5] Abbreviations used: *CYP2R1*, 25-hydroxylase; *CYP24A1*, 24-hydroxylase; *CYP27B1*, 1-α-hydroxylase; *C10orf88*, open-reading frame 88 on chromosome 10q26.13; *DHCR7*, 7-dehydrocholesterol reductase; *DHCR7/NADSYN1*, 7-dehydrocholesterol reductase/nicotiamide adenine dinucleotide synthetase-1; *GC*, vitamin D binding protein; GRS, genetic risk score; LD, linkage disequilibrium; PCR, polymerase chain reaction; PPF, pigment protection factor; SED, standard erythema dose; SNAP, SNP Annotation and Proxy Search; SNP, single nucleotide polymorphism; *VDR*, vitamin D receptor; VitDgen, Vitamin D in genes; VitmaD, Food with vitamin D; 25(OH)D, 25-hydroxyvitamin D.

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In humans, vitamin D can be obtained from the following 2 natural sources: *1*) the majority of vitamin D is synthesized in the skin after solar UVB exposure; and *2*) dietary intake contributes with a small amount of vitamin D because few natural foods contain significant amounts of vitamin D (1). Furthermore, vitamin D can be obtained from multivitamin tablets, vitamin D supplements, or fortified food products. In Northern countries, vitamin D concentrations follow the seasonal variation in UVB-fluence rates. Vitamin D cannot be synthesized in the skin during the winter months (from October to March) in latitudes above 40°N because of negligible UVB irradiation (4).

Several studies have indicated that the genetic variation at specific genes involved in vitamin D synthesis, transport, activation, or degradation may influence 25(OH)D concentrations appreciably (5). This effect may explain the observed interindividual variation in 25(OH)D concentrations, which seems to be independent of latitude (6). Two genome-wide association studies of vitamin D (7, 8) confirmed associations of common variants at 3 loci in vitamin D binding protein (*GC*; vitamin D transport), 25-hydroxylase [*CYP2R1*; hydroxylation of vitamin D to 25(OH)D] and 7-dehydrocholesterol reductase (*DHCR7*; involved in cholesterol synthesis from 7-dehydrocholesterol) genes. Risk of vitamin D insufficiency more than doubles for individuals carrying all risk alleles of all 3 loci (8), indicating that 25(OH)D concentrations do not only depend on vitamin D intake and UVB exposure but also on the genetic variation. A better understanding of how genetic variation influences 25(OH)D concentration after UVB exposure or consumption of vitamin D supplements is needed and may help to identify individuals who substantially elevated risk of developing vitamin D deficiency.

In the Vitamin D in genes (VitDgen) study [clinicaltrials.gov; NCT01741233], associations between 25 single nucleotide polymorphisms (SNPs) located in or near genes involved in vitamin D synthesis, transport, activation, or degradation and the increase in 25(OH)D concentration after a given dose of artificial UVB irradiation during a winter period of 10-d were examined in 92 healthy Danish adults. Furthermore, the effect of a genetic variation in *CYP2R1* and *GC* on 25(OH)D concentrations was compared for vitamin D acquired from artificial UVB irradiation (the VitDgen study) or from the food with vitamin D (VitmaD) study consumption of cholecalciferol (vitamin D_3)–fortified bread and milk (clinicaltrials.gov; NCT01184716).

SUBJECTS AND METHODS

Study population and design

The main focus of this article is on the VitDgen study, which analyzes the association between the increase in 25(OH)D concentration after a given dose of artificial UVB irradiation and 25 widely studied SNPs located in or near genes involved in vitamin D synthesis, transport, activation, or degradation. Second, the study aimed to determine whether genetic variations in *CYP2R1* and *GC* have similar effects on 25(OH)D concentrations after artificial UVB irradiation and supplementation by vitamin D_3–fortified bread and milk. Data from the VitmaD study were used to analyze the genetic effect on 25(OH)D after 6 mo of consumption of vitamin D_3–fortified bread and milk, which previously have been described (9–12).

VitmaD study

The VitmaD study, which was a double-blinded, randomized, placebo-controlled intervention trial, was conducted in the Gladsaxe Municipality in Denmark (latitude 56°N) from late summer to the end of winter (September 2010 to April 2011). The study design and methods were described in detail elsewhere (9–12), and thus, this article is not the first presentation of the 25(OH) response to vitamin D_3 fortification on the basis of the VitmaD study (12). In brief, healthy, ethnically Danish families were allocated either vitamin D_3–fortified bread (5.2 ± 0.3 μg vitamin D/100 g in wheat bread and 4.3 ± 0.3 μg vitamin D/100 g in rye bread) and milk (0.40 ± 0.01 mg/100 L) or placebo for 6 mo during the winter from September 2010 to April 2011 (**Supplemental Figure 1**). The study was conducted according to the guidelines in the Declaration of Helsinki, and the protocol was approved by the Danish ethics committee (H-4–2010-020). All participants gave written informed consent.

VitDgen study

The VitDgen study was an open and controlled clinical trial conducted at Bispebjerg University Hospital, Copenhagen, Denmark (latitude 56°N) during late winter and early spring (January to March 2013) when natural solar UVB irradiation is negligible (Supplemental Figure 1). Furthermore, the cold winter temperatures prevent solar exposure except on the face and hands. All recruited participants were healthy Danes (aged 18–60 y; men and women) with residence in Denmark. Power calculations indicated that a sample size of 78 participants should be sufficient to detect a mean difference of 20 nmol/L between a genetic outcome at the 5% significance level and with 80% power. There were 102 participants included, and 92 participants completed the study (**Supplemental Figure 2**).

Inclusion criteria were healthy Caucasians between 18–60 y of age. Exclusion criteria were the following: *1*) having a skin disease, *2*) taking a medication that influenced vitamin D metabolism or caused photosensitive skin, *3*) pregnancy or breastfeeding, *4*) having had a sun or ski vacation 3 mo before the study period, or *5*) having taken vitamin D supplements 3 mo before the study period. Participants were allowed to take a daily food supplement that contained ≤10 μg vitamin D. Participants were instructed not to use cosmetic makeup with UV filters or sunscreen when receiving UVB treatment. The study was conducted according to the guidelines in the Declaration of Helsinki, and the protocol was approved by the Danish ethics committee (H-4–2012-071). All participants gave written informed consent.

Skin type, pigmentation, and redness

In the VitDgen study, a skin reflectance meter (UV-Optimize, Scientific, Chromo-light) (13) was used to measure the percentage of redness (range: 0–100%) and the pigment protection factor (PPF; range: 1.0–24.0) on the forehead, shoulder (facultative pigmentations), and buttock (constitutive skin pigmentation) at baseline and 2 d after the last UVB treatment. This assessment was done to follow the skin response to UVB treatments. The percentage of redness reflects hemoglobin concentrations in the skin, and the PPF reflects melanin concentrations in the skin (14, 15).

Self-reported skin-type according to Fitzpatrick's classifications I–IV (16) was registered at baseline. Classifications of erythema and tanning reactions to first exposure in summer where skin type I represents always burn and never tan, skin type II represents usually burn and less tan than average (with difficulty), skin type III represents sometimes mild burns and tan about average, and skin type IV represents rarely burn and tan more than the average (with ease). There were 9 participants with skin type I, 29 participants with skin type II, 39 participants with skin type III, and 14 participants with skin type IV.

UVB exposure

While wearing underwear (underpants and bra for female participants), participants' body surfaces were equally exposed to UV radiation in a UV cabin (Waldmann UV1000L; Waldmann GmbH) equipped with a broadband UVB source consisting of 26 UV6 tubes (Waldmann GmbH) emitting radiation mainly between 290 and 350 nm. During the treatment period, the UV intensity was weekly controlled by using a Sola-Hazard spectroradiometer (Solatell).

A total of 92 participants completed the VitDgen study. During a 10-d period, participants received artificial UVB irradiation 4 times with a 2- or 3-d interval (Monday, Wednesday, Friday, and Monday). Standard erythema doses (SEDs) are a standardized measure of the accumulated erythemally weighted UV energy. One SED is equivalent to an erythemal effective radiant exposure of 100 Jm^{-2} at 298 nm by using the International Committee of Illumination erythema action spectrum and corresponds to a UV dose that causes perceptible erythema in the most–sun-sensitive individuals (17, 18). For example, 1.5 SEDs are equivalent to ~15 min sun exposure in the middle of a clear summer day in Denmark (56°N). A total of 23 participants received a total dose of 7.5 SEDs (1 × 3 SEDs for the upper body and 3 × 1.5 SEDs for the whole body). After the first UVB exposure, 4 participants experienced erythema and withdrew from the study. Therefore, the SED dose was subsequently lowered to 1.5 SEDs and given on the whole body to minimize risk of erythema. Whole-body 1.5 SEDs were well tolerated, and none of the participants experienced erythema after these changes. Seventy-nine participants received a total dose of 6 SEDs (4 × 1.5 SEDs for the whole body). At the end of the study, an additional 6 participants withdrew from the study because of personal and other reasons (Supplemental Figure 2).

DNA extraction and genotyping

DNA was purified from buffy coats as described by Miller et al. (19). SNPs were genotyped by using a Sequenom platform and iPLEX Gold reaction. SNPs and the primers used are listed in **Supplemental Table 1**. Polymerase chain reaction (PCR) amplifications were carried out in 5-μL volumes containing the following: 10 ng genomic DNA, 0.5 U HotStart Taq (Qiagen), 1.25 × Enzyme Buffer (Qiagen), 3.5 mmol/L $MgCL_2$, and 1 mmol/L of each deoxynucleotide, and a final primer concentration of 500 mmol/L for each primer was added (Supplemental Table 1). PCRs were performed at the following cycling variables: a 15-min preheat to 94°C, 45 cycles (20 s at 94°C, 30 s at 56°C, and 1 min at 72°C) followed by 3 min at 72°C, and storage at −20°C. PCR products were treated with shrimp alkaline phosphatase, and the dephosphorylation of unincorporated deoxyribonucleotide triphosphates and an extension with molecular weight-modified nucleotides were performed in accordance with the manufacturer's recommendations. PCR reactions were cleaned with resin and dispended on SpectoCHIP bioarrays (Sequenom). The SpectroCHIP bioarrays were placed in a Matrix-assisted laser desorption/ionization Time of Flight mass spectrometer, and the results were analyzed by using MassARRAY Type 4.0 SNP genotyping (Sequenom) (9).

All SNPs analyzed were located in or near genes involved in vitamin D synthesis, transport, activation, or degradation. The following SNPs were selected because of evidence of a significant association in previous studies: *CYP2R1* (rs7116978, rs10741657, rs1562902, and rs10766197), 24-hydroxylase (*CYO24A1*) (rs6013897, rs4809960, rs2296241, rs17219315, and rs2426496), 1-α-hydroxylase (*CYP27B1*) (rs10877012), open-reading frame 88 on chromosome 10q26.13 (*C10orf88*) (rs6599638), 7-dehydrocholesterol reductase/nicotiamide adenine dinucleotide synthetase-1 (*DHCR7/NADSYN1*) (rs1790349 and rs12785878), *GC* (rs16846876, rs12512631, rs17467825, rs2882679, rs842999-triallelic, rs4588, rs222020, and rs2298849), and vitamin D receptor (*VDR*) [rs731236 (*Taq*I), rs757343 (*Tru*I), rs10783219, and rs7139166). For the triallelic rs842999, there was a dose-dependent relation between 25(OH)D concentrations and carriers of no, 1, or 2 copies of the G allele, and genotypes are presented as GG, GX, and XX, where X represents C or A alleles (9). The linkage disequilibrium (LD) structure was evaluated by using Pearsons' r and the SNP Annotation and Proxy Search (SNAP) version 2.2 (http://www.broadinstitute.org/mpg/snap/ldsearchpw.php).

Genotyping was successful in the 102 recruited participants. For quality control, 10%-duplicated samples were randomly placed throughout each of the 384-well plates, and the reproducibility was 100%. No deviation from the Hardy-Weinberg equilibrium was observed (chi-square test; Bonferroni P of 0.05/25 SNPs = 0.002).

Measurement of 25(OH)D concentrations

Blood samples were obtained without previous fasting, and sera were stored in aliquots at −20°C until analysis. Measurements of 25(OH)D concentrations relied on the determination of both $25(OH)D_2$ and $25(OH)D_3$ and were conducted by isotope-dilution liquid-chromatography–tandem mass spectrometry at the Clinical Biochemical Department, Holbæk Hospital, Holbæk, Denmark. 25(OH)D concentrations were measured at baseline and 48 h after the last UVB treatment.

Standard reference material, vitamin D in humans (SRM972), from the National Institute of Standards and Technology (United States) was used as the primary calibrator. The analytic quality of the 25(OH)D assay was assured by Vitamin D External Quality Assessment Scheme certification, and the mean bias was 5.7%.

Statistical analyses

Statistical analyses were performed with the SAS Enterprise Guide 6.1 application (SAS Institute Inc.). 25(OH)D concentrations were log transformed to approximate a normal distribution, and all means are presented as geometric means. A nominal P value of 0.05 was considered statistically significant. Data from the 2 study-populations VitDgen and VitmaD were analyzed in the same manner to compare how vitamin D status is affected by the genetic risk score (GRS) after UVB exposure or vitamin D supplementation.

In the VitDgen study, univariate models were performed to assess the association between baseline 25(OH)D concentrations and each of the following sun- and vitamin D–related variables: ski or sun vacation in the preceding 6-mo period (yes or no), sun preference (prefers sun, sometimes in the sun, or avoids the sun), sun bathing (yes, sometimes, or no), sunscreen use (always, most of the times, sometimes, or seldom/never), outdoor stay in light clothes (most of the time, often, sometimes, or seldom/never), outdoor transport to work (<15, 15–30, 30–60, or >60 min/d), preferring outdoor life (yes, sometimes, or no), working outdoor (always indoor, sometimes outdoor, or outdoor some of the day), sunbed use during the preceding year (yes or no), PPF buttock, Fitzpatrick's skin type (I–IV), and consuming fish (yes or no). Significant baseline ($P < 0.05$) sun- and vitamin D–related variables were included in a linear mixed model, with the following covariates: sex (male and female), age (18–58 y), BMI (underweight, normal weight, overweight, and obese) according to WHO international standards for adults (20), multivitamin use (yes or no), and vitamin D supplement use in the preceding 6 mo (yes or no). Several of the recruited participants were family members (couples: $n = 30$; parent/children: $n = 9$) and all linear mixed models were analyzed with family as a random factor to account for the nonindependency of these participants. Data on sun- and vitamin D–related variables and, in addition, age, sex, BMI, and multivitamin- and vitamin D–supplement use were obtained from a self-administered web-based questionnaire.

No difference in the increase in 25(OH)D concentrations after UVB treatments between the 2 different UVB treatment groups and sex ($P = 0.8871$, data not shown) was shown, and linear mixed models were combined for the 2 UVB treatment groups and adjusted for the following covariates: age, sex, BMI, family as a random factor, and baseline serum 25(OH)D concentration.

In both studies, a GRS was calculated as the sum of the number of risk alleles. The GRS (range: 0–4) was calculated as the sum of the number of G alleles of rs10741657 and A alleles of rs4588. A linear mixed model, which was adjusted for age, sex, BMI, baseline 25(OH)D concentration, and family as a random factor and, in addition, vacation, vitamin D intake, and vitamin D-supplementation use for the VitmaD study, was fitted to log 25(OH)D concentrations with the GRS as an explanatory factor. Adjusted mean concentrations of 25(OH)D were calculated for each GRS. For the VitmaD study, the GRS was calculated for the adult population (18-60 y) at baseline ($n = 414$) and end of the study only for the adult population who consumed vitamin D_3–fortified bread and milk ($n = 208$). The percentage decrease in vitamin D status in relation to the GRS was analyzed in the adult population who participated in the fortification group.

RESULTS

Out of a total of 102 recruited participants in the VitDgen study, 92 participants completed the study fully (submitted blood samples and genotypes and completed the questionnaire). Baseline characteristics of participants are shown in **Table 1**. At baseline, 51% of subjects had adequate concentrations of vitamin D (>50 nmol/L), 43% of subjects were vitamin D insufficient (25–50 nmol/L), and 5% of subjects were vitamin D deficient (<25 nmol/L). At the end of the study, 97% of subjects had adequate concentrations of vitamin D, 3% of subjects were vitamin D insufficient, and none of the subjects were vitamin D deficient. On average, 25(OH)D concentrations increased 28 nmol/L (95% CI: 24.1, 31.1 nmol/L; data not shown) in response to the 4 UVB sessions.

In univariate models, the baseline 25(OH)D concentration was significantly associated with BMI ($P = 0.032$), multivitamin use ($P = 0.011$), and vitamin D–supplement use (supplementation ≤ 10 μg/d was allowed; $P = 0.0014$) and borderline significantly associated with outdoor stay in light clothes ($P = 0.063$), outdoor transport to work ($P = 0.051$), and sun bathing ($P = 0.051$). No associations were shown between baseline 25(OH)D concentrations and skiing or a sun vacation (compared with no vacation; $P = 0.23$), Fitzpatrick's skin-type classifications I–IV ($P = 0.78$), PPF buttock ($P = 0.60$), fish intake ($P = 0.34$), sunbed use ($P = 0.78$), sun preference ($P = 0.14$), sunscreen use ($P = 0.96$), working indoors ($P = 0.16$), employment ($P = 0.17$), or preferring outdoor life ($P = 0.27$), and thus, these variables were not included in the linear mixed models.

In a linear mixed model, there was no significant difference between the baseline 25(OH)D concentration in analyzed genotypes, except for rs12512631 in *GC,* after adjustment for the following variables: age, sex, BMI, use of multivitamin and vitamin D supplement, outdoor stay in light clothes, and sun bathing (**Table 2**). No significant difference was shown for age, sex, and outdoor transport to work for all analyzed genotypes (data not shown).

In a linear mixed model adjusted for age, sex, BMI, and baseline 25(OH)D, there was a significant association between end-of-study 25(OH)D concentrations and genotypes of rs10741657 in *CYP2R1* and rs16846876, rs17467825, rs2282679 and rs4588 in *GC* after 4 UVB treatments (Table 2). All 4 SNPs in *GC* were in strong LD. SNP rs4588 was in strong LD with rs2282679 (Pearson's $r = 0.99$, SNAP $R^2 = 0.98$, $D' = 1.00$) and rs17467825 (Pearson's $r = 0.99$, SNAP $R^2 = 1.00$, $D' = 1.00$). Furthermore, rs17467825 and rs2282679 (Pearson's $r = 1.00$, SNAP $R^2 = 1.00$, $D' = 1.00$) as well as rs2282679 and rs16846876 (Pearson's $r = 0.69$, SNAP $R^2 = 0.44$, $D' = 0.68$) were in LD. We previously showed that rs4588 had the strongest association with 25(OH)D concentrations (9). Additional analyses only included rs4588 in *GC* and rs10741657 in *CYP2R1*. None of the analyzed SNPs in *CYP24A1*, *CYP27B1*, *C10orf88*, *DHCR7/NADSYN1*, or *VDR* genes were significantly associated with the final 25(OH)D concentration.

For the rs10741657 polymorphism, highest end-of-study 25(OH)D concentrations were shown for participants carrying the rs10741657 AA genotype (93.7 nmol/L; 95% CI: 84.0, 104.6 nmol/L), intermediate concentrations were shown in participants carrying the rs10741657 GA genotype (81.9 nmol/L; 95% CI: 75.5, 88.9 nmol/L), and lowest concentrations were shown in participants carrying the rs10741657 GG genotype (77.0 nmol/L; 95% CI: 70.9, 83.5 nmol/L). For the rs4588 genotype, highest end-of-study 25(OH)D concentrations were shown in participants carrying the rs4588CC genotype (84.1 nmol/L; 95% CI: 78.3, 90.4 nmol/L), intermediate concentrations were shown in participants carrying the rs4588 CA genotype (83.5 nmol/L; 95% CI: 77.0, 90.6 nmol/L), and lowest concentrations were shown in participants carrying the rs4588 AA genotype (65.7 nmol/L; 95% CI: 54.5, 79.3 nmol/L) (Table 2).

To determinate combined effects of rs10741657 and rs4588 in the VitDgen study, a GRS was calculated as the sum of the

TABLE 1
Characteristics of the VitDgen study population[1]

	All (n = 92)		F (n = 60)		M (n = 32)	
	n	Value	n	Value	n	Value
Age, y	92	38.6 ± 12.0[2]	60	38.1 ± 11.6	32	39.6 ± 12.9
BMI,[3] kg/m^2						
Underweight (<18.5)	3	18.0 ± 0.4	2	18.0 ± 0.6	1	18.0
Normal weight (18.5–24.9)	57	22.1 ± 1.8	41	22.1 ± 1.9	16	22.3 ± 1.8
Overweight (25.0–29.9)	23	26.7 ± 1.3	11	26.8 ± 1.2	12	26.6 ± 1.5
Obese (>30.0)	9	33.6 ± 3.9	6	32.8 ± 4.1	3	35.5 ± 3.5
Baseline 25(OH)D, nmol/L						
>50	47	78.1 ± 21.8	32	80.6 ± 22.6	15	72.8 ± 19.6
25–50	40	38.2 ± 7.1	25	38.2 ± 7.2	15	38.2 ± 7.1
<25	5	20.4 ± 4.2	3	18.3 ± 4.4	2	23.5 ± 0.7
End 25(OH)D, nmol/L						
>50	89	86.5 ± 22.5	57	87.4 ± 25.7	32	84.8 ± 15.4
25–50	3	46.3 ± 2.9	3	46.3 ± 2.9	—	—
<25	—	—	—	—	—	—
Sun or ski vacation,[4] n (%)		45 (49)		31 (52)		14 (44)
Supplement users 6 mo before the intervention, n (%)						
Multivitamins		19 (21)		14 (23)		5 (16)
Vitamin D		8 (9)		5 (8)		3 (9)
Consuming fish, n (%)						
Yes, total		86 (95)		58 (97)		28 (90)
1–2 times/wk		60 (66)		40 (67)		20 (65)
≥3 times/wk		26 (29)		18 (30)		8 (26)
No		5 (5)		2 (3)		3 (10)
Fitzpatrick skin type, n (%)[5]						
I		9 (10)		6 (10)		3 (9)
II		29 (32)		20 (34)		9 (28)
III		39 (43)		25 (42)		14 (44)
IV		14 (15)		8 (14)		6 (19)
PPF[6]						
Forehead	92	5.5 ± 1.5	60	5.5 ± 1.6	32	5.5 ± 1.3
Shoulder	92	5.1 ± 1.4	60	5.4 ± 1.3	32	4.6 ± 1.4
Buttock	92	3.4 ± 1.1	60	3.6 ± 1.1	32	3.1 ± 1.0
Sunbed use in 2012, n (%)						
Did not use a sunbed		83 (90)		52 (87)		31 (97)
1–4 times		3 (3)		2 (3)		1 (3)
≥5 times		6 (7)		6 (10)		—

[1]PPF, pigment protection factor; VitDgen, Vitamin D in genes; 25(OH)D, 25-hydroxyvitamin D.
[2]Geometric mean ± SD (all such values).
[3]On the basis of WHO international standards for adults (20).
[4]Ski or sun vacation 6 mo before the study in places where dermal vitamin D production was expected.
[5]Fitzpatrick skin type categorization on the basis of sun-reactive types I–IV (16).
[6]PPF (range: 1.0–24.0) reflects melanin concentrations in the skin at baseline.

number of G alleles of rs10741657 and A alleles of rs4588 (range: 0–4) at baseline and final (**Figure 1**A). Coefficients of rs10741657 and rs4588 were very similar in a mixed regression model including both SNPs, and therefore, it was not necessary to weight risk alleles by the correlation coefficient (data not shown). At baseline, there were no associations between GRS and 25(OH)D concentrations (P = 0.16). At the end of the study, there was a linear negative trend between the 25(OH)D concentration and number of risk alleles (0-4 risk alleles; P = 0.0045). Overall, there was a mean difference in 25(OH)D concentrations of 20.9 nmol/L between carriers of no risk alleles and carriers of all 4 risk alleles. Furthermore, there was a significant linear negative trend between the increase in 25(OH)D concentration and the GRS (P = 0.042) (Figure 1B). The lowest increase in 25(OH)D concentrations was observed for carriers of all 4 risk alleles.

To evaluate the effect of rs10741657 and rs4588 on 25(OH)D concentrations at baseline and after 6 mo consumption of vitamin D_3–fortified bread and milk, data from the adult population of the VitmaD study (15, 20, 21) were used and analyzed in the same manner as previously described for the VitDgen study. At baseline (late summer; all adults: n = 414), there was a linear negative trend between the 25(OH)D concentration and carriage of 0–4 risk alleles ($P < 0.0001$) (Figure 1C). After a 6-mo consumption of vitamin D_3–fortified bread and milk (only adults in the fortification group: n = 208), there was still a linear negative trend between the 25(OH)D concentration and carriage of 0–4 risk alleles (P = 0.0270). With the use of a realistic vitamin D_3–fortification model, a decrease in 25(OH)D concentrations was observed during the winter, and the largest percentage decrease was observed for carriers of all 4 risk alleles (Figure 1D).

TABLE 2
Basic characteristics of individual SNPs and associations with 25(OH)D concentrations in the VitDgen study population (n = 92)[1]

						Baseline (day 0)		End (day 10)	Increase in 25(OH)D[2]	
SNP	HWE, P	MAF, %	M/m	Genotype	n	25(OH)D	P-adjusted[3]	25(OH)D	25(OH)D	P-adjusted[4]
CYP2R1										
rs7116978	0.11	39.5	C/T	CC	37	50.8 (43.8, 58.9)[5]	0.32	78.4 (72.3, 85.0)	22.6 (18.3, 27.8)	0.10
				CT	35	50.5 (43.4, 58.7)		80.5 (74.0, 87.5)	27.2 (21.8, 34.1)	
				TT	18	58.1 (47.0, 71.7)		93.4 (83.1, 104.9)	29.8 (21.7, 40.9)	
rs10741657	0.07	41.4	G/A	GG	36	50.2 (43.1, 58.4)	0.28	77.0 (70.9, 83.5)	21.7 (17.7, 26.8)	0.024[6]
				GA	36	50.2 (43.2, 58.5)		81.9 (75.5, 88.9)	28.6 (23.0, 35.5)	
				AA	20	57.9 (47.3, 71.0)		93.7 (84.0, 104.6)	30.7 (22.9, 41.2)	
rs1562902	0.35	43.8	T/C	TT	32	49.8 (42.4, 58.4)	0.84	79.0 (72.3, 86.3)	25.6 (20.4, 32.1)	0.32
				TC	40	49.8 (43.1, 57.4)		81.2 (75.0, 87.9)	26.8 (21.7, 33.2)	
				CC	20	59.9 (48.9, 73.3)		90.4 (80.1, 101.1)	24.6 (18.4, 33.0)	
rs10766197	0.39	48.9	G/A	GG	22	56.0 (46.1, 67.9)	0.40	87.3 (78.5, 97.1)	25.2 (19.0, 33.3)	0.13
				AG	49	52.5 (46.1, 59.7)		83.7 (77.9, 89.9)	26.5 (21.9, 32.1)	
				AA	21	46.4 (38.0, 56.5)		74.5 (66.8, 83.0)	25.3 (19.1, 33.7)	
CYP24A1										
rs6013897	0.83	20.5	T/A	TT	60	50.4 (44.8, 56.7)	0.07	81.9 (76.7, 87.5)	27.3 (23.1, 32.2)	0.70
				AT	28	53.6 (45.1, 63.7)		83.0 (75.4, 91.5)	25.2 (19.8, 32.0)	
				AA	4	61.1 (38.7, 96.5)		83.1 (83.1, 107.3)	12.3 (6.0, 25.2)	
rs4809960	0.11	23.7	T/C	TT	58	51.8 (46.0, 58.4)	0.45	82.5 (77.1, 88.2)	25.8 (21.7, 30.5)	0.26
				TC	31	53.1 (45.1, 62.6)		81.2 (74.1, 89.0)	24.4 (19.4, 30.9)	
				CC	8	39.9 (23.6, 67.6)		91.0 (67.8, 122.2)	49.6 (24.0, 102.5)	
rs2296241	0.26	46.0	G/A	GG	24	44.5 (37.1, 53.5)	0.16	77.5 (70.0, 80.1)	25.2 (19.5, 32.7)	0.39
				AG	52	55.7 (49.1, 63.0)		82.8 (77.2, 88.8)	24.5 (20.3, 29.4)	
				AA	16	51.5 (41.2, 64.5)		88.1 (77.6, 99.9)	31.8 (23.2, 43.6)	
rs17219315	0.78	2.8	A/G	AA	87	51.7 (46.8, 57.0)	0.53	82.0 (77.6, 86.6)	25.7 (22.3, 29.6)	0.29
				AG	5	54.4 (36.1, 82.0)		87.5 (69.6, 110.0)	29.2 (16.5, 51.7)	
rs2426496	0.29	23.3	G/T	GG	54	48.9 (43.2, 55.3)	0.44	78.8 (73.6, 84.3)	24.3 (20.4, 28.9)	0.25
				GT	35	56.7 (48.6, 66.1)		86.9 (79.9, 94.6)	27.8 (22.2, 34.8)	
				TT	3	51.8 (30.7, 87.4)		95.9 (71.9, 128.1)	37.4 (18.0, 77.7)	
CYP27B1										
rs10877012	0.97	35.2	G/T	GG	41	50.4 (43.7, 58.1)	0.38	81.3 (75.1, 88.1)	23.8 (19.4, 29.2)	0.91
				GT	40	50.7 (43.9, 58.6)		82.0 (75.7, 88.9)	28.3 (23.0, 34.7)	
				TT	11	61.9 (47.1, 81.4)		87.1 (74.7, 101.6)	25.8 (17.2, 38.5)	
C10orf88										
rs6599638	0.29	49.4	G/A	GG	20	52.5 (42.8, 64.5)	0.48	80.3 (71.7, 90.0)	23.3 (17.4, 31.2)	0.31
				GA	51	52.5 (46.2, 59.7)		84.2 (78.4, 90.4)	28.4 (23.6, 34.0)	
				AA	21	49.6 (40.6, 60.5)		79.7 (71.3, 89.0)	23.0 (17.3, 30.5)	
DHCR7/NADSYN1										
rs1790349	0.02	15.3	A/G	AA	69	50.6 (45.4, 56.5)	0.35	82.2 (77.3, 87.4)	26.5 (22.7, 31.1)	0.70
				GA	18	56.8 (45.8, 70.5)		84.7 (75.1, 95.5)	23.8 (17.3, 32.8)	
				GG	5	51.0 (33.9, 76.7)		76.0 (60.5, 95.5)	24.2 (13.7, 42.9)	
rs12785878	0.32	28.4	T/G	TT	49	51.2 (44.9, 58.3)	0.77	81.6 (75.9, 87.8)	27.4 (22.7, 33.1)	0.97
				GT	34	52.4 (44.7, 61.3)		83.3 (76.3, 91.0)	24.2 (19.3, 30.3)	
				GG	9	53.2 (39.2, 72.2)		82.2 (69.3, 97.5)	24.6 (16.1, 37.7)	
GC										
rs16846876	0.16	38.6	A/T	AA	32	58.8 (50.2, 68.8)	0.41	92.2 (84.6, 100.4)	26.6 (21.1, 33.4)	0.026[6]
				AT	50	50.0 (44.1, 56.8)		78.8 (73.6, 84.3)	25.5 (21.1, 30.8)	
				TT	10	41.2 (31.3, 54.6)		71.2 (61.2, 83.0)	25.7 (17.2, 38.6)	
rs12512631	0.07	31.6	T/C	TT	38	43.4 (37.7, 49.9)	0.025[6]	74.6 (69.1, 80.6)	26.3 (21.2, 32.5)	0.13
				TC	49	57.3 (50.6, 64.8)		86.1 (80.5, 92.1)	25.2 (20.9, 30.4)	
				CC	5	79.8 (50.9, 110.0)		111.3 (90.1, 137.5)	30.1 (17.0, 53.4)	
rs17467825	0.96	28.4	A/G	AA	49	53.0 (46.5, 60.4)	0.50	83.9 (78.2, 90.1)	24.4 (20.3, 29.4)	0.020[6]
				GA	36	51.3 (44.1, 59.8)		83.7 (77.1, 90.9)	29.1 (23.5, 36.2)	
				GG	7	46.2 (32.7, 65.3)		65.7 (54.5, 79.3)	20.9 (12.4, 35.0)	
rs2282679	0.96	28.4	A/C	AA	49	53.0 (46.5, 60.4)	0.50	83.9 (78.2, 90.1)	24.4 (20.3, 29.4)	0.020[6]
				CA	36	51.3 (44.1, 59.8)		83.7 (77.1, 90.9)	29.1 (23.5, 36.2)	
				CC	7	46.2 (32.7, 65.3)		65.7 (54.5, 79.3)	20.9 (12.4, 35.0)	

(Continued)

TABLE 2 (*Continued*)

SNP	HWE, *P*	MAF, %	M/m	Genotype	*n*	Baseline (day 0) 25(OH)D	Baseline (day 0) *P*-adjusted[3]	End (day 10) 25(OH)D	Increase in 25(OH)D[2] 25(OH)D	Increase in 25(OH)D[2] *P*-adjusted[4]
rs842999	0.14	44.1	G/C/A	GG	25	54.3 (45.4, 65.1)	0.42	82.5 (74.4, 91.4)	24.0 (18.6, 30.9)	0.17
				GX[7]	50	53.1 (46.7, 60.3)		84.2 (78.3, 90.5)	25.8 (21.4, 31.1)	
				XX[8]	13	49.7 (38.7, 63.9)		75.7 (65.7, 87.3)	25.9 (17.9, 37.4)	
rs4588	0.84	29.0	C/A	CC	48	53.3 (46.7, 60.8)	0.57	84.1 (78.3, 90.4)	24.3 (20.1, 29.2)	0.020[6]
				CA	37	51.0 (43.9, 59.3)		83.5 (77.0, 90.6)	29.3 (23.6, 36.2)	
				AA	7	46.2 (32.7, 65.3)		65.7 (54.5, 79.3)	20.9 (12.5, 34.9)	
rs222020	0.84	22.2	T/C	TT	55	54.7 (48.5, 61.6)	0.068	86.2 (80.7, 92.0)	27.2 (22.8, 32.5)	0.31
				TC	33	45.1 (38.7, 52.6)		74.4 (68.4, 81.0)	24.2 (19.3, 30.4)	
				CC	4	77.7 (50.0, 120.9)		100.6 (78.9, 128.2)	22.4 (11.8, 42.3)	
rs2298849	0.80	25.3	T/C	TT	51	53.4 (47.0, 60.6)	0.31	85.5 (79.8, 91.7)	29.0 (24.2, 34.8)	0.33
				CT	35	47.7 (40.9, 55.5)		76.5 (70.3, 83.2)	22.2 (17.8, 27.6)	
				CC	6	65.4 (45.2, 94.6)		91.2 (74.4, 11.8)	24.3 (14.6, 40.6)	
VDR										
rs731236	0.08	42.6	T/C	TT	34	52.2 (44.6, 61.0)	0.35	83.4 (76.5, 91.0)	24.9 (19.9, 31.2)	0.66
				TC	38	49.3 (42.5, 57.1)		79.5 (73.2, 86.4)	27.3 (22.0, 33.9)	
				CC	20	56.4 (46.0, 69.1)		86.8 (76.6, 96.1)	25.0 (18.8, 33.3)	
rs757343	0.98	10.8	G/A	GG	74	52.8 (47.5, 58.8)	0.76	83.2 (78.4, 88.2)	26.8 (23.0, 31.3)	0.56
				AG	17	47.8 (38.3, 59.7)		79.1 (69.9, 89.6)	22.3 (16.4, 30.4)	
				AA	1	47.6 (19.1, 118.7)		74.9 (45.0, 124.7)	27.3 (17.7, 97.5)	
rs10783219	1.00	36.9	A/T	AA	36	53.0 (45.5, 61.8)	0.82	82.1 (75.5, 89.4)	25.4 (20.4, 31.6)	0.69
				TA	43	50.5 (43.9, 58.1)		81.2 (75.2, 87.8)	25.6 (20.9, 31.2)	
				TT	13	52.8 (41.0, 68.1)		86.4 (75.0, 99.5)	28.5 (19.7, 41.2)	
rs7139166	0.24	40.3	C/G	CC	37	53.6 (46.1, 62.3)	0.53	84.4 (77.7, 91.8)	26.1 (21.0, 32.4)	0.81
				CG	37	51.6 (44.4, 59.9)		82.1 (75.5, 89.3)	25.6 (20.6, 31.7)	
				GG	18	48.7 (39.3, 60.5)		78.5 (69.6, 88.5)	26.2 (19.2, 35.7)	

[1]*CYP2R1*, 25-hydroxylase; *CYP24A1*, 24-hydroxylase; *CYP27B1*, 1-α-hydroxylase; *C10orf88*, open-reading frame 88 on chromosome 10q26.13; *DHCR7/NADSYN1*, 7-dehydrocholesterol reductase/nicotiamide adenine dinucleotide synthetase-1; *GC*, vitamin D binding protein; HWE, Hardy-Weinberg equilibrium in the unrelated population; MAF, minor allele frequency for the unrelated population; M/m, major/minor alleles; SNP, single nucleotide polymorphism (ordered by position); *VDR*, vitamin D receptor; VitDgen, Vitamin D in genes; 25(OH)D, 25-hydroxyvitamin D.

[2]Increase in 25(OH)D concentration after 4 UVB treatments with a total of 6 or 7.5 standard erythema doses during a 10-d period.

[3]Linear mixed models with family as a random factor adjusted for age, sex, BMI, use of multivitamin and vitamin D supplementation, outdoor stay in light clothes, outdoor transport to work, and sun bathing.

[4]Linear mixed models with family as a random factor adjusted for age, sex, BMI, and baseline serum 25(OH)D concentration.

[5]Raw serum 25(OH)D concentrations were log transformed to approximate a normal distribution and are presented as geometric means (nmol/L); 95% CIs in parentheses (all such values).

[6]Significant *P* value (<0.05).

[7]GX, GC/GA.

[8]XX, CC/CA/AA.

DISCUSSION

To our knowledge, this is the first study to evaluate the increase in 25(OH)D concentrations after artificial UVB treatments in relation to *GC* and *CYP2R1* genotypes. There was a gene-dose–dependent relation between the UVB-dependent increase in serum 25(OH)D concentrations and the GRS. Genetically predisposed individuals carrying all 4 risk alleles of rs10741657 and rs4588 had the lowest baseline mean 25(OH)D concentration and the smallest increase in 25(OH)D concentrations after 4 UVB treatments during the winter compared with those of carriers of a lower GRS. Furthermore, there was a gene-dose–dependent relation between the percentage decrease in the 25(OH)D concentration and GRS after a 6-mo consumption of vitamin D_3–fortified bread and milk. The largest percentage decrease in 25(OH)D concentrations was also observed in individuals carrying all 4 risk alleles of rs10741657 and rs4588 compared with carriers of a lower GRS. Nimitphong et al. (21) also observed a significantly smaller increase in 25(OH)D_3 and total 25(OH)D concentrations after oral intake of 400 IU vitamin D_3/d (10 μg vitamin D_3/d) for 3 mo in individuals carrying CA or AA genotypes of rs4588.

This study is important for public health recommendations and vitamin D food-fortification programs because it showed that the genetic predisposition in the *CYP2R1* and *GC* genes may have a large impact on 25(OH)D concentrations. During winter, individuals carrying all 4 risk alleles of rs10741657 and rs4588 benefitted the least from either UVB treatments or the consumption of vitamin D_3–fortified bread and milk. In agreement with our findings, Engelman et al. (22) performed a GRS encompassing rs4588 in *GC* and rs2060793 in *CYP2R1* and showed that the mean 25(OH)D concentration was highest in the group with no copies of rs4588 and rs2060793 risk alleles who also had high external sources of vitamin D (>10 μg/d). Furthermore, Engelman et al. (22) showed that the lowest mean 25(OH)D concentration was shown in the group with 3 risk alleles and low external sources of vitamin D (<10 μg/d) or 4 risk alleles regardless of the external sources of vitamin D.

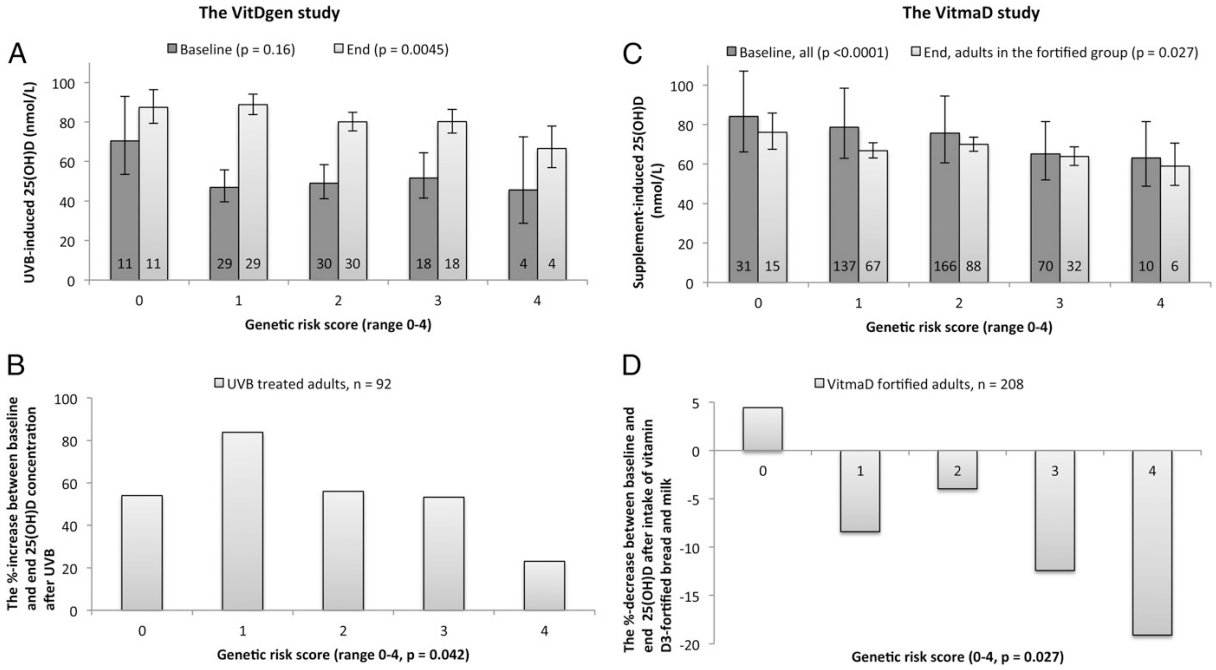

FIGURE 1 Adjusted mean (95% CI) 25(OH)D concentrations at baseline and end of the study were calculated for each GRS category of rs10742657 and rs4588 stratified by UVB treatment in the VitDgen study (A) or by consumption of vitamin D_3–fortified bread and milk in the VitmaD study (C). The GRS (range: 0–4) was calculated as the sum of the number of G alleles of rs10741657 and A alleles of rs4588. The percentage increase in 25(OH)D concentrations after UVB treatment in the VitDgen study (B) or percentage decrease in 25(OH)D concentration after a 6-mo consumption of vitamin D_3–fortified bread and milk during winter in the VitmaD study (D) for each GRS category of rs10742657 and rs4588. The percentage decrease in vitamin D status in relation to the GRS was analyzed in the adult population who participated in the fortification group (n = 208) in the VitmaD study. In both studies, linear mixed models were adjusted for age, sex, BMI, baseline 25(OH)D concentration, and family as a random factor and, in addition, for ski and sun vacations, vitamin D intake, and supplementation for the VitmaD study. Linear mixed models were fitted to log 25(OH)D concentrations with the GRS as an explanatory factor. For the VitmaD study, the GRS was calculated for the adult population (18–60 y) at baseline (n = 414) and at the end of the study only for the adult population who consumed vitamin D_3–fortified bread and milk (n = 208). Numbers in the columns present total numbers of participants carrying the GRS. Error bars indicate 95% CIs. GRS, genetic risk score; VitDgen, Vitamin D in genes; VitmaD, Food with vitamin D; 25(OH)D, 25-hydroxyvitamin D.

Our study indicated that individuals carrying a high GRS may need a longer UVB-exposure time or a higher amount of vitamin D supplementation to achieve a given 25(OH)D concentration than do individuals carrying a lower GRS, or perhaps the results suggest that there is variability in the physiologically normal range of 25(OH)D concentration. Regardless of the method used to increase or maintain a serum 25(OH)D concentration during winter, the effects of UVB treatments or vitamin D supplementation on 25(OH)D concentrations seemed remarkably similar. This study emphasizes the findings that individuals with genetically determined low 25(OH)D concentrations may need different health recommendations to improve their vitamin D status or that there is physiologic variation in the normal range of 25(OH)D concentration, showing that a one-size-fits-all approach may not work well for vitamin D. If the genetically determined low 25(OH)D concentration poses health risk, then carriers of all 4 risk alleles of rs10741657 and rs4588 should be at increased risk of developing vitamin D deficiency or at risk for adverse health outcomes associated with vitamin D deficiency or insufficiency. The genetic variation in rs10741657 has been associated with risk of type 1 diabetes (23). Several studies have reported an association between *GC* genotypes rs7041 and rs4588 and adverse health outcomes including premenopausal bone fracture, postmenopausal breast cancer, endometriosis, diabetes, severity of obstructive pulmonary disease, asthma susceptibility, and rheumatic fever (24–28).

At baseline, there was no significant difference between 25(OH)D concentrations for the analyzed SNPs except for rs12512631 in *GC*. The association between rs12512631 and 25(OH)D concentrations disappeared after 4 UVB treatments. For every 20 statistical tests made for associations with 25(OH)D concentrations at baseline, it was expected to have one false-positive result at the $P < 0.05$ concentration, which the rs12512631 finding may have been. Otherwise, our findings are in agreement with those of previous studies that showed no effects of genetic variation on 25(OH)D concentrations during winter (12, 21, 22). During winter, the vitamin D stored during summer is used, and thus, the genetic variation in biosynthesis genes cannot predict 25(OH)D concentrations.

At the end of the VitDgen study, rs10741657 in *CYP2R1* and rs4588 in *GC* predicted the UVB-induced 25(OH)D concentration. The same polymorphisms have previously been shown to predict 25(OH)D concentrations at late summer and after a 6-mo consumption of vitamin D_3–fortified bread and milk in the VitmaD study (9, 12). In contrast, 2 other polymorphisms, rs10766197 in *CYP2R1* and rs842999 in *GC*, did not predict the UVB-induced 25(OH)D concentration at the end of the VitDgen study, whereas both polymorphisms were associated with 25(OH)D concentrations at late summer and after a 6-mo consumption of vitamin D_3–fortified bread and milk in the VitmaD study (12). The lack of replication of the 2 SNPs in the VitDgen study was likely due to the small sample size.

A strength of the VitDgen study design was that it was conducted in presumably healthy Caucasians aged 18–60 y, and thus, the potential impact of diseases was minimized. Moreover, the increase in 25(OH)D concentration was well controlled by using an artificial UVB source. All blood samples were drawn within a 10-d period during the winter, when the solar influence was minimized. Vitamin D status relied on a single measurement of 25(OH)D concentrations and was analyzed in a single batch-with isotope-dilution liquid-chromatography–tandem mass spectrometry. A disadvantage was that some of the known predictors of 25(OH)D concentrations were quantified by using self-reported questionnaires. It would have been interesting to have measured parathyroid hormone concentrations to assess if there was a recessive effect of rs4588 AA on parathyroid hormone concentrations after UVB treatment as observed after vitamin D supplementation in the VitmaD study (12) and by Pekkinen et al. (29). Moreover, it would have been interesting to analyze possible effects of rs7041 and rs4588 on free and bioavailable 25(OH)D concentrations because genetic differences in the vitamin D binding protein gene may affect the binding of 25(OH)D and, thereby, the amount of free and bioavailable 25(OH)D (30, 31).

In conclusion, common genetic variants in *CYP2R1* and *GC* are predictive of 25(OH)D concentrations in a healthy Caucasian population. Carriers of all 4 risk alleles of rs10741657 in *CYP2R1* and rs4588 in *GC* had the lowest baseline mean 25(OH)D concentration, smallest increase in 25(OH)D concentrations after 4 UVB treatments, and largest percentage decrease in 25(OH)D concentrations after consumption of vitamin D_3–fortified bread and milk during winter compared with in carriers of no risk alleles. This study is important for public health recommendations and vitamin D–food fortification programs because it shows that a genetic predisposition in *CYP2R1* and *GC* genes may have a large impact on 25(OH)D concentrations. Genetic variability may be associated with different response to UVB exposure or vitamin D supplementation perhaps suggesting that some individuals may need different health recommendations to improve their vitamin D status or that there is a physiologic variability in the normal range of 25(OH)D concentrations.

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The authors' responsibilities were as follows—RA, HM, KHM, LBR, GR-H, and JN: designed the VitmaD study; HM, KHM, LBR, RA, and JN: conducted the VitmaD study; JN, UV, GR-H, LBR, and HCW: designed the VitDgen study; JN: conducted the VitDgen study; BAN and PJB: were responsible for the laboratory analysis; JN, UV, and EWA: analyzed data; JN: wrote the first draft of the manuscript; and all authors: critically reviewed and approved the manuscript. Arla Foods A/S, Lantmännen Cerealia A/S, and The Association of Danish Trade Mills partially sponsored the study foods and had no influence on study design, analysis, or interpretation of results. None of the authors had a conflict of interest.

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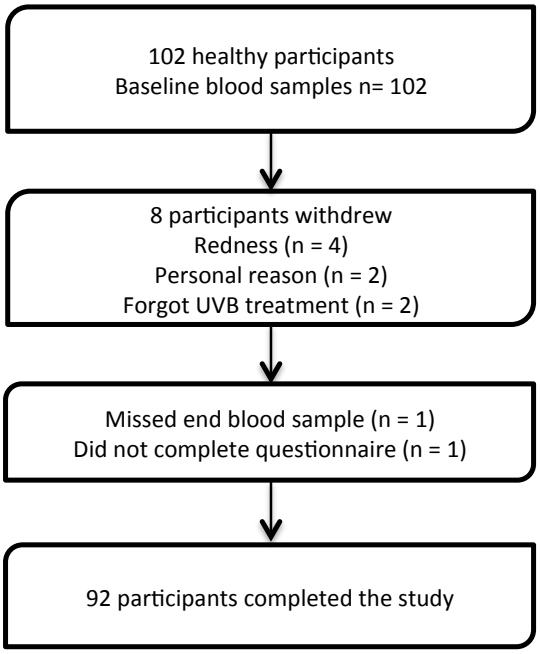
Online Supplemental Material

Supplemental Figure 1: Timescale

Online Supplemental Material

Supplemental Figure 2: Flow diaggram, VitDgen



Online Supplemental Material

Supplemental Table 1: SNP primers

Gene	SNP	iPlex primer 1	iPlex primer 2	Extension primer
CYP2R1	rs7116978	ACGTTGGATGGAAGTCTTTAAGGAATACAC	ACGTTGGATGCATTTAAGTGCTTAAGTCACC	ACCTTTTATAGGTAAAAGATTATCTAA
	rs10741657	ACGTTGGATGGGTGGTTGGGGAGATACTTT	ACGTTGGATGCAGCTCCAATGTCATCTTCC	TTCCTTGACAGCCCT
	rs1562902	ACGTTGGATGACCAGCTTATATCCAGGGAC	ACGTTGGATGGAGACCAGTTGATAGGGAAG	TAACATCTTCCATGAACA C
	rs10766197	ACGTTGGATGAGCTTGGTCCTTTCTGTATC	ACGTTGGATGGTACAATTTGGAACACTCCAG	ACGCCAGTTAATTAGAGATCTTTAAACT
CYP24A1	rs6013897	ACGTTGGATGGTTCAGAAAACTCGTAAATGC	ACGTTGGATGGGGGATAATGAAAGTACCTA	ATAATGAAAGTACCTACTTCAG
	rs4809960	ACGTTGGATGGCCTGTTTACAAAAGAGTTG	ACGTTGGATGGTCACAGACTTGCTCACTGA	GGTGGGTGATTTTGCGGATAAAAC
	rs2296241	ACGTTGGATGGCGGTTGTTTTCTTTGAAGG	ACGTTGGATGTCAACGTGGCCTCTTTCATC	TCATCTATTCTGCCCATAAAATC
	rs17219315	ACGTTGGATGCACCTCAAAATCCCTGAACC	ACGTTGGATGAAGCACCTTTCCTCCTAGTC	ACTAGTCAAAGATTGCACCA
	rs2426496	ACGTTGGATGCTTCTCTGAGTCTAGTTTCC	ACGTTGGATGTCTTGACCTTCCTGAGACAC	GGTACTGAGACACAGGTATAGTAA
CYP27B1	rs10877012	ACGTTGGATGAGAGAGGGCCTGTCTCTAAA	ACGTTGGATGAATGAGGGAGTAAGGAGCAG	GGTAAACTGTGGGAGATT
C10orf88	rs6599638	ACGTTGGATGAAACACTGATTCCTGGACCC	ACGTTGGATGGGAAGGTCTTCAAAATGCAG	TCCTGGCCCTCACTAT
DHCR7/	rs1790349	ACGTTGGATGGCCTGAAAGCCAAGCTATCC	ACGTTGGATGGATCCATCAGAGGGAAGTGC	CCAAACAGCAAGACAAG
NADSYN1	rs12785878	ACGTTGGATGTTGAGTCCAGCCCAGGAGAA	ACGTTGGATGTCTGGGCTGTCTGATATCAC	CCCCATGTCTGATATCACAAAGCTTC
GC	rs16846876	ACGTTGGATGCAAGTTTAGGAGTTCTGTTC	ACGTTGGATGTATCCCTACCTGCACATGTC	CCCTTGCACATGTCTGTGAACTTT
	rs12512631	ACGTTGGATGAACTAGTAGCCTTGTGGTGG	ACGTTGGATGTCTTTTCTCTCTATTAGGC	CTCTCTCTATTAGGCCAAGAAA
	rs17467825	ACGTTGGATGCAATATTTCTGTCAGCGATTC	ACGTTGGATGTTCCAGCACACTCTAAACAC	CCCCTCTAAACACATTTCACCA
	rs2282679	ACGTTGGATGGGGACTACTACTTGCTTCCA	ACGTTGGATGCCCAGCAAATCTCTGTCTCT	CATCTCTGTCTCTTAATTATCTCACA
	rs842999	ACGTTGGATGTGAGAATATTAAGCACCGAG	ACGTTGGATGCTAGTCTTACATATATCAG	CTAGTCTTACATATATCAGAAATTG
	rs4588	ACGTTGGATGTTTTTCAGACTGGCAGAGCG	ACGTTGGATGCTTGTTAACCAGCTTTGCC	GAAAGCTTTGCCAGTTCC
	rs222020	ACGTTGGATGAACCAGAGGAGACAACCTTG	ACGTTGGATGGATAGCAGCAGGAAAAACTC	ATGGGCAAAAAATTCAATGG
	rs2298849	ACGTTGGATGCCACTGGCAAAACACATTAC	ACGTTGGATGAGTGCTGTCAGTTAACAGCC	GCCTCACCTAATTCGTACA
VDR	rs731236	ACGTTGGATGTTCTCTATCCCCGTGCCCA	ACGTTGGATGTTGGACAGGCGGTCCTGGAT	AGTAGGTCCTGGATGGCCTC
	rs757343	ACGTTGGATGTTCCTCTTCGGCCTTTTCTC	ACGTTGGATGATTTTGGAGGCAATGTGCAG	ATGTGCAGTGACCCTT
	rs10783219	ACGTTGGATGTTCTGTGGGATAGTGTGGTC	ACGTTGGATGCCTCTTCCTCCATATCTACA	CCATATCTACAGCCTCC
	rs7139166	ACGTTGGATGCCTCTTATGCTTTTCTTCCC	ACGTTGGATGAAGTAATAGGAAGGATCCCC	GGCTCCCCTTGCCCAAAGCAT

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