CPEG Fellowship Progress Report
Manousaki, Despoina

Dear CPEG Fellowship Committee,

Thank you for your generous support, awarding me with the CPEG Fellowship in 2014.This Fellowship enabled me to continue my Master's degree in the Department of Human Genetics in McGill University, and to transition to a PhD program in the same Department under the supervision of Dr Brent Richards. Dr Richards has been an amazing mentor to me and a perfect example of a clinician researcher and an academic center professor. Under his mentorship, I was involved in research projects in translational genomics that made impactful changes in clinical care. Thus, my postgraduate training provided me and keeps providing me with the skills to conduct my own future research. This will enable me to launch my career as an independent researcher and clinician in the field of pediatric endocrinology, and to continue working for a better care of our patients.

Sincerely,

Despoina Manousaki

This report summarizes the research work I undertook during the one-year period funded by the CPEG Fellowship. Of note, this funding period started on May 1st 2016 (after an 8-month maternity leave), and ended in December 31st 2017 (interrupted by 8 months of a second maternity leave between January 1st 2017 and August 31st 2017). The primary focus of my research work during this year was the project summarized below, which is also the main axis of my PhD Thesis:

PROJECT TITLE: The Role of Rare and Low-Frequency Genetic Variation in Vitamin D status

Overview: The research proposal of the CPEG 2014 Fellowship application summarizes the rationale of this research project. The aim of this work was to describe novel rare genetic variants affecting 25 hydroxy-vitamin D levels in Europeans, through a large scale meta-analysis of genome-wide association studies on 25 hydroxy-vitamin D. The detailed methods and results of this project appear in **Appendix 3.**

Project timeline:

o May 2015 – October 2015 • Data collection (individual summary results from vitamin D GWAS, preliminary meta-analyses)

o November 2015 – July 2016 • Data analysis (quality control, meta-analysis of individual summary results, conditional analyses, secondary analyses: gene-

environment interaction study, effect of novel variants on vitamin D insufficiency and vitamin-D related clinical outcome (multiple sclerosis))

o November 2016-April 2017 • Manuscript completion and submission to peer reviewed journals

o June 2017 • Manuscript accepted for publication (American Journal of Human Genetics)

Project status: This study was accomplished during the first 18 months of my Master's training, and stemmed a publication in the American Journal of Human Genetics (PMID: 28757204), and two conference presentations: 2017 ASBMR annual meeting (poster presentation) and 2017 ASHG annual meeting (oral presentation). Below is the abstract submitted for the ASBMR and ASHG meetings, which reports the basic findings of this research work (for more details see the AJHG publication in **Appendix 3**):

Title: Low Frequency Synonymous Coding Variation in CYP2R1 has Large Effects on Vitamin D Level and Risk of Multiple Sclerosis.
Authors: Despoina Manousaki, Brent Richards and the SUNLIGHT_Seq Consortium

Introduction: Vitamin D insufficiency is common, correctable and influenced by genetic factors, and it has been associated to risk of several diseases. We sought to identify low-frequency genetic variants that strongly increased the risk of vitamin D insufficiency and tested their effect on risk of multiple sclerosis, a disease influenced by low vitamin D concentrations.

Methods/Results: We used whole-genome sequencing data from 2,619 individuals through the UK10K program and deep imputation data from 39,655 genome-wide genotyped individuals. Meta-analysis of the summary statistics from 19 cohorts identified a low-frequency synonymous coding variant (rs117913124[A], minor allele frequency=2.5%) in the *CYP2R1* gene which conferred a large effect on 25-hydroxyvitamin D (25OHD) levels (-0.43 standard deviations of natural log-transformed 25OHD, per A allele, P-value = 1.5 x10-88). The effect on 25OHD was four-times larger and independent of the effect of a previously described common variant near CYP2R1. By analyzing 8,711 individuals we showed that heterozygote carriers of this low-frequency variant have an increased risk of vitamin D insufficiency (OR=2.2, 95% CI 1.78-2.78, P=1.26 x 10-12). Individuals carrying one copy of this variant had also an increased odds of multiple sclerosis (OR=1.4, 95%CI 1.19-1.64, P=2.63 x 10-5) in a sample of 5,927 cases and 5,599 controls.

Conclusions: We describe a novel low-frequency coding variant in the CYP2R1 gene, which exerts the largest effect upon 25OHD levels identified to date in the general European population. Since *CYP2R1* is known to encode a critical enzyme in the production of the active form of vitamin D, these findings implicate vitamin D in the etiology of multiple sclerosis.

Adjunctively to the above principal research project, a number of separate projects were completed or initiated during the funding period of the CPEG fellowship under the supervision of Dr Richards. Below is a list of completed projects (in parentheses are the PMIDs of the related publications). A more detailed description is provided for an ongoing pharmacogenetic project. A complete list of my publications during the last year is provided in Appendix 1.

List of completed/ongoing projects:

- Mendelian randomization studies, investigating the role of Vitamin D in atopic disease (PMID:28486474, first author), coronary artery disease (PMID: 27418593, first author) and Alzheimer disease (PMID: 27856775, co-author).

-Literature review for an editorial published in BMJ on the role of Vitamin D in cancer (PMID: 29089329, first author).

- Study investigating the role of a common genetic variant in type 2 diabetes in Inuit (PMID: 27561922, first author).

- Pharmacogenetic trial investigating the role of a vitamin D rare genetic variant in CYP2R1 on the response to vitamin D oral supplementation (in collaboration with the CARTAGENE biobank).

PROJECT TITLE: Pharmacogenetic study exploring the impact of a genetic variant in the *CYP2R1* gene on the response to vitamin D replacement therapy

Overview: This study aims to evaluate the change in blood 25 hydroxyvitamin D levels (the biomarker for vitamin D levels in humans) in individuals carrying a specific vitamin D–related genetic variant in the CYP2R1 gene (discovered as part of the aforementioned GWAS meta-analysis), compared to individuals not carrying this variant, after administration of 10,000 international units of oral vitamin D3 once a week over 3 months. The detailed study protocol appears in **Appendix 2.**

Project timeline:

o December 2017 • Approval by Health Canada and the LDI REC

o February 2018 • Signature of the Data Access contract with CARTAGENE

o June 2018-May 2019 • Recruitment of participants, data collection

o June 2019-July 2019 • Data analysis

o August 2019 • Manuscript completion and submission to peer reviewed journal

Project status: The project was approved by Health Canada and the REC of the Lady Davis Institute at the Jewish General Hospital of Montreal. The data access contract with CARTAGENE is currently under approval for signature by the LDI officers. We anticipate starting the recruitment of the participants in summer 2018.

Appendix 1

List of accepted publications (May 2016-December 2017)

1. Manousaki D, Richards JB Low vitamin D levels as a risk factor for cancer. BMJ. 2017 Oct 31;359:j4952. doi: 10.1136/bmj.j4952. PMID: 29089329

2.Despoina Manousaki, Tom Dudding , Simon Haworth, Yi-Hsiang Hsu, Ching-Ti Liu, Carolina Medina-Gómez Trudy Voortman, Nathalie van der Velde, Håkan Melhus, Cassiane Robinson-Cohen, Diana L. Cousminer Maria Nethander, Liesbeth Vandenput, Raymond Noordam, Vincenzo Forgetta, Celia MT Greenwood, Mary L. Biggs, Bruce M. Psaty, Jerome I. Rotter, Babette S. Zemel, Jonathan A. Mitchell, Bruce Taylor, Mattias Lorentzon, Magnus Karlsson, Vincent V.W. Jaddoe, Henning Tiemeier, Natalia Campos-Obando, Oscar H.Franco, Andre G. Utterlinden, Linda Broer, Natasja M. van Schoor, Annelies C. Ham, M. Arfan Ikram, David Karasik, Renée de Mutsert, Frits R. Rosendaal, Martin den Heijer ,Thomas J. Wang, Lars Lind, Eric S.Orwoll, Dennis O. Mook-Kanamori, Karl Michaëlsson, Bryan Kestenbaum, Claes Ohlsson, Dan Mellström, Lisette CPGM de Groot, Struan F.A. Grant,Douglas P. Kiel, M. Carola Zillikens, Fernando Rivadeneira, Stephen Sawcer , Nicholas J Timpson and J. Brent Richards (2017) Low Frequency Coding Variation in CYP2R1 has Large Effects on Vitamin D Level and Risk of Multiple Sclerosis. Am J Hum Genet. 2017 Aug 3;101(2):227-238. doi: 10.1016/j.ajhg.2017.06.014. Epub 2017 Jul 27. PMID: 28757204

3. Despoina Manousaki, Lavinia Paternoster, Marie Standl, Miriam F. Moffatt, Martin Farrall, Emmanuelle Bouzigon, David P Strachan, Florence Demenais, Mark Lathrop, William O.C.M. Cookson, J. Brent Richards (2016) Mendelian randomization shows no role for low vitamin D levels in asthma, atopic dermatitis or IgE levels. Plos Medicine PMID:28486474

4. Despoina Manousaki, Johnny Deladoey, Louis Geoffroy, and Patricia Olivier (2017) Continuous Subcutaneous Insulin Infusion in Children: a Pilot Study Validating a Protocol to Avoid Hypoglycemia at Initiation. Frontiers Endocrinol (Lausanne) 2017 Apr 24;8:84 PMID: 28484424

5. Despoina Manousaki, Frank Rauch, Josée Dubois, Gilles Chabot, Nathalie Alos. (2016). Pediatric Reference Data for Dual X-Ray Absorptiometric Measures of Normal Lumbar Bone Mineral Density in Young Children: Impact of the Mechanostat. J Musculoskelet Neuronal Interact. 2016 Sep 7;16(3):247-55.

6. Despoina Manousaki, Jack Kent, Karin Haack, Sirui Zhou, Pingxing Xie, Celia M. Greenwood, Paul Brassard, Deborah Newman, Shelley Cole, Jason G. Umans, Guy Rouleau, Anthony G. Comuzzie and J. Brent Richards. (2016). Towards Precision Medicine: TBC1D4 Disruption is Common in The Inuit and Leads to Under-Diagnosis of Type 2 diabetes. Diabetes Care. 2016 Aug 25. pii: dc160769.

7. Despoina Manousaki, Lauren E. Mokry, Stephanie Ross, David Goltzman , J. Brent Richards. (2016). Mendelian Randomization Studies do not Support a Role for Vitamin D in Coronary Artery Disease. Circulation: Cardiovascular Genetics. ePub:116.001396

8. Lauren E. Mokry, Stephanie Ross, John A. Morris, Despoina Manousaki, Vince Forgetta, J. Brent Richards (2016). Genetically decreased vitamin D and risk of Alzheimer's disease. Neurology. 2016 Dec 13;87(24):2567-2574. Epub 2016 Nov 16.

Appendix 2

Protocol Title: Pharmacogenetic study exploring the impact of a genetic variant in the CYP2R1 gene on the response to vitamin D replacement therapy

Description: This protocol describes a study to evaluate the change in blood 25 hydroxyvitamin D (25OHD) levels (the biomarker for vitamin D levels in humans) in individuals carrying a specific vitamin D–related genetic variant in the *CYP2R1* gene, compared to individuals not carrying this variant, after administration of 10,000 international units of oral vitamin D3 once a week over 3 months.

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ABBREVIATIONS

AE	Adverse Event
BMI	Body mass index
CI	Confidence Interval
CRF	Case Report Form
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
FFQ	Food Frequency Questionnaire
GWAS	Genome-Wide Association Study
IEC	Independent Ethics Committee
IRB	Institutional Review Board
IU	International Units
L	Liter
mcg	Micrograms
mL	Milliliter
OR	Odds Ratio
PI	Primary Investigator
PTH	Parathyroid Hormone
QD	Once daily
R	R Statistical Package
REB	Research Ethics Board
SNP	Single Nucleotide Polymorphism
25OHD	25-hydroxyvitamin D

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Illumina OMNI 2.5 array
Affymetrix Axiom array

1. INTRODUCTION

A recent Genome-Wide Association Study (GWAS)[1] has shown that a novel low-frequency genetic variant in a gene called *CYP2R1* confers a substantial decrease in the blood levels of 25-hydroxy-vitamin D (25OHD), the biomarker of vitamin D in humans, and is associated with a two-fold increase in their risk of vitamin D insufficiency. We hypothesize that this genetic variant blocks the hepatic step of hydroxylation of vitamin D, one of the steps in the production of vitamin D's active form. In order to test this hypothesis, we will measure the response in 25OHD levels to oral vitamin D supplementation of individuals carrying this variant compared to non-carriers.

1.1. Background: Vitamin D Deficiency and Genetic Variation associated with 25OHD levels

Vitamin D deficiency affects up to 50% of otherwise healthy adults with potential public health consequences[2]. Almost half of the variability in the levels of 25OHD has been attributed to genetic factors [3,4]. In 2010, the SUNLIGHT consortium[5], a large multicentre genome-wide association study (GWAS) of 15 cohorts from Europe, Canada and the USA on 33,996 individuals, co-led by Dr. Richards, showed that four common genetic variants (in loci related to vitamin D synthesis, metabolism and transport) explain little of the heritability of 25OHD levels (less than 2.5%). We therefore hypothesized that low frequency or rare variants (defined as variants having a minor allele frequency (MAF) <5% and 1% respectively) can impart a much larger effect on 25OHD levels, and to test this hypothesis, we conducted a large meta-analysis of 19 GWAS studies (N=42,326 individuals of European ancestry)[1], where we tested the effect of rare and low frequency genetic variants on 25OHD levels. We found that a low-frequency variant (rs117913124, minor allele frequency =2.5%) in a gene coding region of *CYP2R1* (which controls hepatic hydroxylation of cholecalciferol, the form of vitamin D taken by diet or synthetized in the skin) confers a much larger effect on 25OHD level than the common variant already described in the same locus, and that this effect is independent of the effect of the common variant. By analyzing 8,711 individuals from four of the participating studies, we demonstrated that carriers of one allele of this low-frequency variant have an increased risk of vitamin D insufficiency (OR=2.2, 95% CI 1.8-2.8, P-value=1.25 x 10^{-12}) (**Figure 1**). We also showed that carrying one copy of this variant increases significantly the risk of multiple sclerosis, (OR=1.4, 95%CI 1.19-1.64, P=2.63 x 10^{-5}), a disease shown to be strongly associated with low 25OHD levels[6].

Figure 1: Forest plot with the odds ratios of vitamin D insufficiency in carriers of the *CYP2R1* variant from 4 studies, as well as the meta-analytic odds ratio (modified from the original figure in the paper by Manousaki et al[1]).

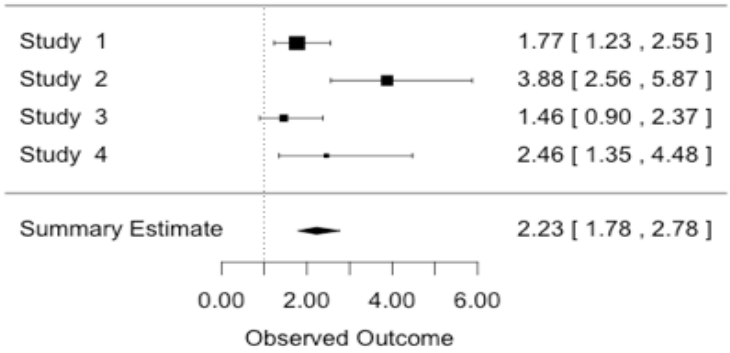
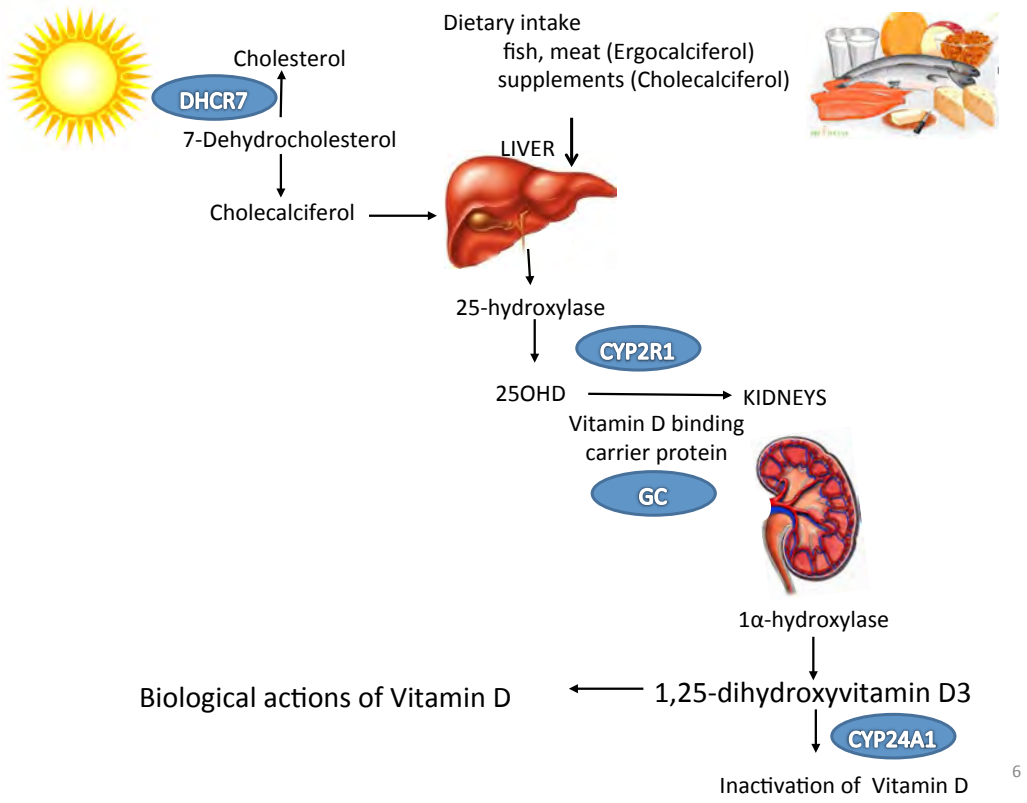


Figure 2 shows the vitamin D pathway. *CYP2R1* encodes one of the 25 hydoxylases that transform cholecalciferol, (the form of vitamin D produced in the skin after sun exposure or taken from the diet) to 25OHD. Thus *CYP2R1* is involved in a major step of 25OHD synthesis.25OHD is then further metabolized to 1,25 dihydroxyvitamin D, the biological active form of vitamin D.
Figure 2: The vitamin D pathway



Given that: 1) the *CYP2R1* encodes the major 25-hydroxylase for vitamin D[7] and 2) hepatic hydroxylation is a necessary step in the conversion of dietary vitamin D to its active form, we hypothesize that individuals heterozygous or homozygous for the *CYP2R1* variant are likely to have less of an increase in 25OHD after

administration of cholecalciferol, than individuals without this genetic variant. This is because these individuals have a coding genetic variant in a key enzyme, *CYP2R1,* that is associated with lowered 25OHD level and impairment of this enzyme would lead to less conversion of dietary vitamin D to 25OHD. Given the large effect of this variant on multiple sclerosis, restoring normal 25OHD levels in the carriers could be crucial in preventing this debilitating disease in those individuals.
This study may have direct clinical relevance, since these individuals have a low level of vitamin D and efforts to increase their 25OHD level are likely to be hindered by their genetic variant in *CYP2R1.* Consequently, these individuals should have their low vitamin D levels treated with the active form of the vitamin D pathway, 1,25 dihydroxyvitamin D, rather than cholecalciferol, which is the current standard of care. Therapy with 1,25 hydroxyvitamin D would enable individuals with the genetic variant to bypass the *CYP2R1* and have the biologic effect of the active form of vitamin D. Importantly this genetic variant affects ~5% of individuals of European descent, which could influence the clinical care of hundreds of thousands of Canadians.
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1.2. Rationale

Data from this study will be used to evaluate if individuals carrying the low-frequency variant in the *CYP2R1* gene do not respond well to oral cholecalciferol. If this is shown, these individuals, which represent ~5% of the European population, should likely be treated with the active form of vitamin D (1,25 hydroxyvitamin D).

1.3. Summary of Risk Management

1.3.1. Risks Related to Vitamin D Use

There is little risk associated with taking vitamin D supplements at the dose proposed in our study (10,000 IU/week), as the tolerable upper intake level to protect against vitamin D toxicity for adults according to data published by the Institute of Medicine is 4,000 IU/day[8], equivalent to 28,000 IU/week.

Also note that according to the product monograph produced by Orimed Pharma[9], only daily doses of vitamin D ranging from 1.25 to 2.5 mg (50,000 to 100,000 IU) in adults may result in hypervitaminosis. Thus, there is no risk of hypervitaminosis with our proposed dosage of 10,000 IU/week.

1.3.2. Risks related to 25OHD Sampling

Subjects eligible to participate will undergo a blood-draw before and after the 3-month period of oral cholecalciferol supplementation. 20 of these subjects will undergo additional blood-draws after 3, 7 and 14 days of oral cholecalciferol supplementation. Whole blood will be collected from an antecubital vein, after the

site of puncture has been cleansed and sanitized with iodine-alcohol or chlorhexidine-based solutions. Two 5 mL EDTA-tubes of whole blood will be collected. The risks from blood draw include pain, bruising and a rare risk of infection. These risks are clearly described in the informed consent document.

2. OBJECTIVE

To study both the acute and the chronic changes in 25OHD levels after 3, 7 and 14 days and after 3 months of oral cholecalciferol supplementation in carriers and non-carriers of the *CYP2R1* low-frequency variant.

3. ENDPOINT

Both acute and chronic changes (delta) in 25OHD levels before and after supplementation with oral vitamin D in carriers and non-carriers of the *CYP2R1* low frequency variant, controlling for relevant covariates.

4. INVESTIGATIONAL PLAN

4.1. Study Design

Recruitment of study subjects will be undertaken at the Jewish General Hospital, as outlined below. We will seek out approximately 150 participants recruited through the CARTaGENE Study[10], using genotyped-based recall, for which patients in this cohort have been consented. We expect enrolment to be completed within 9 months of trial initiation and the trial to close 3 months thereafter (**Timeline Table).**

Timeline: Pharmacogenetic study exploring the impact of a low-frequency CYP2R1 variant on the response to vitamin D replacement therapy.				
December 2016	October 2017	November 2017	December 2017 – October 2018	November 2018
REB (ethics) approval	Protocol amendment & CTA submission	CTA approved (NOL issued) & REB re-approval	Patient visits, follow-up etc.	Completion of study, results made available
			Begin patient recruitment	Preparation of manuscript

All subjects included in this pharmacogenetic trial will receive 3 months of oral cholecalciferol at a dose of 10,000 IU per week. Half of the participants will be carriers of at least one copy of the *CYP2R1* low-frequency variant (exposure), whereas the other half will not carry any copy of the *CYP2R1* low-frequency variant. The exposure will be assessed by imputing the *CYP2R1* variant using available genome-wide genotyping data from approximately 3,000 participants of the CARTaGENE project. The status of carriers and non-carriers will be attributed to each one of the 3,000 participants prior invitation to enrol in the study.
Sampling for 25OHD levels will take place before and after the 3-month period of cholecalciferol supplementation for all subjects, and after 3, 7 and 14 days for 20 of those subjects (10 carriers and 10 non-carriers). The samples will then be stored (as outlined below) at the laboratory of Dr. Brent Richards of the Lady Davis Institute. The level of 25OHD will be measured at the Jewish General Hospital. Analysis of the data will be carried out in the Richards laboratory at the Lady Davis Institute. In addition to 25OHD measurements, individual data will be collected at Visit 1 to allow for statistical control of relevant covariates. These include: age, sex, body mass index (BMI), ethnicity, history of intake of vitamin D supplements. Information on dietary vitamin D intake will be retrieved from already available food questionnaires provided by the CARTaGENE project and new food questionnaires filled-out by the participants at enrolment to the study.

4.1.1. Screening

To determine subject eligibility for enrolment in the study, a screening visit will be performed. For the purposes of subject eligibility for enrolment in the study, screening assessments are defined as any assessments performed prior to the first dose of vitamin D, including baseline assessments on Day -1 that are used to qualify the subject for enrolment.
If a subject is not eligible for the study based on the Inclusion and Exclusion Criteria at the initial attempt, but becomes eligible at a later date during the enrolment period, the investigator may re-screen the subject.

4.1.2. Treatment regimen

On Visit 1, subjects will be admitted to the clinical research unit of the Endocrine division of the Jewish General Hospital and will be allocated to the carrier or non-carrier group, after assessment to ensure that they satisfy the inclusion criteria and that they present no exclusion criteria. The allocation to the two groups will be based on of the presence of at least one copy of the *CYP2R1* low-frequency variant, revealed by genotyping undertaken prior to invitation of the individuals to participate. Additionally, 20 subjects will be further randomly allocated to the acute trial group; 10 subjects will be selected at random from each the carrier and the non-carrier group.

Individuals will undergo the informed consent process in the presence of a research assistant and of a study nurse. They will have the opportunity to ask as many questions about the study, as they see fit and the research assistant and the nurse will be bilingual. Individuals will be asked to bring in all medications and dietary supplements in their containers for accurate tracking of sources of vitamin D supplementation. They will be asked to fill out questionnaires enabling to assess medical history, demographics, prior vitamin D supplementation and current dietary vitamin D intake, and standard anthropometric measurements (height, weight) will be performed.

The first dose of vitamin D will be taken on Day 1 after discharge from the clinical research unit. Discharge on Day 1 will occur after blood testing for 25OHD levels. Oral vitamin D supplements (^{Pr}ViDextra) at a dose of 10,000 IU of cholecalciferol weekly will be prescribed to all study participants for a 3-month period. The containers with the appropriate amount of vitamin D pills for the study period will be provided to the participants upon enrolment to the study. The study nurse will give specific instructions to the participants in regards to the time of the day and the amounts of pills taken weekly. Specifically, it will be asked from the participants to take their weekly vitamin D pill with a meal, on the same day and at the same time every week, for a period of three months. Written instructions will appear on the containers as well. Patients will return to the hospital twice before the end of the 3-month period, after 1 month and after 2 months, for pill counts to monitor compliance.

In case an individual is on vitamin D supplementation at the screen visit, he/she will be asked to stop taking vitamin D supplements for a 3-month period (wash-out period). Visits 1 and 2 will be deferred to Day 90 and Day 180 respectively.

4.1.3. Follow-up Visits

All subjects will attend three follow-up visit 30 days (Visit 2), 60 days (Visit 3) and 90 days (Visit 4) following Visit 1. Pill counts will be conducted during Visits 2 and 3. A blood draw will be conducted during Visit 4. Subjects in the acute trial group will attend 3 additional follow-up visits at 3 days (Visit 1a), 7 days (Visit 1b) and 14 days (Visit 1c) after Visit 1. Any subject withdrawing from the trial prematurely should also be asked to complete follow-up procedures 7-10 days after withdrawal. See Section 4.4 (Time and Events Table) for details regarding all study procedures performed during the course of this trial.

4.2. Investigational Product Dosage/Administration

Vitamin D supplements will be provided by Orimed Pharma Inc. Details on the product appear in the Table below.

Product Name:	**PrViDextra**
Ingredient description:	Vitamin D3 (Cholecalciferol) 250 mcg/10,000 IU.
Dosage form:	Tablet
Unit dose strength(s)/Dosage level(s):	10,000 IU
Route/Administration/Duration:	Oral
Dosing/Storage instructions:	**For Vitamin D deficiency:** 5 000 IU (125 µg) daily until a biochemical and radiographic response is achieved. **For Vitamin D-resistant rickets:** 12 000 to 500 000 IU (0.3 to 12.5 mg) daily. **For hypoparathyroidism**: 50 000 to 200 000 IU (1.25 to 5 mg) daily. Calcium supplementation is also required. Store in well-closed container at room temperature (15 – 30 °C). Protect from light.
Device:	None
Manufacturer/source of procurement:	Orimed Pharma Inc.

4.3. Dose Adjustment Criteria

In all cases, ethics boards will be informed of dosage changes prior to administration to the subjects. There are no *a priori* plans to change the dose.

4.4. Time and Events Table

	Participants not undergoing washout period							Participants undergoing washout period			
Visit	**Screen (V1)**	**V1a– only for acute trial participants**	**V1b- only for acute trial participants**	**V1c- only for acute trial participants**	**V2**	**V3**	**V4**	**Screen (V1)**	**V2**	**V3**	**V4**
Timing	Day -1	Day 3	Day 7	Day 14	Day 30	Day 60	Day 90	Day 90	Day 120	Day 150	Day 180
Informed Consent	✓							✓			
Anthropomorphic measurements	✓							✓			
Demographics	✓							✓			
Concomitant Medication	✓						✓	✓			✓
Medical History	✓							✓			
Genotyping of the CYP2R1 variant*	✓							✓			
25OHD levels**	✓	✓	✓	✓			✓	✓			✓
Pill Count					✓	✓			✓	✓	
Study Drug Dispensation	✓						✓	✓			✓
Study Drug Reconciliation							✓				✓
Adverse Event Monitoring							✓				✓

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1. At screening, medications taken in the 90 days prior should be collected. All subjects will be asked to bring in all pill bottles, packets, drops and inhaled medications from their medicine cabinets. All medications will be reviewed on Days 90 (and 180 for individuals undergoing a wash-out period).
2. Assessment of anthropomentric measures (weight, height) will be performed on Day-1.
3. Blood samples for 25OHD levels will be collected on Day -1and Day 90 after enrolment and in Day 3, Day 7, Day 14 after enrolment for the acute trial group(can be non-fasting). For individuals being previously on Vitamin D supplementation, a wash-out period of three months will be required prior to start of vitamin D supplementation. These individuals will have their 25OHD measurement deferred from Day -1 and Day 90 to day 90 and day 180 respectively.
4. Vitamin D3 (cholecalciferol) will be administered PO once weekly concomitantly with the largest meal of the day, starting on Day 1 and continuing through Day 90 (or from day 90 to day 180 for individuals undergoing a wash-out period).
5. Monitoring for adverse events will take place on Day 90 (or 180 for individuals undergoing a wash-out period)

5. STUDY POPULATION

5.1. Number of Subjects

We will seek out approximately 150 participants recruited through the CARTaGENE Study[10]. The CARTaGENE study is a research platform that consists of both biological samples and data on the health and lifestyles of more than 42,000 Quebecers aged between 40 and 69 years old. Its mission is to create and maintain long-term database and biobank representative of the genomic diversity of Quebec's population[10]. Candidate participants will be identified through imputation of the *CYP2R1* variant based on existing genotype data as described below. Potential subjects will be initially contacted by CARTaGENE by email, and asked if they are interested to be approached for participation in our study. If yes, they will be then sent a letter, signed by the principal investigator of this project, inviting them to participate in the study and providing them with contact information to contact study investigators should they wish to participate. Subjects will be sent a follow-up letter 1 month after the first letter and will be contacted by telephone by the research assistant of the Richards lab after the second letter if they have not contacted the team to answer any questions about the study.

If subjects prematurely discontinue the study, additional subjects may be enrolled as replacement subjects and assigned to the same treatment at the discretion of the Investigator.

5.2. Eligibility Criteria

5.2.1. Inclusion Criteria

A subject will be eligible for inclusion in this study if all of the following criteria apply:

1. Prior participation in the CARTaGENE study
2. Age 18-71 years
3. Available dietary data from the CARTaGENE study.
4. Available genotyping results allowing to assess carrier/non-carrier status for the A allele at the rs117913124 SNP.

5.2.2. Exclusion Criteria

A subject will not be eligible for inclusion in this study if any of the following criteria, (which will be assessed by taking a medical history at the screening visit and will be documented in the case report form [CRF]) apply:

1. Pregnancy and breastfeeding
2. Currently hospitalized patients
3. History of gastric bypass surgery or gastrectomy
5. Subjects with clinically significant or unstable medical conditions that might limit their ability to absorb or metabolize vitamin D, or to comply with the protocol, including: dermatologic, hepatic, gastrointestinal, renal and psychiatric disease

6. Current use of anti-epileptic or anti-tuberculosis medications (given that these medications lead to impaired vitamin D metabolism)

6. DATA ANALYSIS AND STATISTICAL CONSIDERATIONS

6.1. Hypothesis

We hypothesize that the *CYP2R1* low-frequency variant carriers will have lower response to vitamin D treatment, as expressed by the change in 25OHD levels. This is because this variant likely profoundly disrupts the action of *CYP2R1*, which is the key hepatic hydroxylation step in the generation of 25OHD. This SNP is known to have a profound effect on 25OHD levels, since we have shown that its minor allele is associated with 2.2-fold increase in the odds of vitamin D insufficiency (95% CI 1.8-2.8, P-value=1.25 x 10^{-12}) and since this same SNP decreases log 25OHD levels by 0.43 standard deviations, (95% CI -0.47/-0.39, P-value = 1.5 x10^{-88})[1].

6.2. Sample Size Considerations

Among the ~3,000 individuals of the CARTaGENE project with available genotyping data and food questionnaires, there are approximately 150 individuals carrying the low-frequency variant of interest (heterozygotes prevalence was calculated from 2 x MAF x (1-MAF), homozygote prevalence was calculated from MAF^2, where MAF is 0.025; this gives a total of 5% individuals carrying at least one *CYP2R1* low-frequency variant allele in a given population). With a recall rate of 50%, we anticipate recruiting ~75 individuals carrying the *CYP2R1* variant, which will constitute the exposed group of our study. An equal number of non-carriers of the *CYP2R1* variant will constitute the non-exposed group.

Based on the existing literature[11], an increase of the 25OHD levels of 47 nmol/L has been described in a group taking 600UI of vitamin D daily for 4 months. The baseline value of 25OHD of this group was 23 nmol/L. Based on the same literature, the standard deviation for the above delta 25OHD was of 41.5 nmol/L. According to the above estimates and assuming that the delta 25OHD of the *CYP2R1* variant carriers post vitamin D supplementation is 23 nmol/L instead of 47 nmol/L, (a reasonable assumption, given its profound effect on 25OHD levels), a sample size of 75 *CYP2R1* variant carriers and 75 non-carriers gives our study a statistical power of 96%. Clearly, the true effect size of this variant is not known, but this is, of course, the reason why we are doing this study.

6.3. Data Analysis Considerations

6.3.1 *CYP2R1* Genotyping

Genome-wide genotyping data from CARTaGENE participants will be used for assessing for the presence of the *CYP2R1* variant by imputing this variant (rs117913124 on chromosome 11) to the latest imputation panel (Haplotype Reference Consortium)[12].

Genotyping data for approximately 2000 participants (Illumina OMNI 2.5 array) is already available through the CARTaGENE study. We expect that the CARTaGENE will release genotyping data for another 1000 participants (Affymetrix axiom array) before end of 2017. The genotyping data will be provided to us by the CARTaGENE study investigators. They will be coded according to the CARTaGENE coding system and stored at the Lady Davis Institute until the imputation process is completed. Once the status of carrier/non-carrier is allocated to each of the 3,000 samples, the CARTaGENE investigators will decode the samples and provide us with the necessary information to contact the CARTaGENE participants to ask them to participate in our study.

6.3.2 Change in 25OHD levels

After running linear regression models to generate 25OHD levels adjusted for covariates (age, sex, BMI, ethnicity, oral vitamin D intake and season of measurement), we will calculate the change in 25OHD levels (25OHD levels post treatment- 25OHD levels pre-treatment), adjusted for baseline 25OHD levels. Appropriate transformation of this change in 25OHD level will be used if distributions are found to be skewed. Participants of the two groups will be matched (ie carriers against non-carriers), based on their baseline 25OHD level, given that individuals with the lowest levels of 25OHD, are expected to respond the most to vitamin D replacement. A Student's paired t-test will be used to compare the change in 25OHD level between matched carriers and non-carriers of the *CYP2R1* variant.

7. STUDY ASSESSMENTS AND PROCEDURES

This section lists the parameters of each planned study assessment. The exact timing of each assessment is listed in the Time and Events Tables (Section 4.4).

7.1. Demographic/Medical History Assessments

The following demographic parameters will be captured: date of birth, gender, country of birth of grandparents, self-reported race and ethnicity.
Medical/medication history will be assessed as related to the eligibility criteria listed in Section 5.2.

7.2. Anthropometrics

Height, weight, will be measured and recorded. No physical examination is required for this study.

7.3. Sample Analysis

25OHD measurements will be performed using the field-standard radio-immunoassay, (LIAISON® 25-hydroxyvitamin D Assay by DiaSorin) at the biochemistry lab of the Jewish General Hospital. Raw data will be stored in secure format by the Analysis Team. Our server room at the Richard's lab is monitored by video, has appropriate air-

conditioning, flood control and access in restricted by monitored key cards. The entire cluster is behind a firewall and login is only permitted via a single login node.

7.4. Dietary Questionnaires

Dietary data will be collected via an interviewer-administered, semi-quantitative food frequency questionnaire (FFQ) at the time of participants' recruitment. The FFQ is based on an existing FFQ developed for the participants in the Canadian Multicenter Osteoporosis Study (CaMos)[13]. For each food item, the frequency and serving sizes are included. The content in vitamin D of each food item is calculated using Canada's Food and Drugs regulations on fortification standards and vitamin D quantity from food labels. The FFQ will be filled out by the participants during the screen visit with the help of the research assistant.

8. CONCOMITANT MEDICATIONS AND NON-DRUG THERAPIES

8.1. Permitted Medications

All concomitant medications taken during the study will be recorded in the Case Report Form (CRF).
Antipyretic and analgesic drugs may be used on an as-needed basis provided the total daily dose does not exceed the maximum dose recommended by the manufacturer.

Lipid lowering medications, anti-hypertensives and thyroid hormones will be permitted.

Permitted concomitant medications should be taken as prescribed and the usual time(s), and may be taken prior to scheduled study clinic visits.
Other medications may be permitted if they are not considered to affect subject safety or the objectives of the study.

8.2. Prohibited Medications

If participants are on medications listed in this section, or if they should be started on these medications during the 3 months of the study, they will be deemed non-eligible to participate or to continue their participation in this study. A detailed list of the prohibited medications will be provided to the participants at the screen visit.

- Potent inhibitors of CYP3A4, including but not limited to diltiazem, verapamil, ketoconazole, cyclosporine.
- Potent inducers of CYP3A4, including but not limited to rifampicin (and other anti-tuberculosis drugs), carbamazepine, phenobarbital, phenytoin (anti-epileptic drugs).
- Oral or injectable corticosteroids (inhaled, intranasal and topical are permitted).
- Methotrexate, for autoimmune disease such as rheumatoid arthritis or psoriasis.
- Atypical antipsychotic medications (e.g., aripiprazole, risperidone, clozapine, olanzapine, quetiapine, and ziprasidone).

8.3. Non-Drug Therapies

- Vitamins (incuding multi-vitamins), herbal and dietary supplements (including St John's Wort) are prohibited within 7 days or 5 half-lives (whichever is longer) prior to the first dose of study medication and through final discharge from the study.

9. COMPLETION OR EARLY WITHDRAWAL OF SUBJECTS

9.1. Subject Completion

A completed subject is one who has completed all phases of the study including the follow-up visit.
The end of the study is defined as the last subject's last visit.

9.2. Subject Withdrawal Criteria

A subject may withdraw from investigational product at any time at his/her own request, or may be withdrawn at any time at the discretion of the investigator for safety, behavioural or administrative reasons.

9.3. Subject Withdrawal Procedures

Subjects may withdraw from the study, at his/her own request, at any time and for any reason. They are not obliged to state the reason for withdrawal. However, the reasons for withdrawal, or failure to provide a reason, must be documented by the physician on the CRF. In case of a withdrawal from the study, further analyses will end and the participant has the right to ask to have his/hers samples destroyed. However, all information collected up to the point of withdrawal may be used in order to preserve the scientific integrity of the study.

9.4. Return of Results to Participants and Treatment After the End of the Study

Subjects, upon consent, will receive a letter with information in regards to their 25OHD levels before and after the vitamin D treatment after the completion of the study. Since the clinical relevance of carrying the *CYP2R1* low frequency variant in their response to vitamin D treatment has not been tested yet, information on the participants' carrier/non-carrier status will not be disclosed. We do not anticipate generating any incidental findings from this study. The participants will not receive any additional treatment after completion of the study. Nevertheless, if a participant responds poorly to conventional vitamin D replacement therapy and wishes to be aware of his response to this treatment, we will discuss with the patient potential follow-up medical care and offer consultation with an Endocrinologist.

9.5. Screen and Baseline Failures

Data for screen and baseline failures will be collected in source documentation at the site.

10. INVESTIGATIONAL PRODUCT(S)

Vitamin D dosage and administration details are listed in Section 4.2.

10.1. Packaging and Labelling

A label with sample collection date and study number will be adhered to the blood sample tube. The samples will be stored at room temperature.

10.2. Preparation/Handling/Storage/Desctruction

The blood samples drawn from the participants in our study will be kept for 25 years, in order to allow measurements of additional markers of vitamin D metabolism (e.g. PTH, Phosphate, 1,25 hydroxyvitamin D) if needed. Upon consent of the participants, these samples could be used in future pharmacogenetics studies investigating the response to vitamin D treatment, including other genetic variants than the one analyzed in this study. We will follow the below standard procedures for storage of samples:

1. Identification of samples only through 2D bar-codes. These bar-codes are located inside the tube and therefore cannot be removed. They contain no numbers or letters and are only recognizable by a 2D bar-code scanner.
2. The samples are therefore coded and can only be decoded.
3. All access to the laboratory requires a magnetic swipe card.
4. Samples will be stored in our dedicated locked freezers located in the Richards laboratory. These samples are under the responsibility of Dr. Richards and his delegates, which include the students and laboratory technicians assigned to handle these samples. Only Dr Richards and his students will be able to decode the participant's samples.
5. After 25 years, the samples will be destroyed by incineration by a facility holding a certificate of authorization for the operation of a biomedical waste incineration such as Stericycle Canada. Samples will be transported to the disposal site in a rigid, sealed and watertight container for non-anatomical biomedical waste as required by the Quebec Regulation respecting biomedical waste.

11. STUDY CONDUCT CONSIDERATIONS

11.1. Posting of Information on Publicly Available Clinical Trial Registers

Study information from this protocol will be posted on publicly available clinical trial registers before enrolment of subjects begins.

11.2. Regulatory and Ethical Considerations, Including the Informed Consent Process

Prior to initiation of a study site, the investigator will obtain favourable opinion/approval of the Jewish General Hospital Ethics Committee.
The study will be conducted in accordance with all applicable regulatory requirements. This includes approval by Health Canada prior to the initiation of the study. This is required since Vitamin D3 at doses exceeding 1,000 IU falls under schedule F (prescription drug) according to the *Food and Drugs Act.*
The study will also be conducted in accordance with ICH Good Clinical Practice (GCP), all applicable subject privacy requirements, and, the guiding principles of the 2008 Declaration of Helsinki. This includes, but is not limited to, the following:

- IRB review and favorable opinion/approval to conduct the study and of any subsequent relevant amended documents
- Written informed consent (and any amendments) to be obtained for each subject before participation in the study
- Investigator reporting requirements (e.g. reporting of AE/protocol deviations to IRB)

Written informed consent must be obtained from each subject prior to participation in the study.
If an event occurs that is related to the conduct of the study, and this new event is likely to affect the safety of subjects, the investigator will take appropriate urgent safety measures to protect subjects against any immediate hazard.
The investigator will notify the IRB and Health Canada.

11.3. Records Retention

Following closure of the study, the principal investigator (PI), Dr Richards, must maintain all site study records in a safe and secure location at the Lady Davis Institute for Medical Research. The records must be maintained to allow easy and timely retrieval, when needed (e.g., audit or inspection), and, whenever feasible, to allow any subsequent review of data in conjunction with assessment of the facility, supporting systems, and staff. The retention period will default to 25 years.

11.4. Provision of Study Results to Investigators, Posting of Information on Publically Available Clinical Trials Registers and Publication

Where required by applicable regulatory requirements, an investigator signatory will be identified for the approval of the clinical study report. The investigator will be provided reasonable access to statistical tables, figures, and relevant reports and will have the opportunity to review the complete study results at a mutually-agreeable location.
A manuscript will be progressed for publication in the scientific literature if the results provide important scientific or medical knowledge.

11.5. Data Management

All information obtained about during this study will be treated confidentially within the limits of the law. Subjects' names will not appear in any reports published as a result of this study.

Samples will be coded and will not contain any identifying information (such as name, date of birth, RAMQ number, etc…). The only identification that will be on blood samples will be study number, which will be generated by a random digit generator. It will therefore not be possible to identify subjects from the samples. The PI, or designate, can only perform decoding of the samples. All electronic documents will be password-protected and hard copies kept under lock. The Richards' lab contains swipe-card only access and all freezers are locked. The data facility is accessible only to individuals with swipe cards and the server room only to the PI. All data will be anonymized using random digits as identifiers. All data containing such information is stored on our server, which has all firewalls installed, is behind the Lady Davis Institute firewall and has all file-transfer protocol (FTP) ports blocked.

11.6 Return of Results to CARTaGENE

Among the data collected during this study, the following information will be returned to the CARTaGENE study:

- 25OHD levels and baseline and at the end of the 3-month supplementation period
- Imputed genotypes of the 150 participants (each genotype containing information for approximately 40,000,000 genetic variants).

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APPENDIX 3

American Journal of Human Genetics manuscript

Low-Frequency Synonymous Coding Variation in *CYP2R1* Has Large Effects on Vitamin D Levels and Risk of Multiple Sclerosis

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Vitamin D insufficiency is common, correctable, and influenced by genetic factors, and it has been associated with risk of several diseases. We sought to identify low-frequency genetic variants that strongly increase the risk of vitamin D insufficiency and tested their effect on risk of multiple sclerosis, a disease influenced by low vitamin D concentrations. We used whole-genome sequencing data from 2,619 individuals through the UK10K program and deep-imputation data from 39,655 individuals genotyped genome-wide. Meta-analysis of the summary statistics from 19 cohorts identified in *CYP2R1* the low-frequency (minor allele frequency = 2.5%) synonymous coding variant g.14900931G>A (p.Asp120Asp) (rs117913124[A]), which conferred a large effect on 25-hydroxyvitamin D (25OHD) levels (−0.43 SD of standardized natural log-transformed 25OHD per A allele; p value = 1.5×10^{-88}). The effect on 25OHD was four times larger and independent of the effect of a previously described common variant near *CYP2R1*. By analyzing 8,711 individuals, we showed that heterozygote carriers of this low-frequency variant have an increased risk of vitamin D insufficiency (odds ratio [OR] = 2.2, 95% confidence interval [CI] = 1.78–2.78, p = 1.26×10^{-12}). Individuals carrying one copy of this variant also had increased odds of multiple sclerosis (OR = 1.4, 95% CI = 1.19–1.64, p = 2.63×10^{-5}) in a sample of 5,927 case and 5,599 control subjects. In conclusion, we describe a low-frequency *CYP2R1* coding variant that exerts the largest effect upon 25OHD levels identified to date in the general European population and implicates vitamin D in the etiology of multiple sclerosis.

Introduction

Vitamin D insufficiency affects approximately 40% of the general population in developed countries.[1] This could have important public health consequences, given that vitamin D insufficiency has been associated with musculoskeletal consequences and several common diseases, such as multiple sclerosis (MIM: 126200), type 1 diabetes (MIM: 222100), type 2 diabetes (MIM: 125853), and several cancers.[2] Further, repletion of vitamin D status can be achieved safely and inexpensively. Thus, understanding the determinants of vitamin D insufficiency, and their effects, can provide a better understanding of the role of vitamin D in disease susceptibility with potentially important public health benefits.

Approximately half of the variability in the concentration of the widely accepted biomarker for vitamin D status, 25-hydroxyvitamin D (25OHD), has been attributed to genetic factors in twin and family studies.[3,4] Four common (minor allele frequency [MAF] > 5%) genetic variants in loci near four genes known to be involved in cholesterol synthesis (*DHCR7* [MIM: 602858]), hydroxylation (*CYP2R1* [MIM: 608713]), vitamin D transport (*GC* [MIM: 139200]), and catabolism (*CYP24A1* [MIM: 126065]) are strongly associated with 25OHD levels yet explain little of its heritability.[5] Low-frequency and rare genetic variants (defined as those with a MAF ≤ 5% and 1%, respectively) have recently been found to have large effects on clinically relevant traits,[6–8] providing an opportunity to better understand the

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biologic mechanisms influencing disease susceptibility in the general population.

Therefore, the principal objective of the present study was to detect low-frequency and rare variants with large effects on 25OHD levels through a large-scale meta-analysis and describe their biological and clinical relevance. Similar to an earlier genome-wide association study (GWAS) examining common (MAF $\geq$ 5%) genetic variation by the SUNLIGHT consortium,[5] we sought to increase understanding of the genetic etiology of vitamin D variation within the general population; however, our current study focused on genetic variation with a MAF $<$ 5%. This has only recently been made possible through whole-genome sequencing (WGS) and the use of improved genotype imputation for low-frequency and rare variants with the recent availability of large WGS reference panels.[9] The second objective of this study was to better understand whether low-frequency genetic variants with large effects on 25OHD could predict a higher risk of vitamin D insufficiency in their carriers and whether vitamin D intake through diet might interact with such genetic factors to prevent, or magnify, vitamin D insufficiency. Finally, we sought to understand whether these genetic determinants of 25OHD levels are implicated in multiple sclerosis, a disease influenced by low 25OHD levels.[10]

To do so, we first undertook an association study of WGS data and deeply imputed genome-wide genotypes to identify novel genetic determinants of vitamin D in 42,274 individuals. We next tested if these genetic variants conferred a higher risk of vitamin D insufficiency in 8,711 subjects and whether this insufficiency showed effect modification by dietary intake. Last we assessed their effect on multiple sclerosis in a separate sample of 5,927 case and 5,599 control subjects.

Material and Methods

Cohorts

All human studies were approved by each respective institutional or national ethics review committee, and all participants provided written informed consent. To investigate the role of rare and low-frequency genetic variation on 25OHD levels in individuals of European descent, we used WGS data at a mean read depth of 6.7× in 2,619 subjects from two cohorts with available 25OHD phenotypes in the UK10K project[11] (Table 1). We also used imputation reference panels to impute variants that were missing, or poorly captured, from previous GWASs of 39,655 subjects (Table 1 and Figure 1). The participating individuals were drawn from independent cohorts of individuals of European descent. A detailed description of each of the participating studies is provided in Table S1.

25OHD Measurements

The methods applied for measuring 25OHD levels differed among the participating cohorts (Tables S1 and S6). The four methods

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Table 1. Participating Cohorts and Number of DNA Samples per Cohort

Study	Imputed	Whole-Genome Sequenced
ALSPAC	3,679	1,606
TUK	1,919	1,013
Generation R	1,442	–
BPROOF	2,514	–
FHS	5,402	–
MrOS	3,265	–
RSI	3,320	–
RSII	2,022	–
RSIII	2,913	–
CHS	1,792	–
BMDCS	863	–
MrOS GBG	945	–
GOOD	921	–
MrOS Malmo	893	–
PIVUS	943	–
ULSAM	1,095	–
NEO	5,727	–
Total	**39,655**	**2,619**

used were tandem mass spectrometry (in the Bone Mineral Density in Childhood Study [BMDCS], Osteoporotic Fractures in Men USA [MrOS], and B-Vitamins for the Prevention of Osteoporotic Fractures [BPROOF]), combined high-performance liquid chromatography and mass spectrometry (in the Avon Longitudinal Study of Parents and Children [ALSPAC], BPROOF, Cardiovascular Health Study [CHS], Upssala Longitudinal Study of Adult Men [ULSAM], Netherlands Epidemiology of Obesity [NEO], and Generation R Study [Generation R]), chemiluminescence immunoassay (DiaSorin) (in TwinsUK [TUK], the Prospective Investigation of the Vasculature in Upssala Seniors [PIVUS], the Framingham Heart Study [FHS], Osteoporotic Fractures in Men Malmo [MrOS Malmo], Osteoporotic Fractures in Men Gothenburg [MrOS GBG], and Gothenburg Osteoporosis and Obesity Determinants [GOOD]), and electrochemiluminescence immunoassay (COBAS, Roche Diagnostics) (in Rotterdam Studies I [RSI], II [RSII], and III [RSIII]). Detection limits for the different methods are provided in Table S6.

WGS, Genotyping, and Imputation

ALSPAC WGS and TUK WGS cohorts had been sequenced at an average read depth of 6.7× through the UK10K consortium on the Illumina HiSeq platform and aligned to the GRCh37 human reference sequence with Burrows-Wheeler Aligner 31.[12] Single-nucleotide variant (SNV) calls were completed with SAMtools/BCFtools,[13] and VQSR[14] and GATK were used to recall these variants. WGS for the ALSPAC and TUK cohorts has been described in detail in a previous publication from our group.[7] Table S8 summarizes the data-generation method for sequencing-based cohorts.

Participating studies separately genotyped samples and imputed them to WGS-based reference panels. The most recent imputation panels, such as the UK10K and 1000 Genomes Project (v.3) combined panel (7,562 haplotypes from the UK10K project and 2,184 haplotypes from the 1000 Genomes Project[9]) and the Haplotype Reference Consortium (HRC) panel (64,976 haplotypes[15]), enabled more accurate imputation of low-frequency variants than the UK10K or 1000 Genomes reference panel alone.[9] Specifically, 11 of the 17 participating cohorts were imputed to the combined UK10K and 1000 Genomes reference panel (total number of imputed individuals included in the meta-analysis = 25,589). Three of the participating cohorts were imputed with the HRC panel (n = 5,717). Finally, two cohorts were imputed to the 1000 Genomes panel (n = 7,536), and one cohort was imputed to the UK10K panel (n = 863) (Table S1). Details on genotyping methods and imputation for the 17 participating cohorts are presented in Table S6. Info scores for the imputed SNVs per participating cohort are presented in Table S7. To assess the quality of imputation, we tested the non-reference discordance rate for the low-frequency genome-wide-significant SNVs and found this to be 0% (Table S9).

Association Testing for 25OHD Levels and Meta-analysis

We conducted a GWAS separately for each cohort by using an additive genetic model for 25OHD levels. Because 25OHD concentrations were measured by different methods, log-transformed 25OHD levels were standardized to Z scores after adjustment for age, sex, BMI, and season of measurement. Specifically, the phenotype for each GWAS was prepared according to the following steps: (1) We log transformed 25OHD levels to ensure normality. (2) We used linear regression models to generate cohort-specific residuals of log-transformed 25OHD levels adjusted for covariates (age, sex, BMI, and season). Season was treated as a non-ordinal categorical variable (summer: July to September; fall: October to December; winter: January to March; and spring: April to June). (3) We added the mean of log-transformed 25OHD levels to the residuals to create the adjusted 25OHD phenotype. (4) We then normalized the above phenotype within each cohort (mean of 0 with 1 SD) to make the phenotype consistent across cohorts, given that our consortium has measured 25OHD levels in different cohorts by different methods. (5) Finally, we removed outliers beyond 5 SD from step 4.

For comparison purposes, we computed the average 25OHD levels, adjusted for age, sex, BMI, and season of measurement, in one cohort of our meta-analysis (TUK WGS) in carriers and non-carriers of the lead SNV(s).

The software used for each cohort's GWAS is listed in Table S1. We performed single-variant tests for variants with MAF > 0.1% by using an additive effect of the minor allele at each variant in each cohort. The type of software employed for single-variant testing for each cohort is shown in Table S1. Studies with related individuals used software that accounted for relatedness. Cohort-specific genomic inflation factors (lambda values) are also shown in Table S1 (the mean lambda value was 1.015).

We then meta-analyzed association results from all discovery cohorts (n = 42,274). This stage included validation of the results file format, filtering files by the above quality-control (QC) criteria, comparison of trait distributions among different studies, and identification of potential biases (large beta values and/or standard errors, inconsistent effect allele frequencies, and/or extreme lambda values). Meta-analysis QC of the GWAS data included the following SNV-level exclusion criteria: (1) information score < 0.4, (2) Hardy-Weinberg equilibrium (HWE) p value < 10^{-6}, (3) missingness > 0.05, and (4) MAF < 0.5%.

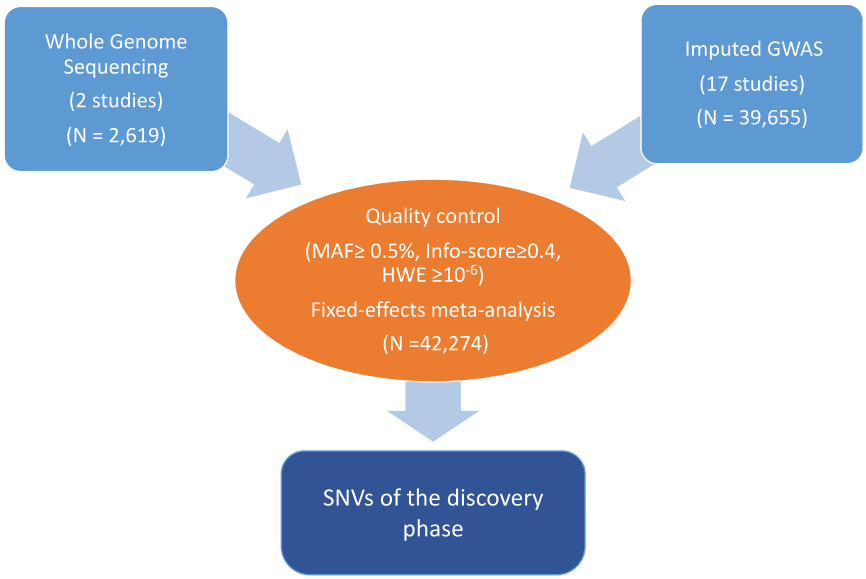

Figure 1. Schematic of the Discovery Single-Variant Meta-analysis

SNV alignment across studies was done with the chromosome and position information for each variant according to genome build hg19 (UCSC Genome Browser). SNVs in the X chromosome were not included in the meta-analysis. Fixed-effects meta-analysis was performed with the software package GWAMA[16] with adjustment for genomic control. We tested bi-allelic SNVs with MAF $\geq$ 0.5% for association and declared genome-wide statistical significance at $p \leq 1.2 \times 10^{-8}$ for variants present in more than one study. This stringent p value threshold was set to adjust for all independent SNVs above the MAF threshold of 0.5%.[17]

Conditional analysis was undertaken for the four previously described lead vitamin D SNVs from the SUNLIGHT consortium with the Genome-wide Complex Trait Analysis (GCTA) package.[18] This method uses an approximate conditional-analysis approach from summary-level statistics from the meta-analysis and inter-SNV linkage-disequilibrium corrections estimated from a reference sample. We used UK10K individuals as the reference sample to calculate the linkage disequilibrium of SNVs. The associated regions flanking within 400 kb of the top SNVs from SUNLIGHT were extracted, and the conditional analyses were conducted within these regions. Conditional analyses of individual variants presented in Tables 2 and S5 were conducted with GCTA v.0.93.9 and default parameters.

We used analyses of haplotype blocks for the candidate variants of interest by deriving phased haplotypes from 1,013 individuals from the TUK WGS cohort with a custom R package.

Effects on Vitamin D Insufficiency

To investigate the effect of genome-wide-significant SNVs on vitamin D insufficiency (defined as 25OHD levels below 50 nmol/L), we used data from four cohorts: TUK imputed, TUK WGS, BPROOF, and MrOS (n = 8,711). We performed logistic regression of this binary phenotype against the SNVs by adjusting for the following covariates: age, sex, BMI, and season of measurement. Meta-analysis of cohort-level summary statistics was performed in R[19] with the epitools[20] and metafor[21] packages.

Interaction Analysis with Vitamin D Intake

We analyzed interactions between our candidate SNV(s) and vitamin D dietary intake (continuous and tertiles) in 9,224 individuals from five of the cohorts (FHS, PIVUS, ULSAM, BPROOF, and RSIII) participating in our discovery phase. A detailed description of the method for capturing vitamin D intake in each of the participating cohorts appears in Table S6. Linear regression was conducted in each of these studies under an additive genetic model. The following variables and co-variables were included in the model: log-transformed serum 25OHD as the dependent variable; SNV genotype (coded as 0, 1, or 2) as an independent variable; SNV (genotype) × dietary vitamin D intake (continuous or tertiles) as an interaction term; and age, sex, BMI, season of 25OHD measurement, dietary vitamin D intake (continuous or tertiles), supplemented vitamin D (yes or no), and total energy intake as covariates. The results from the five studies were meta-analyzed by a fixed-effects model with the metafor tool of the R statistical package.

Effects on Multiple Sclerosis

We tested the effect of the genome-wide-significant SNVs on the risk of multiple sclerosis in 5,927 case and 5,599 control samples by assuming an additive genetic model. Control samples were obtained from the UK Biobank[22] by random selection of participants without multiple sclerosis. Case samples were obtained from the UK Biobank,[22] previously published multiple sclerosis GWASs,[23,24] and newly genotyped UK subjects. Before genotype imputation of the genotyped case samples, we applied numerous QC criteria to ensure unbiased genotype calls between cohorts. These included retaining only SNVs with a MAF > 1% and excluding SNVs or samples with high missingness.[25] Further, samples were assessed for population stratification with EIGENSTRAT,[26,27] and outliers were removed. Genotype data were then imputed by the Sanger Imputation Service[15] with the combined UK10K and 1000 Genomes Phase 3 reference panels,[9,28] the same reference panel used for the UK Biobank control samples. Genotype data were phased with EAGLE2[29] and imputed with PBWT.[30] SNPTEST[31] was used for association testing on the combined case-control dataset, which included testing the additive effect of each allele on multiple sclerosis status and using the top ten principal components from EIGENSTRAT[26,27] to adjust for population stratification and batch effects.

Results

GWAS

After strict QC, the genomic inflation factor for the meta-analysis of 19 GWASs was 0.99, suggesting a lack of bias due to population stratification (Figure 2). Through meta-analysis of 11,026,511 sequenced and imputed variants from our discovery cohorts (Table 1), we identified a signal at the chromosomal locus 11p.15.2, which harbors variants associated with 25OHD levels (lead low-frequency

Table 2. Association Results for Genome-wide-Significant Low-Frequency Variants from Discovery 25OHD Meta-analysis before and after Conditioning on the Lead Common *CYP2R1* SNP, rs10741657, and the Lead Low-Frequency *CYP2R1* Variant, rs117913124

SNV	Chr	Position	EA[a]	EAF[b]	Candidate Gene	Function	Beta[c]	p Value	Conditional on rs10741657		Conditional on rs117913124		n
									Beta[c]	p Value	Beta[c]	p Value	
rs117913124	11	14900931	A	0.025	*CYP2R1*	exon 4 (synonymous codon)	−0.43	1.5×10^{-88}	−0.39	2.4×10^{-78}	NA	NA	41,336
rs116970203	11	14876718	A	0.025	*CYP2R1*[d]	intron 11 variant	−0.43	2.2×10^{-90}	−0.40	3.3×10^{-80}	NA	NA	41,138
rs117361591	11	14861957	T	0.014	*CYP2R1*[d]	intron 11 variant	−0.44	9.1×10^{-51}	−0.40	2.2×10^{-44}	−0.05	0.017	38,286
rs117621176	11	14861320	G	0.014	*CYP2R1*[d]	intron 11 variant	−0.44	8.7×10^{-51}	−0.40	2.1×10^{-44}	−0.05	0.016	38,273
rs142830933	11	14838760	C	0.014	*CYP2R1*[d]	intron 5 variant	−0.44	1.4×10^{-48}	−0.40	1.7×10^{-42}	−0.05	0.03	37,541
rs117672174	11	14746404	T	0.014	*CYP2R1*[d]	intron 1 variant	−0.43	2.8×10^{-45}	−0.39	2.9×10^{-39}	−0.04	0.062	37,209

Abbreviations are as follows: Chr, chromosome; EA, effect allele; EAF, effect allele frequency; NA, not applicable; SNV, single-nucleotide variant.
[a]Effect allele is the 25OHD decreasing allele.
[b]Effect allele frequency.
[c]Beta values represent changes in standard deviations of the standardized log-transformed 25OHD levels.
[d]Nearest gene: *PDE3B*.

SNV g.14900931G>A [p.Asp120Asp] [rs117913124(A)] [GenBank: NC_000011.9]; MAF = 2.5%, allelic effect size = −0.43 SD of the standardized log-transformed 25OHD levels [SD], $p = 1.5 \times 10^{-88}$; Figure 3 and Table 2). The direction of effect was consistent across all discovery cohorts (Table 3 and Figure 3A), and the mean imputation information score for the imputed studies was 0.97. This low-frequency synonymous coding variant is in exon 4 of *CYP2R1* and is ~14 kb from the previously identified common *CYP2R1* variant rs10741657 (r^2 between these two SNVs = 0.03) (Figure 4). To our knowledge, rs117913124 has not previously been associated with any vitamin-D-related traits in humans.

Figure S1 shows a comparison of the average 25OHD levels, adjusted for age, sex, BMI, and season of measurement, in non-carriers and heterozygous carriers of the A allele of rs117913124 in the TUK WGS cohort. The average 25OHD levels, adjusted for age, sex, BMI, and season of measurement were computed in 542 individuals from the TUK WGS cohort, among which 510 were not carriers and 32 were heterozygous carriers of the A allele of rs117913124 (no homozygous carriers were present in this cohort). After removing outliers (adjusted 25OHD levels ±3 SD from the mean), we included in our analysis 449 non-carriers and 30 heterozygous carriers (for a total of 479 individuals). A linear-regression model with the adjusted 25OHD levels as the dependent variable and the dose of the A allele of rs117913124 (numeric factor 1 or 0) as the independent variable demonstrated an 8.3 nmol/L decrease in the adjusted 25OHD levels per A allele. The mean adjusted 25OHD levels were 64.3 nmol/L in non-carriers and 56.0 nmol/L in heterozygous carriers.

Two-way conditional analysis between the *CYP2R1* common (rs10741657) and low-frequency (rs117913124) variants revealed that the two association signals are largely independent. Specifically, after conditioning on rs10741657, rs117913124 remained strongly associated with 25OHD levels ($p_{cond} = 2.4 \times 10^{-78}$); after conditioning on rs11791324, the effect of rs10741657 on 25OHD levels remained significant ($p_{cond} = 4.0 \times 10^{-33}$ versus $p_{pre\text{-}cond} = 8.8 \times 10^{-45}$; Tables 2 and S5). Further, no other low-frequency variant in the region remained significant after conditioning on rs117913124 (Table 2). To further disentangle the role of rs117913124 from that of rs10741657 on 25OHD levels, we undertook a haplotype analysis based on WGS data from 3,781 individuals from the TUK WGS and ALSPAC WGS cohorts. We found that the 25OHD decreasing A allele of rs117913124 was always transmitted in the same haplotype block with the 25OHD decreasing G allele of the common *CYP2R1* variant rs10741657. By using 25OHD data from the TUK WGS cohort, we compared the 25OHD levels among carriers of the various haplotype blocks. We observed lower levels of 25OHD in carriers of the A allele of rs117913124 than in non-carriers, independently of the presence of the effect allele G of the common *CYP2R1* variant (Table 4).

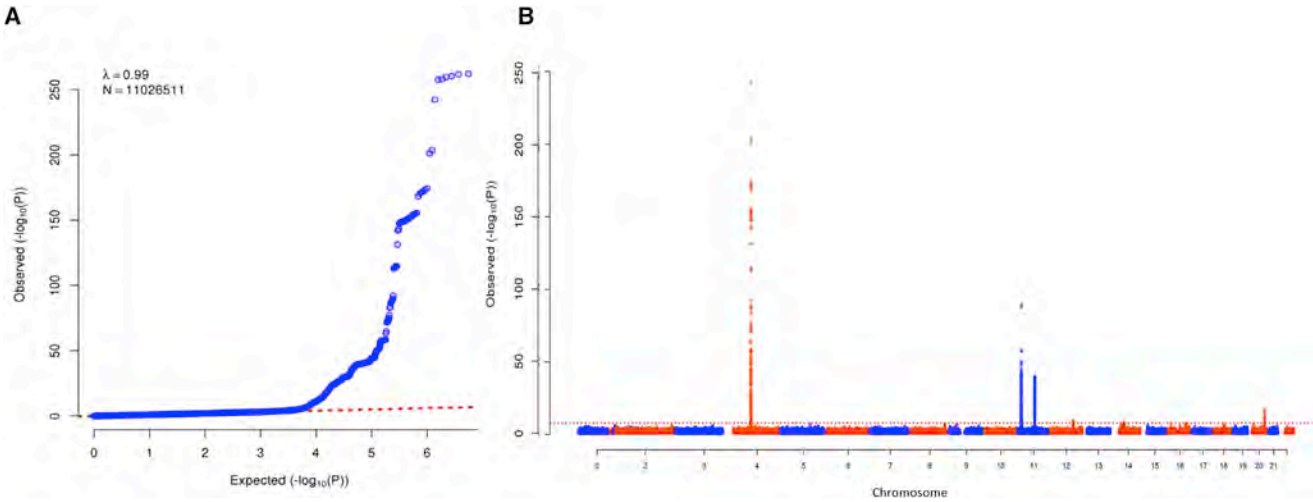

Figure 2. Discovery Single-Variant Meta-analysis
(A) Quantile-quantile plot for the single SNV meta-analysis.
(B) Manhattan plot of the meta-analysis depicts variants with MAF > 0.5% across the 22 autosomes against the $-\log_{10}$ p value from the meta-analysis of 19 cohorts, which included 42,274 individuals.

No other low-frequency or rare variants were identified in the three previously described vitamin-D-related loci at *DHCR7*, *GC*, and *CYP24A1*. The mean effect size of the four previously reported common (MAF ≥ 5%) genome-wide-significant SNVs from the SUNLIGHT consortium was −0.13 SD, and the largest effect size was −0.25 SD (for the *GC* variant) in our meta-analysis (Table S3 and Figure 3B). The effect size of rs10741657(G), the known common *CYP2R1* variant, was −0.09 SD. Hence, the observed effect size of rs117913124 is 3-fold larger than the above mean, 4-fold larger than that of the common *CYP2R1* variant, and almost twice that of the largest previously reported effect of the *GC* variant. Last, the percentage of the 25OHD phenotype variance explained by the low-frequency *CYP2R1* variant (0.9%) was more than double the percentage of the variance explained by the *CYP2R1* common variant (0.4%).

We also identified 18 genome-wide-significant low-frequency and rare SNVs on the same chromosome 11 region as rs117914124 in the neighboring *PDE3B* (MIM: 602047)

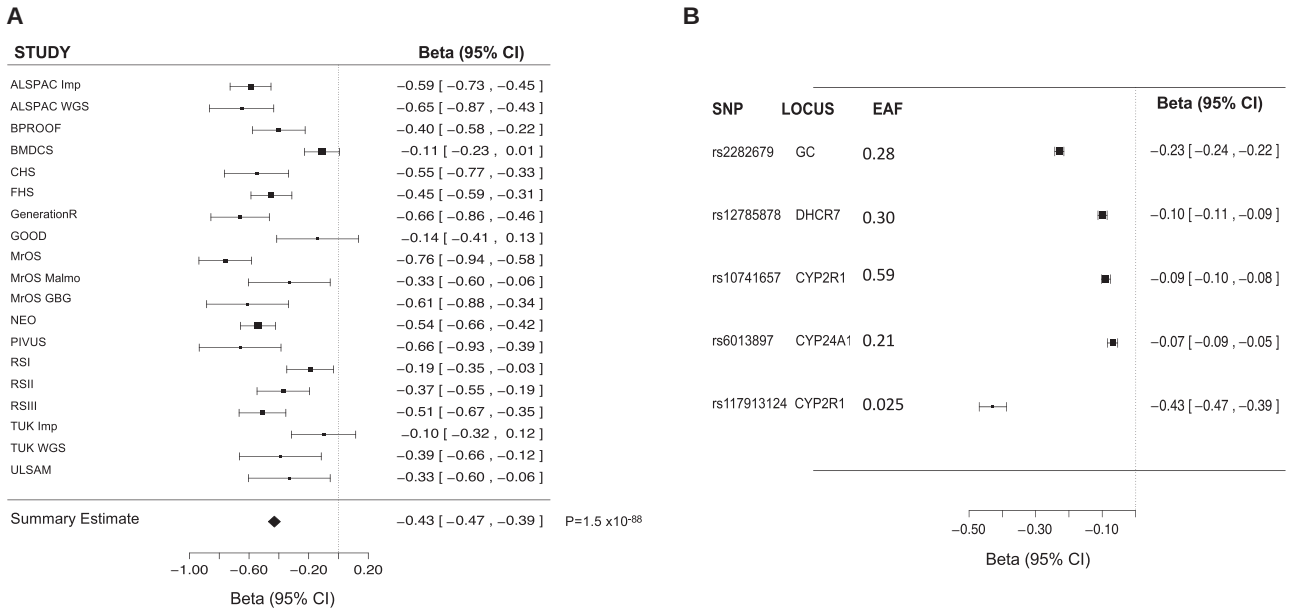

Figure 3. Forest Plot by Cohort for rs117913124 and Forest Plot for rs117913124 and the Previously Described Common 25OHD-Related Variants from Discovery Meta-analysis
(A) Forest plot of estimates from all 19 studies for the low-frequency *CYP2R1* variant rs117913124.
(B) Forest plot of the effect of the four common SUNLIGHT variants and the *CYP2R1* low-frequency variant rs117913124 on log-transformed 25OHD levels.
Squares represent beta values in the 19 studies, and bars around the squares represent 95% confidence intervals (CIs).

Table 3. Summary Statistics for the *CYP2R1* Low-Frequency Variant, rs117913124, from 19 Studies

Study	25OHD Measurement Method	n	EAF (A Allele[a])	Beta[b]	Standard Error	p Value	Information Score
ALSPAC imputed	MS	3,675	0.028	−0.59	0.07	3.43×10^{-18}	0.99
ALSPAC WGS	MS	1,606	0.028	−0.65	0.11	8.23×10^{-10}	NA
BPROOF	MS	2,512	0.027	−0.4	0.09	4.99×10^{-6}	0.97
BMDCS	MS	863	0.019	−0.11	0.06	0.058	0.98
CHS	MS	1,581	0.022	−0.55	0.11	5.15×10^{-7}	0.88
FHS	CLIA	5,402	0.021	−0.45	0.07	2.32×10^{-10}	0.97
GenerationR	MS	1,442	0.033	−0.66	0.1	1.78×10^{-6}	1
GOOD	CLIA	921	0.028	−0.14	0.14	0.31	0.96
MrOS	MS	3,265	0.018	−0.76	0.09	5.63×10^{-16}	0.96
MrOS Malmo	CLIA	893	0.033	−0.33	0.14	0.016	0.94
MrOS GBG	CLIA	945	0.026	−0.61	0.14	7.87×10^{-6}	1
NEO	MS	5,727	0.025	−0.54	0.06	2.73×10^{-19}	1
PIVUS	CLIA	943	0.028	−0.66	0.14	2.56×10^{-6}	0.99
RSI	ECLIA	3,320	0.025	−0.19	0.08	0.019	0.98
RSII	ECLIA	2,022	0.033	−0.37	0.09	2.38×10^{-5}	0.99
RSIII	ECLIA	2,913	0.027	−0.51	0.08	4.61×10^{-10}	0.98
TUK imputed	CLIA	1,919	0.021	−0.1	0.11	0.35	0.98
TUK WGS	CLIA	1,013	0.025	−0.39	0.14	0.006	NA
ULSAM	MS	1,095	0.025	−0.33	0.14	0.02	1

Abbreviations are as follows: CLIA, chemiluminescence immunoassay; EAF, effect allele frequency; ECLIA, electrochemiluminescence immunoassay; MS, mass spectrometry; NA, not applicable; 25OHD, 25-hydroxyvitamin D.
[a]Effect allele is the 25OHD decreasing allele.
[b]Beta values represent changes in standard deviations of the standardized log-transformed 25OHD levels.

(Tables 2 and S4 and Figure 4B). Signals from these SNVs in *PDE3B* were independent of the common variant at *CYP2R1* (Table 2). We then created haplotype blocks with rs117913124 and SNVs at *PDE3B* on the basis of haplotype information from the 3,781 individuals from the TUK WGS and ALSPAC WGS cohorts (Table S2). We found that the 25OHD decreasing allele (A) of rs117913124 was always inherited with the 25OHD decreasing allele (A) of its perfect proxy rs116970203 ($r^2 = 1$). Therefore, rs116970203 is not likely to have a distinct effect from that of rs117913124 on 25OHD levels. On the other hand, the 25OHD decreasing alleles of the remaining four low-frequency variants (all with a MAF of approximately 1.4%) were not always inherited in the same haplotype block as rs117913124 and rs116970203 and were in moderate linkage disequilibrium with rs117913124 (all $r^2 < 0.6$; Figures 4B and 4C). Each of the four alleles was in almost perfect linkage disequilibrium with the remaining three (all $r^2 > 0.96$). This implies that these four SNVs might influence 25OHD levels independently of rs117913124. Nevertheless, as mentioned above, after conditioning on the lead low-frequency *CYP2R1* SNV rs117913124, the p values of the four *PDE3B* SNVs became non-significant and their beta values decreased substantially (Table 2), demonstrating that they probably do not represent an independent signal at the chromosome 11 locus.

rs117913124 and Risk of Vitamin D Insufficiency

To further investigate the clinical significance of the low-frequency *CYP2R1* variant rs117913124, we tested its effect on a binary outcome for vitamin D insufficiency (defined as 25OHD levels < 50 nmol/L) in 8,711 individuals from four studies (TUK WGS, TUK IMP, BPROOF, and MrOS). rs117913124 was strongly associated with an increased risk of vitamin D insufficiency (odds ratio [OR] = 2.20, 95% confidence interval [CI] = 1.8–2.8, p = 1.2×10^{-12}) (Figure 5) after control for relevant covariates as described in the Material and Methods.

Common 25OHD-Associated SNVs

We report two additional loci associated with 25OHD levels (Table 5). Variants leading these associations were common and exerted a rather small effect on 25OHD: (1) a variant in chromosome 12 (rs3819817[C], intronic to *HAL* [MIM: 609457]) with a MAF of 45%, a beta value of 0.04, and a p value of 3.2×10^{-10}; and (2) a variant in chromosome 14 (rs2277458[G], intronic to *GEMIN2* [MIM: 602595]) with a MAF of 21%, a beta value of −0.05, and

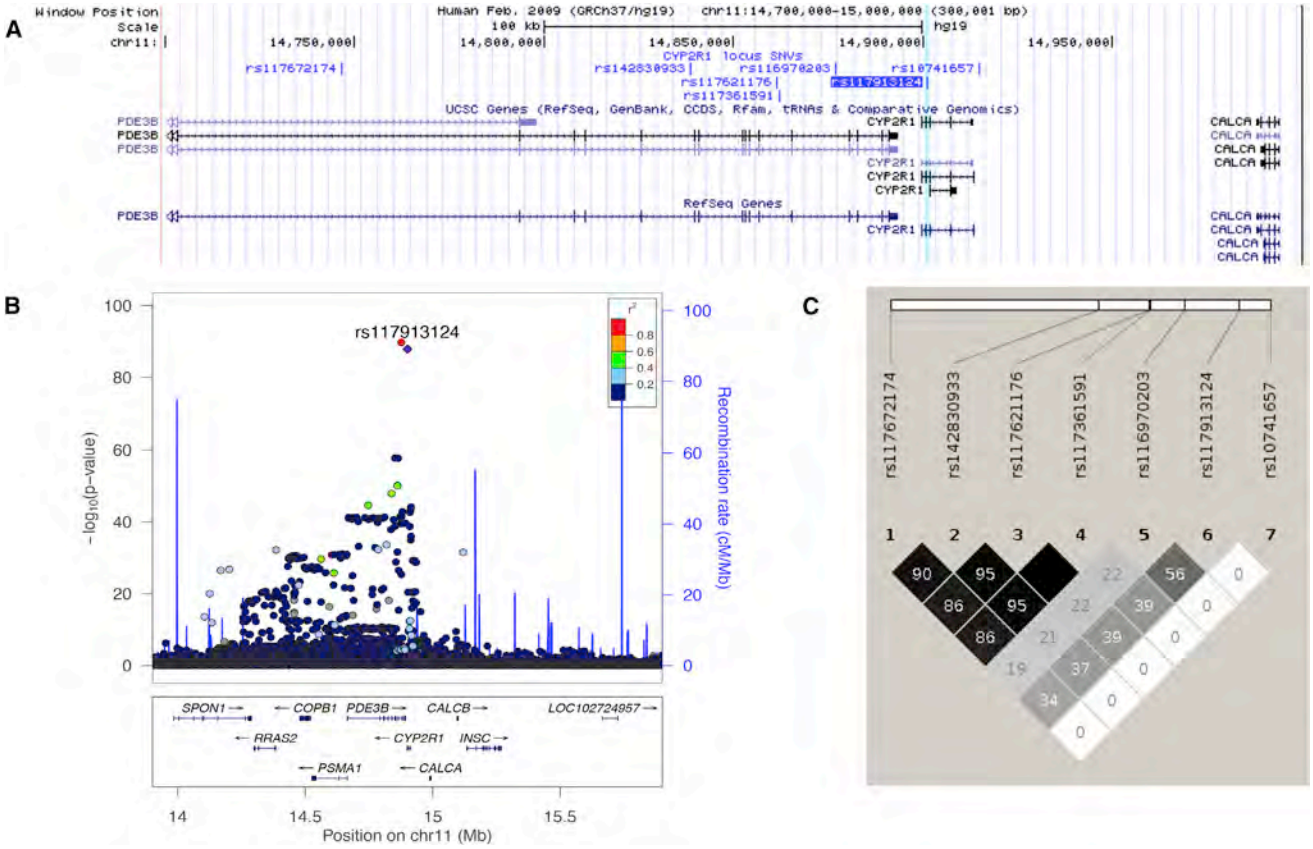

Figure 4. Association Signals from 11p.15.2
(A) UCSC Genome Browser snapshot including the top low-frequency SNVs (see Table 2) and the lead common variant rs10741657 in *CYP2R1*. The position of rs117913124 is highlighted in light blue.
(B) Regional disequilibrium plot showing rs117913124 (purple dot), its perfect proxy rs11670203 (red dot), and the other genome-wide-significant SNVs in the same locus (blue and green dots). The plot depicts SNVs within 1 Mb of a locus's lead SNV (x axis) and their associated meta-analysis p value ($-\log_{10}$) (see Table S10 for more details). SNVs are color coded according to r^2 with the lead SNV (labeled; r^2 was calculated from the UK10K WGS dataset). The recombination rate (blue line), position of genes and their exons, and direction of transcription are also displayed (below plot).
(C) Linkage-disequilibrium plot indicating the r^2 values between the SNVs of Table 2 (top low-frequency variants) and between these low-frequency SNVs and the lead common variant (rs107416570) at the same *CYP2R1* locus (r^2 calculated from the 1000 Genomes dataset).

a p value of 6.0×10^{-9}. Both variants were present in all 19 studies, and the direction of the effect was the same among the 19 studies (Figure 6). Neither the *HAL* nor the *GEMIN2* locus is previously known to be associated with 25OHD levels. Of note, neither variant was present in the HapMap imputation reference used in the SUNLIGHT study.

Interaction Analysis

CYP2R1 encodes the enzyme responsible for 25-hydroxylation of vitamin D in the liver,[32] a necessary step in the conversion of dietary vitamin D and vitamin D oral supplements to the active metabolite, 1,25 dihydroxy-vitamin D. Therefore, we hypothesized that, in contrast with non-carriers, individuals heterozygous or homozygous for rs117913124 in *CYP2R1* would not show a response in their 25OHD levels to vitamin D intake. In other words, we expected carriers of the effect allele of rs117913124 to have steadily lower 25OHD levels, independently of their vitamin D intake. To investigate this hypothesis, we tested the presence of interaction between rs117913124 and vitamin D dietary intake (continuous values and tertiles) on 25OHD levels in 9,224 individuals from five studies (Figure S2). We found no interaction between rs117913124 and dietary vitamin D intake (beta value = −0.0002 and interaction p value = 0.41 for continuous vitamin D intake; beta value = 0.012 and p value = 0.60 for tertiles of vitamin D intake). Given that the two common 25OHD-associated SNVs are located in genes (*HAL* and *GEMIN2*) with no known role in the processing of dietary vitamin D, we found no biological rationale for undertaking a gene-diet interaction analysis for these variants.

25OHD-Assosiated Variants and Risk of Multiple Sclerosis

We tested whether the *CYP2R1* low-frequency variant rs117913124 and the common variants rsrs3819817 and

Table 4. Effect of Different Haplotype Combinations of the Low-Frequency (rs117913124) and Common (rs10741657) *CYP2R1* Variants on 25OHD Levels

Haplotype[a]	Beta[b]	p Value	n
GA GA	−0.02	0.79	156
AG GA	−0.49	0.02	23
AG GG	−0.3	0.13	27
GA GG	0.01	0.87	477
GG GG	0.05	0.58	330

Results are based on individuals from the TUK WGS cohort.
[a]The first allele in each chromatid corresponds to the low-frequency variant rs117913124; the second allele corresponds to the common variant rs10741657. The two AG blocks contain the 25OHD decreasing allele (A) of the low-frequency variant, which is always inherited with the 25OHD decreasing allele (G) of the common variant.
[b]Beta values represent changes in standard deviations of the standardized log-transformed 25OHD levels.

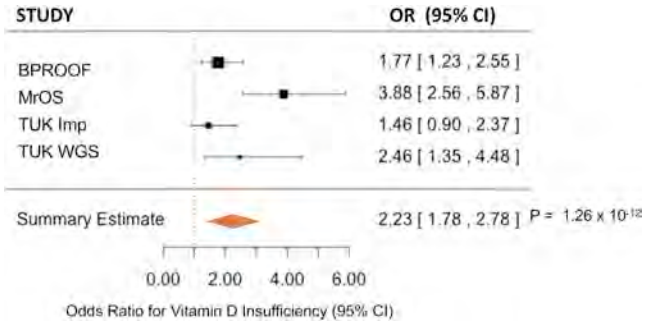

Figure 5. Effect of rs117913124 on Vitamin D Insufficiency
Forest plot of the effect of the low-frequency *CYP2R1* variant rs117913124 on vitamin D insufficiency in four studies. Squares represent odds ratios for vitamin D insufficiency in the four studies, and bars represent 95% CIs.

rs2277458 in *HAL* and *GEMIN2*, respectively, influence the risk of multiple sclerosis. In 5,927 multiple sclerosis samples and 5,599 control samples, we found that the 25OHD decreasing allele at rs117913124[A] was associated with increased odds of multiple sclerosis (OR = 1.40; 95% CI = 1.19–1.64; p value = 2.6×10^{-5}). By way of comparison, the OR of multiple sclerosis for the common *CYP2R1* variant was 1.03 (95% CI = 0.97–1.08; p value = 0.03) in the same study and has previously been reported to be 1.05 (95% CI = 1.02–1.09; p value 0.004) in a separate study.[33] Thus, the effect per allele of rs117913124 on multiple sclerosis was 12.4-fold larger than that attributed to the already known common variant at *CYP2R1*. With regard to the two common SNVs, the 25OHD decreasing allele (T) at the *HAL* variant rs3819817 was not clearly associated with risk of multiple sclerosis; however, there was a trend in the expected direction: OR = 1.05 (95% CI = 1.00–1.11; p value = 0.07). We found no association between the 25OHD decreasing allele (G) at the *GEMIN2* variant rs2277458 and risk of multiple sclerosis: OR = 1.03 (95% CI = 0.96–1.11; p value = 0.34).

Discussion

In the largest GWAS meta-analysis of 25OHD levels in European populations to date, we have identified a low-frequency, synonymous coding genetic variant that has a large effect and strongly associates with 25OHD levels. This variant has an effect size 4-fold larger than that described for the common variant in the same gene (*CYP2R1*) and is associated with a 2-fold increase in risk of vitamin D insufficiency and a 40% increase in the odds of developing multiple sclerosis. The biological plausibility of these findings is supported by the fact that the low-frequency variant is located in *CYP2R1*, encoding the major hepatic 25-hydroxylase for vitamin D.[32] These findings are of clinical relevance given that 5% of the general European population carries this variant in either the homozygous or heterozygous state, and it is associated with a clinically relevant increase in the risk of multiple sclerosis.

Our study was enabled by large imputation reference panels (UK10K-1000 Genomes and HRC) that offer at least 10-fold more European samples than the 1000 Genomes reference panel alone. We did not identify genome-wide-significant variants with a large effect on 25OHD in novel genes in Europeans, although we did find variants with smaller effects in two loci not previously known to be associated with 25OHD. We also identified in a known vitamin-D-related gene low-frequency variants with much larger effects than those of the previously described common variants.

CYP2R1 encodes the enzyme that is responsible for 25-hydroxylation of vitamin D and is one of the two main enzymes responsible for vitamin D hepatic metabolism[32] (Figure 7). Rare mutations in *CYP2R1* have already been described to cause rickets (MIM: 27744).[32,34] Given the important role of *CYP2R1* in the conversion of dietary vitamin D and vitamin D oral supplements to the active form of vitamin D, we hypothesized that carriers of the low-frequency *CYP2R1* variant might respond poorly to vitamin D replacement therapy. We tested this hypothesis by undertaking an interaction analysis between the *CYP2R1* low-frequency variant and dietary vitamin D intake, which showed no clear interaction. However, we note that studies of gene-environment interactions are generally underpowered, measurement error in dietary data is common, and this interaction was further limited by time differences between assessment of dietary intake and measurement of 25OHD levels. Therefore, whether this genetic variant influences 25OHD response to vitamin D administration requires further study.

Although the aim of the present study was to describe variants of low MAF and large effect on 25OHD, we report two common chromosome 12 (*HAL*) and 14 (*GEMIN2*) variants that have a small effect size and reached genome-wide significance in our

Table 5. Main Findings of the GWAS Meta-analysis

SNP	Chr	Candidate Gene	EA	EAF	Beta[a]	p Value	n
rs117913124	11	*CYP2R1*	A	0.025	−0.43	1.5×10^{-88}	41,336
rs3819817	12	*HAL*	C	0.45	0.04	3.2×10^{-10}	41,071
rs2277458	14	*GEMIN2*	G	0.21	−0.05	6.0×10^{-9}	39,746

Abbreviations are as follows: Chr, chromosome; EA, effect allele; EAF, effect allele frequency; SNP, single-nucleotide polymorphism.
[a]Beta values represent changes in standard deviations of the standardized log-transformed 25OHD levels while controlling for age, sex, BMI, and season of measurement.

meta-analysis. Although no existing evidence implicates *GEMIN2* in vitamin-D-related physiological pathways, *HAL* is expressed in the skin and is involved in the formation of urocanic acid, a "natural sunscreen."[35,36] Thus, this could constitute a plausible pathophysiologic mechanism implicating *HAL* in vitamin D synthesis in the skin. Additional functional follow-up of the signals in chromosomes 12 and 14 is needed to characterize the genes and/or mechanisms underlying these associations.

Our findings could have clinical relevance for several reasons. First, individuals carrying at least one copy of the low-frequency *CYP2R1* variant have lower levels of 25OHD by a clinically relevant degree. Specifically, the risk of vitamin D insufficiency is doubled in these individuals. Second, their risk of multiple sclerosis is also increased in accordance with previous evidence supporting a causal role for vitamin D in the risk of multiple sclerosis.[10] Third, these findings affect ~5% of individuals of European descent. Fourth and finally, rs117913124 could be used along with the previously identified common vitamin-D-related variants as an additional genetic predictor of low 25OHD levels in Mendelian randomization studies investigating the causal role of low vitamin D levels in human disease.

Our study also has its limitations. First, although the scope of our study was detection of low-frequency and rare variants, we opted to include in our meta-analysis two WGS studies with a relatively low read depth of 6.7×, as well as three studies imputed to older imputation panels (1000 Genomes and UK10K). These studies have a limited capacity to capture very rare variants, which might explain why we failed to identify such associations. In addition to the limitations arising from the time difference between assessment of dietary vitamin D intake and 25OHD measurements, the analysis of the gene-diet interaction, as mentioned above, might have lacked statistical power. Because our analysis was restricted to populations of European ancestry, we cannot make any assumptions concerning the effect of

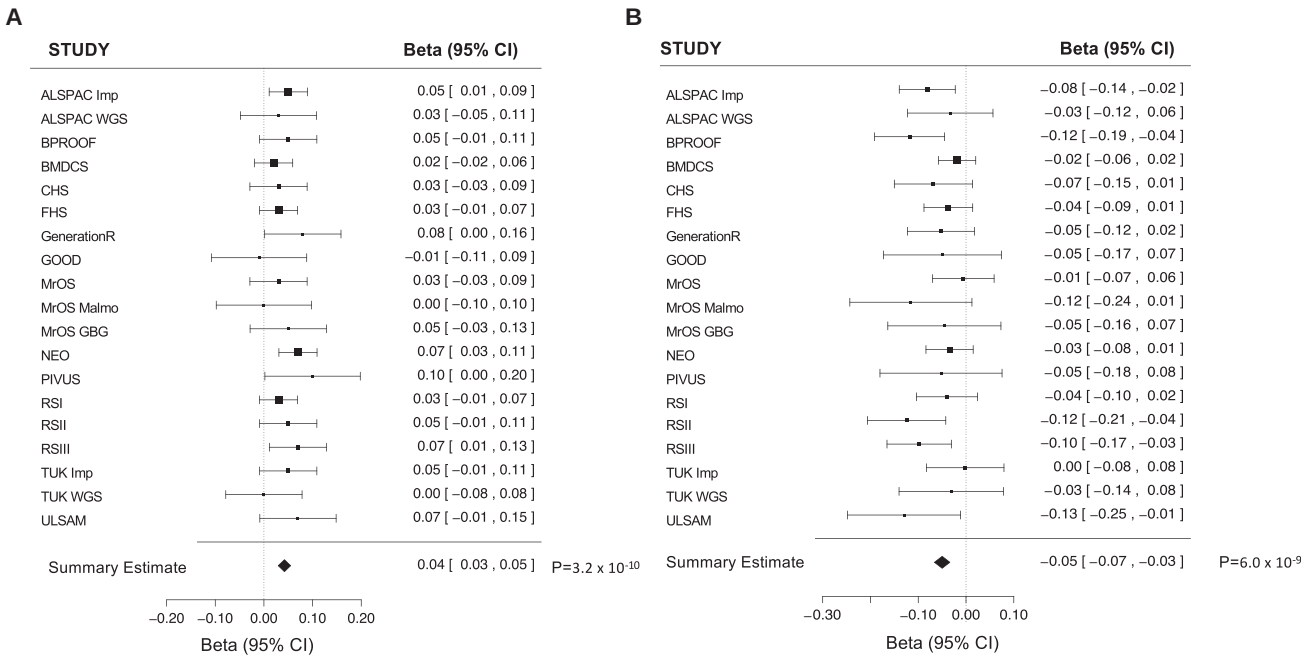

Figure 6. Association Signals from Chromosomes 12 and 14
Forest plots with (A) estimates for the chromosome 12 common variant rs3819817 and (B) estimates for the chromosome 14 common variant rs2277458 from all 19 studies of the meta-analysis where both variants were present. Squares represent beta values in the 19 studies, and bars around the squares represent 95% CIs.

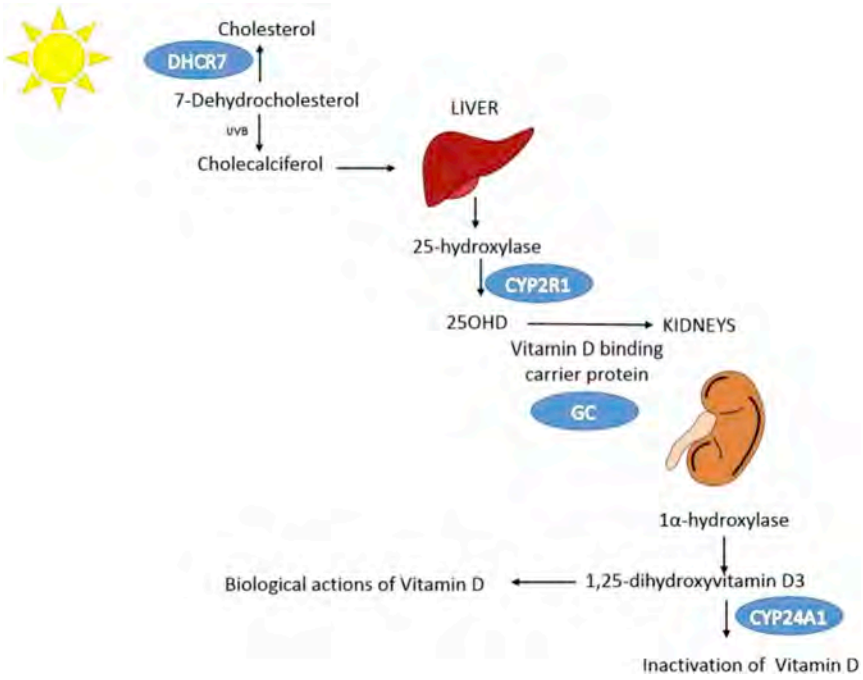

Figure 7. Schematic of the Vitamin D Metabolic Pathway
UVB, ultraviolet B rays.

rs117913124 in non-European populations. Nonetheless, according to the 1000 Genomes reference, this variant is rare in Africans (MAF = 0.3%) and has not been described in East Asians (MAF = 0%). Therefore, describing with any certainty the effect of this variant on 25OHD levels in these populations will require large sample sizes of these populations. Finally, in the absence of functional experiments showing the exact function of rs117913124 in *CYP2R1* and given that this synonymous polymorphism does not affect protein sequence, we cannot unequivocally confirm that this low-frequency variant is causal; however, given that this is a coding variant in a well-documented 25OHD-associated gene, it seems likely that it exerts its effect on *CYP2R1*.

In conclusion, our findings demonstrate the utility of WGS-based discovery and deep imputation for enabling the characterization of genetic associations, offering an improved understanding of the pathophysiology of vitamin D, providing an enriched set of genetic predictors of 25OHD levels for future study, and enabling the identification of groups at increased risk for vitamin D insufficiency and multiple sclerosis.

Accession Numbers

The GWAS summary statistics reported in this paper have been deposited in the Genome-wide Repository of Associations between SNPs and Phenotypes (GRASP).

Supplemental Data

Supplemental Data include 2 figures, 10 tables, and Supplemental Acknowledgments and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2017.06.014.

Web Resources

GCTA, http://cnsgenomics.com/software/gcta/
GenBank, https://www.ncbi.nlm.nih.gov/genbank/
GRASP: Genome-wide Repository of Associations between SNPs and Phenotypes, https://grasp.nhlbi.nih.gov/Overview.aspx
GWAMA, http://www.geenivaramu.ee/en/tools/gwama
OMIM, http://www.omim.org
UCSC Genome Browser, https://genome.ucsc.edu/
UK10K, http://www.uk10k.org
VQSLOD, http://www.broadinstitute.org/gsa/wiki/index.php/Variant_quality_score_recalibration

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