Genetics of vitamin D in an Italian population

Francesca Marini [1], Francesca Giusti [2,3], Gaia Palmini [1], Simone Donati [2], Giada Marroncini [4], Francesco Saverio Martelli [4,5], Maria Luisa Brandi [1,3]

[1] F.I.R.M.O., Italian Foundation for Research on Bone Diseases, Florence, Italy; [2] Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy; [3] Donatello Bone Clinic, Villa Donatello Hospital, Sesto Fiorentino (FI), Italy; [4] Biomolecular Diagnostic Srl, Florence, Italy; [5] IMI-EDN (International Microdentistry Institute- Excellence Dental Network), Florence, Italy.

ABSTRACT

The active form of vitamin D (calcitriol) is a pleiotropic hormone exerting important biological functions, mainly as a key factor in the regulation of correct calcium and bone metabolism. Two hydroxylases are responsible for vitamin D (cholecalciferol) hydroxylation at positions 1 and 25 of the molecule, namely 1-alpha-hydroxylase and 25-hydroxylase, encoded by the genes *CYP27B1* and *CYP2R1*, respectively. Polymorphic variants in these genes are recognized to influence individual vitamin D status.

Here, we looked for associations between five polymorphic variants, three in *CYP2R1* and two in *CYP27B1*, and serum levels of 25-hydroxy-vitamin D [25(OH)D3] in an Italian adult population.

The AA genotype, homozygous for the minor allele of the single nucleotide polymorphism (SNP) rs10741657 in *CYP2R1*, was significantly associated with higher mean levels of 25(OH)D3 in the blood, in accordance with results previously found in other populations.

Our data, confirmed that the AA genotype is a favorable genetic status in reducing individual risk of vitamin D deficiency and insufficiency, and that GG-bearing individuals may benefit more from supplementation with calcifediol rather than cholecalciferol.

KEYWORDS

Vitamin D3, *CYP2R1* gene, *CYP27B1* gene, polymorphic variants.

Introduction

The active form of vitamin D [calcitriol or 1,25-dihydroxy-vitamin D; $1,25(OH)_2D_3$] is a pleiotropic hormone exerting important biological functions. Vitamin D is principally known as a key factor in the regulation of correct calcium and bone metabolism.

$1,25(OH)_2D_3$ is synthesized from cholecalciferol (vitamin D_3), a molecule derived from cholesterol that is produced in the subcutaneous tissue upon exposure to ultraviolet solar radiation. Essentially, cholecalciferol is activated through two distinct hydroxylation reactions that add two -OH groups to the molecule, at position 25 and position 1, respectively. These reactions are mediated by two specific enzymes—25-hydroxylase, encoded by the *CYP2R1* gene, and 1-alpha-hydroxylase, encoded by the *CYP27B1* gene—and they occur in the liver and in the kidney proximal convoluted tubule cells, respectively. Renal 1-alpha-hydroxylase is a key regulator of active vitamin D_3 synthesis and it is inhibited, among other factors, by $1,25(OH)_2D_3$ itself, through a negative feedback mechanism. The functionality of these two hydroxylases is essential in regulating individual bioavailability of $25(OH)D_3$ and $1,25(OH)_2D_3$.

$25(OH)D_3$ is the main circulating metabolite of vitamin D_3, with a half-life of 12-18 days, and it is therefore the biochemical parameter most commonly measured to evaluate vitamin D_3 bioavailability in the organism. Conversely, $1,25(OH)_2D_3$ circulates in the blood at concentrations approximately 1,000 times lower and has an extremely short half-life. According to the international guidelines of the Endocrine Society, vitamin D_3 deficiency corresponds to less than 20 ng/ml of circulating $25(OH)D_3$, and insufficiency to levels of between 21 and 29 ng/ml, while normal levels fall within the 30-200 ng/ml range [1]. Vitamin D_3 deficiency and insufficiency require supplementation of this vitamin, making it very important, also in order to determine the most suitable and effective type of supplement, to identify individuals at greatest risk, especially those whose deficiency may be due to reduced activity, genetically predetermined, of one or both of the hydroxylases involved in the vitamin D_3 activation process.

Rare variants (mutations) in the genes encoding 25-hydroxylase and 1-alpha-hydroxylase have been associated with Mendelian disorders, namely, 25-hydroxylase deficiency-associated rickets (OMIM 600081) and vitamin D-dependent rickets type 1 (OMIM 264700), respectively. In addition, both of these genes, *CYP2R1* and *CYP27B1*, are highly polymorphic, and the less frequent alleles of some polymorphisms (common gene variants with a >1% population frequency) have been shown to

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Contact
Francesca Marini: francesca.marini@fondazionefirmo.com
F.I.R.M.O. - Italian Foundation for Research on Bone Diseases, Florence, Italy

affect enzyme function [2,3], ultimately modifying the circulating levels of 25(OH)D_3 and of available active hormone, thereby contributing to the individual's vitamin D status.

No studies of the allele frequencies of potentially functional polymorphisms on *CYP2R1* and *CYP27B1* in relation to individual vitamin D_3 status have ever been carried out in the general Italian population, except in association with particular pathologies.

Study population and methods

Study population

This study was approved by the Tuscany Regional Ethics Committee for Clinical Trials - Area Vasta Centro section (CEAVC) on September 29, 2024 [Reference number: 25008_oss]. Adults (n=105) of both sexes, aged between 18 and 65 years, were included. The inclusion criteria were:

- age between 18 and 65 years;
- not taking, or not having taken within the six months prior to inclusion in the study, supplementation with cholecalciferol, calcifediol, or calcitriol;
- availability of at least one serum dosage of 25(OH)D_3 in their health record;
- availability of a genomic DNA sample, extracted from venous blood lymphocytes, for genetic analysis.

The exclusion criteria were:

- age under 18 years (to eliminate skeletal growth as a possible confounding influence on vitamin D_3 values and metabolism);
- age over 65 years (because aging is associated with an effective reduction in the activity of 25-hydroxylase and, especially, 1-alpha-hydroxylase [4,5]);
- taking, or having taken in the six months prior to the study, cholecalciferol, calcifediol, or calcitriol supplementation;
- taking, or having taken in the six months prior to the study, drug therapy (teriparatide, natpar, etc.) that can alter vitamin D_3 metabolism;
- presence of disease known to impair vitamin D_3 metabolism (hyperparathyroidism, hypoparathyroidism, hypophosphatemic rickets, etc.);
- presence of intestinal disorders that impair vitamin D_3 absorption (Crohn's disease, ulcerative colitis, celiac disease, irritable bowel syndrome, etc.);
- antibiotic treatment within the three months prior to the study (because this could have altered the normal intestinal microbiome and reduced intestinal absorption of vitamin D_3) [6];
- presence or recent history (within the 24 months prior to the study) of any type of neoplasm;
- body mass index greater than 30 (as fat mass tends to "capture" cholecalciferol and therefore reduce bioavailable vitamin D_3) [7].

Serum levels of vitamin D_3 metabolites

For the purposes of this study, data on circulating levels of 25(OH)D_3, measured in the absence of any supplementation with vitamin D_3 or its hydroxylated metabolites, were retrieved from the health records of the individuals included in the study. Serum levels of 25(OH)D_3 were categorized, in accordance with internationally established reference values, into four groups: 1) vitamin D_3 deficiency (≥ 20 ng/ml), 2) vitamin D_3 insufficiency (21-29 ng/ml), 3) normal vitamin D_3 concentration (30-200 ng/ml), and 4) excess vitamin D_3 (≥201 ng/ml).

Blood sampling and genomic DNA extraction

For genetic analyses, a venous whole blood sample in EDTA was collected from each subject who agreed to participate in the study.

Genomic DNA was extracted from the whole blood samples using an automated MagCore extractor (RBC Bioscience Corp., Taiwan) according to the manufacturer's instructions. Before proceeding with the genetic analyses, extracted DNA was assessed for concentration using spectrophotometric reading with NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA) at 260/280 nm, and for quality and integrity by means of electrophoresis on 0.8% agarose gel.

Analysis of polymorphic variants of *CYP27R1* and *CYP27B1*

We analyzed the following single-nucleotide polymorphisms (SNPs):

CYP2R1:

- rs12794714
- rs10741657
- rs2060793

CYP27B1:

- rs4646536
- rs10877012

These five SNPs were specifically selected for the association analyses since, according to literature data [3], they play a functional role in modulating the enzymatic activity of the two vitamin D hydroxylases, and have been associated with serum vitamin D levels in various populations and/or clinical conditions.

Genetic characterization of the five aforementioned SNPs was performed on each of the genomic DNA samples by TaqMan probe assays through the real-time polymerase chain reaction amplification method. For each SNP, a pair of specific unlabeled primers for the gene loci to be analyzed and two specific TaqMan probes capable of discriminating the two different polymorphic alleles thanks to labeling with two different fluorescent markers (FAMTM and VIC® fluorochromes) were used. Amplification and genotyping conditions of each reaction were as follows: initial denaturation at 95°C for 10 minutes, followed by 50 amplification cycles, each consisting of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for one minute.

Once the amplification reaction for each examined SNP was complete, individuals were categorized, based on analysis of the emitted fluorescence peaks, according to whether they presented the minor allele (based on its frequency, MAF) and/or the most common allele (wild type) found in the general population. The categories were: "homozygous for the minor allele," "heterozygous," and "homozygous for the wild-type allele."

Statistical analyses

Continuous variables were calculated as mean ± standard de-

viation (SD), and median with range of values; Student's t-test was used for statistical analysis.

Categorical (discrete) variables were calculated as percentages and analyzed by the Yates-corrected Chi-square test.

For the three different genotypes of each polymorphic variant analyzed, the association with serum 25(OH)D_3 values was calculated, by considering these levels as categorical variables divided into three analytical groups: deficiency, insufficiency, normal values; the Yates-corrected Chi-square test was used for the correlation analyses.

For all the applied statistical tests, p-values <0.05 were considered statistically significant.

Results

Characteristics of the study population

The study comprised 105 individuals with a mean age of 52.6 ± 10.6 years (median: 55.0; range 18-65 years). Of these, 77 (73.3%) were women (mean age 51.4 ± 11.2 years; median: 54.0; range 18-65) and 28 (26.6%) were men (mean age 55.6 ± 8.5 years; median: 57.0; range 29-65). Divided by age, the population was composed of 37 subjects (35.2%) aged 18-50 years and 68 (64.8%) aged over 50 years (51-65 years).

Serum levels of vitamin D_3 metabolites

Serum levels of 1,25$(OH)_2D_3$ were not available for any of the 105 subjects included in the study, confirming that this metabolite is not normally used as a parameter for evaluating vitamin D_3 bioavailability in the general population, but is measured only to help in assessing specific medical conditions.

The 105 individuals included in the study had a mean serum 25(OH)D_3 level of 28.3 ± 16.4 ng/ml (median: 25.7; range 2-119 ng/ml). The women had a mean serum 25(OH)D_3 level of 28.5 ± 14.4 ng/ml (median: 27.5; range 2-92.4 ng/ml), versus 27.8 ± 21.1 ng/ml (median: 23.5; range 4-119 ng/ml) in the men, with no significant difference between the two sexes [T-test: t-value = 0.200; p = 0.842].

Analysis of the 37 individuals aged ≤ 50 years (18-50 years) showed an mean serum 25(OH)D_3 level of 24.4 ± 16.6 ng/ml (median: 21.0; range 2-92.4 ng/ml), versus 30.4 ± 16.0 ng/ml (median: 29.0; range 9.1-119) in the 68 individuals > 50 years of age (51-65 years), with no significant difference between the two groups [T-test: t-value = -1.818; p = 0.072].
Categorization of serum 25(OH)D_3 levels in the 105 analyzed individuals showed:
- 31 subjects (29.5%) with 25(OH)D_3 deficiency;
- 35 subjects (33.4%) with 25(OH)D_3 insufficiency;
- 39 subjects (37.1%) with normal 25(OH)D_3 levels.

Categorization of serum 25(OH)D_3 levels in the 77 women showed:
- 23 subjects (29.9%) with 25(OH)D_3 deficiency;
- 22 subjects (28.6%) with 25(OH)D_3 insufficiency;
- 32 subjects (41.5%) with normal 25(OH)D_3 levels.

Categorization of serum of 25(OH)D_3 levels in the 28 men showed:
- 8 subjects (28.6%) with 25(OH)D_3 deficiency;
- 13 subjects (46.4%) with 25(OH)D_3 insufficiency;
- 7 subjects (25.0%) with normal 25(OH)D_3 levels.

The overall rate of 25(OH)D_3 deficiency/insufficiency was 58.4% (45/77) in the women and 75.0% (21/28) in the men, with no significant difference between the sexes [Chi-square test 1.75; p = 0.185].
Categorization of serum 25(OH)D_3 levels in the 37 subjects aged ≤ 50 years (18-50 years) showed:
- 17 subjects (46.0%) with 25(OH)D_3 deficiency;
- 13 subjects (35.1%) with 25(OH)D_3 insufficiency;
- 7 subjects (18.9%) with normal 25(OH)D_3 levels.

In the 68 subjects aged over 50 years (51-65 years), categorization of serum 25(OH)D_3 levels showed:
- 14 subjects (20.6%) with 25(OH)D_3 deficiency;
- 22 subjects (32.4%) with 25(OH)D_3 insufficiency;
- 32 subjects (47.0%) with normal 25(OH)D_3 levels.

Subjects aged ≤ 50 years showed a higher 25(OH)D_3 deficiency rate than those aged > 50 years (46.0% vs 20.6%), with a statistically significant difference between the two groups [Chi-square test = Yates; = 6.24; p-value = 0.013].

Overall25(OH)D_3 deficiency/insufficiency in the sample divided by age revealed a rate of 81.1% (30/37) in the subjects aged ≤ 50 years, and 52.9% (36/68) in those aged > 50 years, with a significant difference between the two groups [Chi-square test = 3.97; p = 0.008].

In the 12 individuals aged ≤ 40 years, the mean serum 25(OH)D_3 level was 20.7 ± 9.4 ng/ml (median 20.0; range 4-38.3 ng/ml), with only one individual (8.3%) in the normal range, six (50.0%) showing vitamin D deficiency, and five (41.7%) vitamin D insufficiency. These percentages remained similar in the subjects ≤ 30 years (8 individuals), who recorded a mean serum 25(OH)D_3 level of 20.5 ± 11.7 ng/ml (median 21.1; range 4-38.3 ng/ml), with only one individual (12.5%) showing normal concentration, four (50%) vitamin D deficiency, and three (37.5%) vitamin D insufficiency.

Table I summarizes the distribution of the three vitamin D concentration categories in the overall analyzed population and in gender- and age-based subpopulations.

Genotypes of *CYP2R1* and *CYP27B1* in relation to serum 25(OH)D_3 levels

In our population, the SNP rs12794714 A>G in the *CYP2R1* gene showed the following genotype frequencies:
- 31 subjects (29.5%) with the AA genotype, homozygous for the wild-type allele;
- 55 subjects (52.4%) with the heterozygous AG genotype;
- 19 subjects (18.1%) with the GG genotype, homozygous for the minor allele.

The distribution of genotype frequencies in relation to 25(OH)D_3 concentration is shown in Figure 1, Panel A.

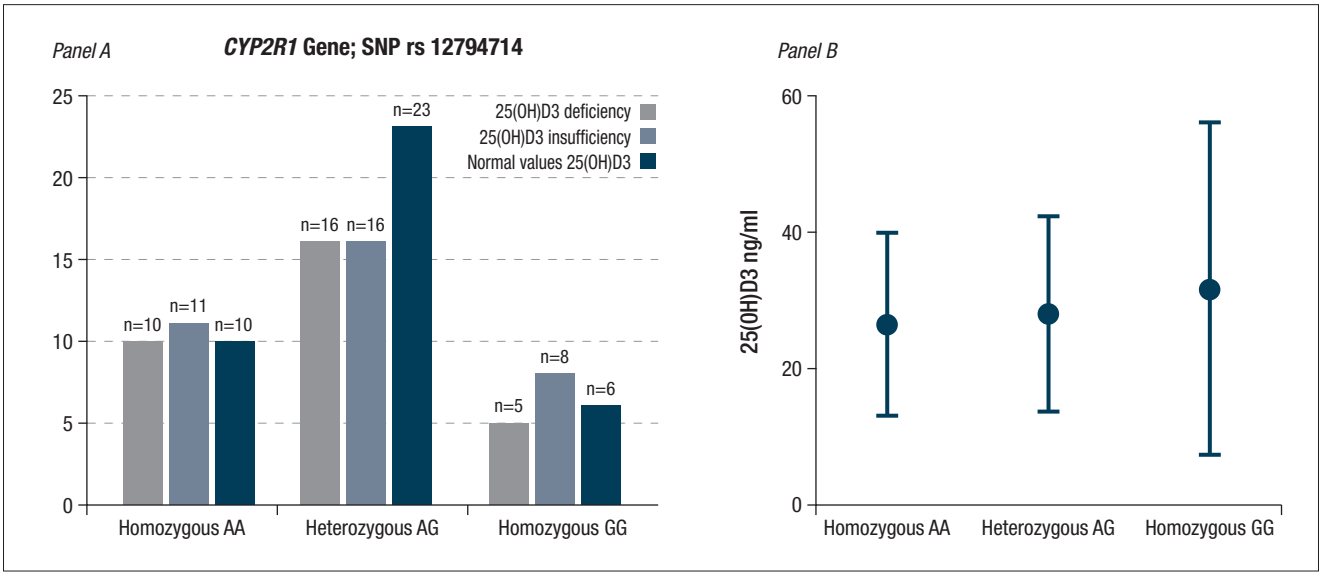
Statistical comparison between opposite homozygous genotypes (AA vs GG) showed no significant differences in rates of 25(OH)D_3 deficiency [Chi-square test = 0.02; p-value = 0.899], 25(OH)D_3 insufficiency [Chi-square test = 0.03; p-value = 0.867], or normal 25(OH)D_3 levels [Chi-square test = 0.07; p = 0.793].

In the statistical comparison between the AA genotype (homozygous for the wild-type allele) and the heterozygous genotype (AG), no significant differences were found in rates

Table I Distribution of vitamin D concentration categories in the overall population and in gender- and age-based subpopulations.

POPULATION (N)	VITAMIN D DEFICIENCY (%)	VITAMIN D INSUFFICIENCY (%)	NORMAL VITAMIN D LEVEL (%)
Overall [105]	29.5%	33.4%	37.1%
Women [77]	29.9%	28.6%	41.5%
Men [28]	28.6%	46.4%	25.0%
Subjects ≤ 50 years [37]	46.0%	35.1%	18.9%
Subjects > 50 years [68]	20.6%	32.4%	47.0%
Subjects ≤ 40 years [12]	50.0%	41.7%	8.3%
Subjects ≤ 30 years [8]	50.0%	37.5%	12.5%

Figure 1 *Panel A:* Graphical representation of the distribution of genotype frequencies of the rs12794714 polymorphic variant in the *CYP2R1* gene. *Panel B:* Graphical representation of mean values + standard deviation of 25(OH)D3 concentration in the three genotypes of the rs12794714 polymorphic variant.



of 25(OH)D_3 deficiency [Chi-square test = 0.00; p = 0.950], 25(OH)D_3 insufficiency [Chi-square test = 0.14; p = 0.710], or normal 25(OH)D_3 levels [Chi-square test = 0.42; p = 0.519].

Comparison of the GG genotype (homozygous for the minor allele) and the heterozygous AG genotype revealed no significant differences in rates of 25(OH)D_3 deficiency [Chi-square test = 0.00; p = 0.949], 25(OH)D_3 insufficiency [Chi-square test = 0.58; p = 0.447], or 25(OH)D_3 normal levels [Chi-square test = 0.27; p = 0.606].

The individuals with the AA genotype (homozygous for the wild-type allele) had a mean 25(OH)D_3 level of 26.5 ± 13.4 ng/ml, versus 28.1 ± 14.4 ng/ml in those with the heterozygous AG genotype, and 31.8 ± 24.6 ng/ml in those with the GG genotype (homozygous for the minor allele) (Figure 1, Panel B). Statistical comparison of the different genotypes did not show significant differences in mean serum 25(OH)D_3 concentration between the homozygous GG and AA genotypes [T-test = -0.992; p = 0.326], between the homozygous GG genotype and the heterozygous GA genotype [T-test = -0.526; p = 0.626], or between the homozygous AA genotype and the heterozygous GA genotype [T-test = -0.788; p = 0.433]. In our population, the SNP rs10741657 G>A in *CYP2R1* showed the following genotype frequencies:

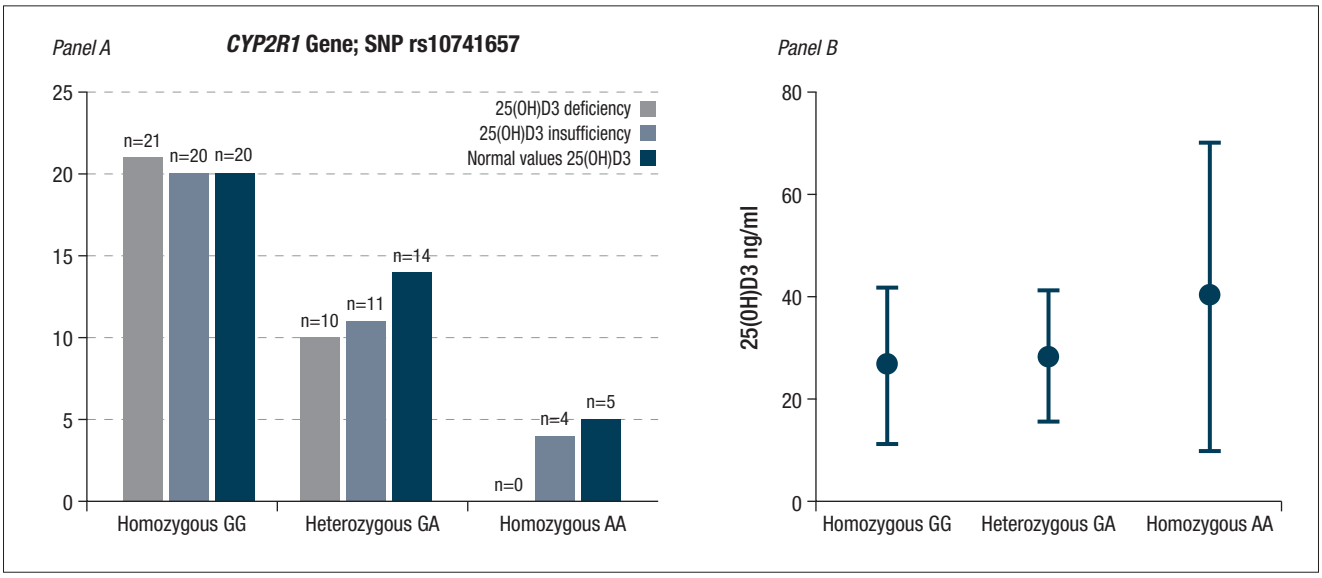
- 61 subjects with the homozygous GG genotype (homozygous for the wild-type allele) (58.1%);
- 35 subjects with the heterozygous GA genotype (33.3%);
- 9 subjects with the homozygous AA genotype (homozygous for the minor allele) (8.6%).

Figure 2, Panel A shows the distribution of the genotype frequencies in relation to serum 25(OH)D_3 concentration.

Statistical comparison of the opposite homozygous genotypes (GG vs AA) showed no significant differences in rates of 25(OH)D_3 deficiency [Chi-square test = 0.2.94; p = 0.087], 25(OH)D_3 insufficiency [Chi-square test = 0.10; p = 0.755], or normal 25(OH)D_3 levels [Chi-square test = 0.92; p = 0.338].

In the statistical comparison between the GG genotype (homozygous for the wild-type genotype) and the heterozygous genotype (GA), no significant differences were found in rates of 25(OH)D_3 deficiency [Chi-square test = 0.13; p = 0.716], 25(OH)D_3 insufficiency [Chi-square test = 0.01; p = 0.929], or

Figure 2 *Panel A:* Graphical representation of the distribution of genotype frequencies of the rs10741657 polymorphic variant of the *CYP2R1* gene. *Panel B:* Graphical representation of mean values + standard deviation of 25(OH)D3 concentration in the three genotypes of the rs10741657 polymorphic variant.



25(OH)D_3 normal levels [Chi-square test = 0.24; p = 0.624].

Statistical comparison of the AA genotype (homozygous for the minor allele) with the heterozygous GA genotype, revealed no significant differences in rates of 25(OH)D_3 deficiency [Chi-square test = 1.90; p = 0.168], 25(OH)D_3 insufficiency [Chi-square test = 0.12; p = 0.734], or 25(OH)D_3 normal levels [Chi-square test = 0.21; p = 0.634].

The mean concentration of 25(OH)D_3 was 26.5 ± 15.1 ng/ml in the individuals with the GG genotype (homozygous for the wild-type allele), 28.4 ± 12.7 ng/ml in the subjects with the heterozygous GA genotype, and 39.7 ± 30.2 ng/ml in those with the AA genotype (homozygous for the minor allele) (Figure 2, Panel B). Statistical comparison of different genotypes did not show significant differences in mean serum 25(OH)D_3 concentration between the homozygous GG genotype and the heterozygous GA genotype [T-test = -0.636; p = 0.526], or between the homozygous AA genotype and the heterozygous GA genotype [T-test = -1.733; p = 0.090]. A statistically significant difference was instead found between the two homozygous genotypes (GG and AA) [T-test = -2.109; p = 0.039], with the latter found to be associated with significantly higher mean serum 25(OH)D_3 concentration. In our population, the SNP rs2060793 G>A in *CYP2R1* showed the following genotype frequencies:

- 53 subjects (50.5%) with the GG genotype (homozygous for the wild-type allele);
- 41 subjects (39.0%) with the heterozygous GA genotype;
- 11 subjects (10.5%) with the AA genotype (homozygous for the minor allele).

Figure 3, Panel A shows the distribution of genotype frequencies in relation to serum 25(OH)D_3 concentration.

Statistical comparison between opposite homozygous genotypes (GG vs AA) showed no significant differences in rates of 25(OH)D_3 deficiency [Chi-square test = 0.45; p = 0.503], 25(OH)D_3 insufficiency [Chi-square test = 0.67; p = 0.414], or normal 25(OH)D_3 concentration [Chi-square test = 0.03; p = 0.865].

When comparing the homozygous GG genotype and the heterozygous GA genotype, no significant differences were found in rates of 25(OH)D_3 deficiency [Chi-square test = 0.27; p = 0.605], 25(OH)D_3 insufficiency [Chi-square test = 0.94; p = 0.333], or normal 25(OH)D_3 concentration [Chi-square test = 2.64; p = 0.104].

In the statistical comparison between the AA genotype (homozygous for the minor allele) and the heterozygous GA genotype, no significant differences were found in rates of 25(OH)D_3 deficiency [Chi-square test = 0.04; p = 0.845], 25(OH)D_3 insufficiency [Chi-square test = 2.42; p = 0.120], or normal 25(OH)D_3 levels [Chi-square test = 0.87; p = 0.351].

The mean 25(OH)D_3 level was 25.9 ± 14.7 ng/ml in individuals with the GG genotype (homozygous for the wild-type allele), 29.8 ± 13.7 ng/ml in subjects with the heterozygous GA genotype, and 34.1 ± 29.0 ng/ml in those with the AA genotype (homozygous for the minor allele) (Figure 3, Panel B). Statistical comparison of different genotypes did not show significant differences in mean serum levels of 25(OH)D_3 between the homozygous GG and AA genotypes [T-test = -1.381; p = 0.172], between the GG genotype and the heterozygous GA genotype [T-test = -1.288; p = 0.201], or between the homozygous AA genotype and the heterozygous GA genotype [T-test = -0.715; p = 0.478]. In our population, the SNP rs4646536 A>G in *CYP27B1* showed the following genotype frequencies:

- 62 subjects (59.1%) with the AA genotype (homozygous for the wild-type allele);
- 39 subjects (37.1%) with the heterozygous AG genotype;
- 4 subjects (3.8%) with the GG genotype (homozygous for the minor allele).

Figure 4, Panel A shows the distribution of the genotype frequencies in relation to serum 25(OH)D_3 concentration.

Statistical comparison between opposite homozygous geno-

Figure 3 *Panel A:* Graphical representation of the distribution of genotype frequencies of the rs2060793 polymorphic variant of the *CYP2R1* gene. *Panel B:* Graphical representation of mean values + standard deviation of 25(OH)D3 concentration in the three genotypes of the rs2060793 polymorphic variant.

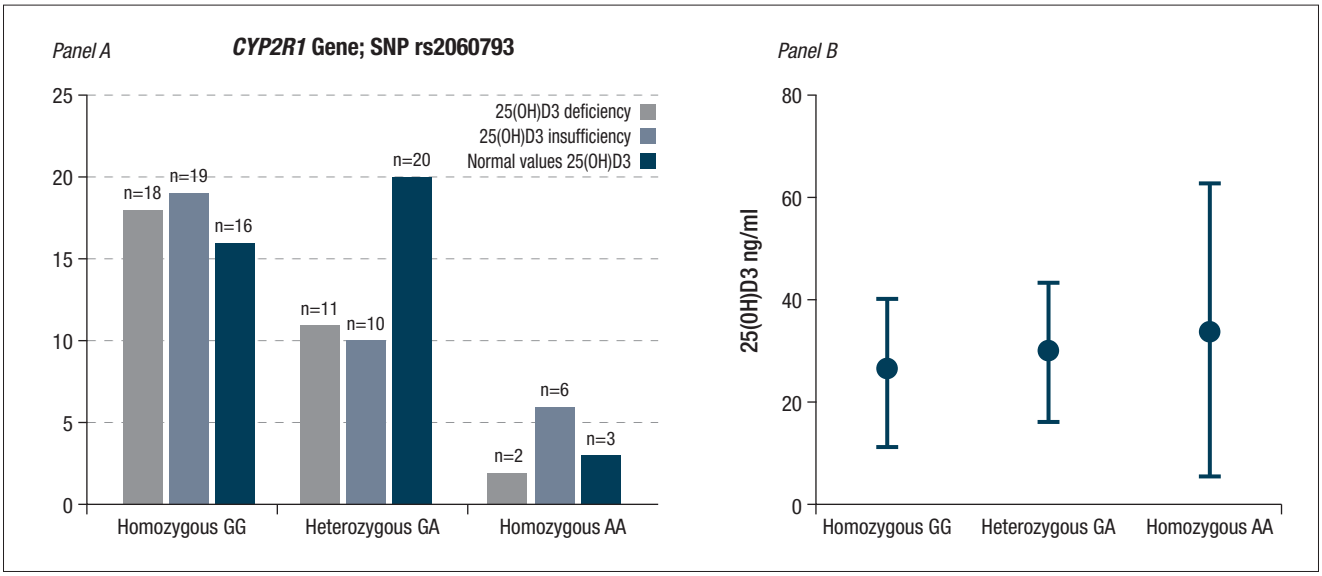
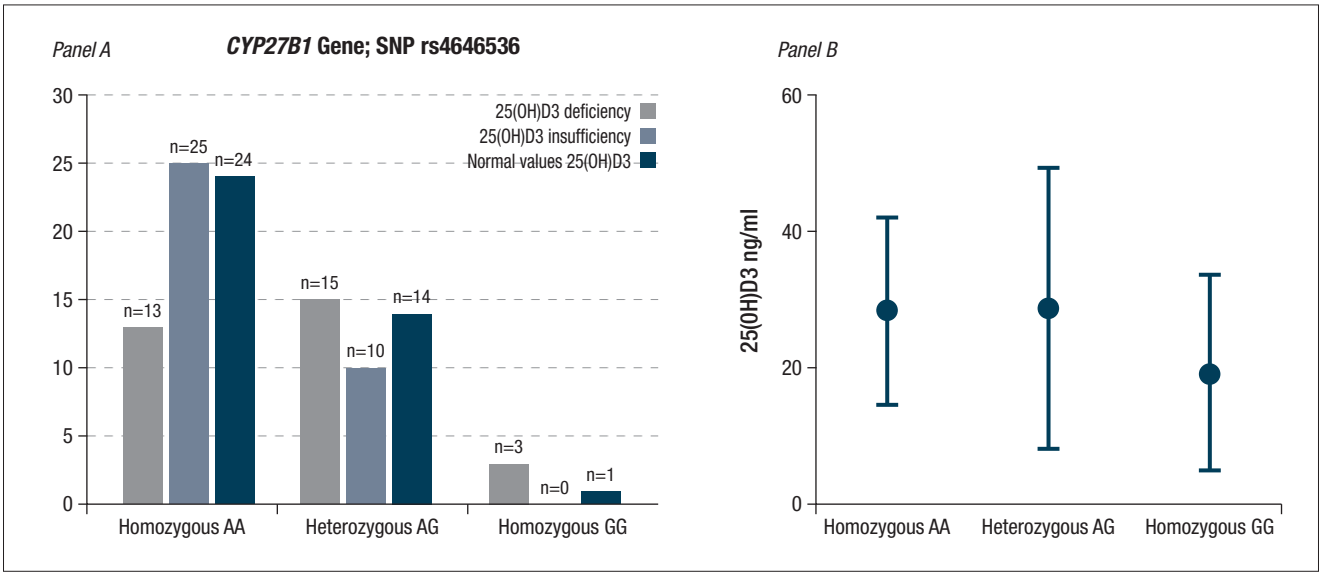


Figure 4 *Panel A:* Graphical representation of the distribution of genotype frequencies of the rs4646536 polymorphic variant of the *CYP27B1* gene. *Panel B:* Graphical representation of mean values + standard deviation of 25(OH)D3 concentration in the three genotypes of the rs4646536 polymorphic variant.



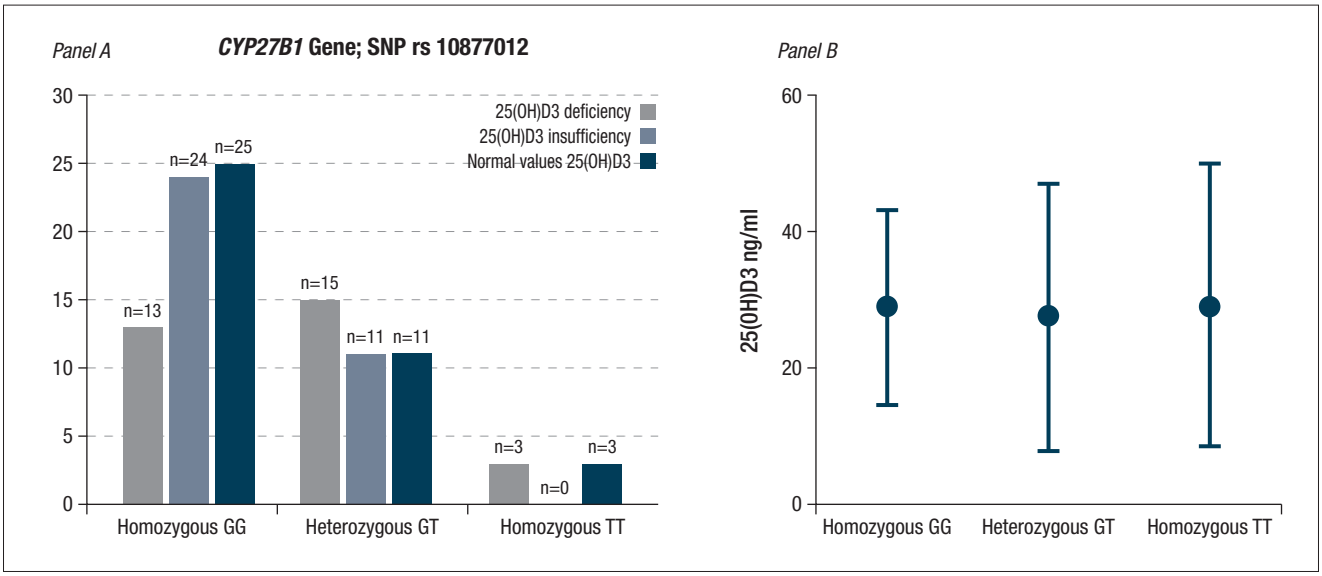
types (AA vs GG) showed no significant differences in rates of 25(OH)D_3 deficiency [Chi-square test = 3.39; p = 0.066], 25(OH)D_3 insufficiency [Chi-square test = 1.17; p = 0.280], or 25(OH)D_3 normal concentration [Chi-square test = 0.00; p = 0.987].

In the statistical comparison between the AA genotype (homozygous for the wild-type allele) and the heterozygous AG genotype, no significant differences were found in rates of 25(OH)D_3 deficiency [Chi-square test = 2.84; p = 0.092], 25(OH)D_3 insufficiency [Chi-square test = 1.68; p = 0.195], or normal 25(OH)D_3 concentration [Chi-square test = 0.01; p = 0.942].

Statistical comparison of the GG genotype (homozygous for the minor allele) and the heterozygous AG genotype revealed no significant differences in rates of 25(OH)D_3 deficiency [Chi-square test = 0.77; p = 0.380], 25(OH)D_3 insufficiency [Chi-square test = 0.29; p = 0.593], or normal (OH)D_3 levels [Chi-square test = 0.01; p = 0.908].

The mean value of 25(OH)D_3 was 28.5 ± 13.8 ng/ml in individuals with the homozygous AA genotype, 28.8 ± 20.8 ng/ml in those with the heterozygous AG genotype, and 19.3 ± 14.4 ng/ml in the homozygous GG genotype group (Figure 4, Panel B). Statistical comparison between different genotypes did not show significant differences in mean serum 25(OH)D_3 between the homozygous genotypes (AA and GG) [T-test =

Figure 5 *Panel A:* Graphical representation of the distribution of genotype frequencies of the rs10877012 concentration polymorphic variant of the *CYP27B1* gene. *Panel B:* Graphical representation of mean values + standard deviation of 25(OH)D3 in the three genotypes of the rs10877012 polymorphic variant.



1.294; p = 0.200], between the homozygous AA genotype and the heterozygous AG genotype [T-test = -0.076; p = 0.939], or between the homozygous GG genotype and the heterozygous AG genotype [T-test = -0.922; p = 0.362]. In our population, the SNP rs10877012 G>T in *CYP27B1* showed the following genotype frequencies:

- 62 subjects with the GG genotype (homozygous for the wild-type allele) (59.1%)
- 37 subjects with the heterozygous GT genotype (35.2%)
- 6 subjects with the TT genotype (homozygous for the minor allele) (5.7%)

Figure 5, Panel A shows the distribution of the genotype frequencies in relation to serum concentration of 25(OH)D_3.

Statistical comparison between opposite homozygous genotypes (GG vs TT) showed no significant differences in rates of 25(OH)D_3 deficiency [Chi-square test = 1.20; p = 0.273], 25(OH)D_3 insufficiency [Chi-square test = 2.09; p = 0.148], or normal levels of 25(OH)D_3 [Chi-square test = 0.00; p = 0.980].

The statistical comparison between the GG genotype (homozygous for the wild-type allele) and the heterozygous GT genotype revealed no significant differences in rates of 25(OH)D_3 deficiency [Chi-square test = 3.46; p = 0.063], 25(OH)D_3 insufficiency [Chi-square test = 0.47; p = 0.492], or normal levels of 25(OH)D_3 [Chi-square test = 0.71; p = 0.399].

In the statistical comparison between the TT genotype (homozygous for the minor allele) and the heterozygous GT genotype, no significant differences were found in rates of 25(OH)D_3 deficiency [Chi-square test = 0.00; p = 0.992], 25(OH)D_3 insufficiency [Chi-square test = 1.09; p = 0.297], or normal levels of 25(OH)D_3 [Chi-square test = 0.26; p = 0.608].

The mean value of 25(OH)D_3 was 28.8 ± 14.0 ng/ml in individuals with the homozygous GG genotype, 27.2 ± 19.4 ng/ml in individuals with the heterozygous GT genotype, and 29.2 ± 20.5 ng/ml in those with the homozygous TT genotype (Figure 5, Panel B). Statistical comparison of the different genotypes did not show significant differences in mean serum levels of 25(OH)D_3 between the homozygous genotypes (GG vs TT) [T-test = -0.064; p = 0.949], between GG and the heterozygous GT genotype [T-test = -0.488; p = 0.626], or between TT and the heterozygous GT genotype [T-test = -0.238; p = 0.813].

Discussion and conclusions

Vitamin D deficiency/insufficiency is a widespread public health challenge affecting a large percentage of the general population worldwide, with significant implications for bone health, immune function, and overall well-being. Therefore, better understanding of the genetic bases of reduced vitamin D bioavailability in different populations is important to better tailor supplementation of this important molecule.

Globally, it is estimated that approximately one billion people have inadequate vitamin D levels, with prevalence rates varying by age, countries and lifestyle. Despite being a Mediterranean country, Italy shows surprisingly high rates of vitamin D deficiency and insufficiency, collectively estimated to range from 50% to 80%, and the elderly, pregnant women, children, and people with chronic diseases are the most affected groups. Various factors—reduced sun exposure due to more indoor lifestyles and greater efforts to protect the skin from the sun through clothing and sunscreen, as well as the fact that the traditional Italian diet is not naturally high in vitamin D-rich foods—are among the causes of the high prevalence vitamin D deficiency and insufficiency in the Italian population, which is thus among those that would stand to benefit the most from widespread vitamin D supplementation.

In our sample population, in particular, deficiency/insufficiency was observed in 62.9% of the subjects analyzed, suggesting that almost two people in three have below-normal levels of 25(OH)D_3 in the blood, and as such require sup-

plementation. The data were even more surprising in the 37 analyzed subjects aged ≤ 50 years, in whom the total rate of 25(OH)D_3 deficiency/insufficiency was 81.1%, with as many as 46% classed as having 25(OH)D_3 deficiency; both of these values differed significantly from the corresponding rates recorded in the 68 subjects aged > 50 years (52.9% and 20.6%, respectively). This confirms the importance of vitamin D supplementation even at a young age. The high incidence of vitamin D deficiency/insufficiency was even more evident when considering the 12 subjects aged ≤ 40 years (91.7%) and the eight aged ≤ 30 years (87.5%).

Statistical comparisons of the associations between the different genotypes of the five polymorphic variants analyzed in this study and the three categories of serum 25(OH)D_3 concentration (deficiency, insufficiency, normal) revealed no significant correlations.

Instead, exploration of the associations between the different genotypes of the selected polymorphic variants and the mean value of circulating 25(OH)D_3 revealed a significant difference (p = 0.039) between the GG genotype (homozygous for the wild-type allele) and the AA genotype (homozygous for the minor allele) of the polymorphic variant rs10741657 in *CYP2R1*, which encodes 25-hydroxylase, with the less frequent AA genotype found to be associated with significantly higher mean levels of 25(OH)D_3. This interesting datum appears to be consistent with the results obtained from a 2018 systematic review and meta-analysis conducted on 16 international studies published between June 2010 and May 2018, which included a total of 52,417 participants in good general health [8]. The meta-analysis found that individuals with the GG genotype of the rs10741657 variant showed a clear decreasing trend in 25(OH)D_3 levels compared with those with the AA genotype, both in the total population included in the analysis and in subgroup analyses of Caucasian and Asian subjects.

This association was statistically significant only in the Caucasian population (15,795 subjects). Furthermore, in this meta-analysis the GG genotype, homozygous for the wild-type allele, was significantly associated with an increased risk of vitamin D deficiency, with quite homogeneous findings emerging across the results of the single studies included in the analysis. The association of the GG genotype with significantly lower 25(OH)D_3 levels was also confirmed in 92 Indian professional and semi-professional athletes [9], and in 30 Bangladeshi adults with low serum 25(OH)D_3 levels [10], while the less frequent allele (A) was significantly associated with higher plasma 25(OH)D_3 levels and lower odds of vitamin D deficiency in adolescents of Arab ethnicity in Kuwait [11], and with three-fold higher 25(OH)D_3 values in institutionalized elderly men not treated with vitamin D in Spain [12]. In a 2019 study [13] conducted in 616 healthy adolescent girls who received nine weeks of supplementation with 50,000 IU of cholecalciferol per week, serum 25(OH)D_3 levels increased in all the participants, but the girls carrying the less frequent allele (A) seemed to respond better to the supplementation, with higher mean percentages of change in serum 25(OH)D_3 found in those with the AA (539.4 ± 443.1) and AG (443.7 ± 384.6) genotypes, compared with the common GG genotype (363.3 ± 354.0). Regression analysis revealed that the probability of an increase in circulating 25(OH)D_3 was 2.5 times greater in treated subjects with the AA genotype than in those with the GG genotype (OR = 2.5 (1.4-4.4); p-value = 0.002).

In conclusion, the data from our study, conducted in a representative sample of the healthy adult Italian population, seem to confirm that the rarer AA genotype of the rs10741657 variant may represent a "positive" genetic factor in reducing the individual risk of vitamin D deficiency. And considering the results of the literature, it can also be hypothesized that subjects carrying this AA genotype might benefit more from supplementation with cholecalciferol, whereas those with the more common GG genotype might be more beneficially treated with calcifediol.

Study limitations

This study has two limitations: A) the relatively low number of analyzed subjects, which certainly needs to be increased in order to replicate and confirm the data obtained, and B) the fact that the genetic screening was restricted to only five selected SNPs in *CYP2R1* and *CYP27B1*; further SNPs should be analyzed, both singly and as SNP associations (haplotypes), for their possible association with circulating 25(OH)D_3.

In addition, association studies between different *CYP2R1* and *CYP27B1* genotypes and serum levels of 25(OH)D_3, measured before and after administration of different vitamin D metabolites, should be performed with the aim of establishing whether certain subgroups might better benefit from a specific type of vitamin D supplementation, based on their genotype.

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