No causal association between 25-hydroxyvitamin D and features of skin aging: evidence from a bidirectional Mendelian randomization study

M.A. Hamer*
R. Noordam*
L.M. Pardo Cortes
T. van der Nat
J.C. Kiefte-de Jong
M. Kayser
P.E. Slagboom
A. Uitterlinden
M.C. Zillikens
M. Beekman
T. Nijsten
D. van Heemst
D.A. Gunn

**Shared first authorship*

J Invest Dermatol. 2017 Nov;137(11):2291-2297

ABSTRACT

Data from in-vitro experiments suggest that vitamin D reduces the rate of skin aging, whereas population studies suggest the opposite, most likely due to confounding by UV-exposure. We investigated whether there are causal associations between 25-hydroxyvitamin D concentrations and features of skin aging in a bidirectional Mendelian randomization study. In the Rotterdam Study (N=3831; 58.2% women, median age 66.5 years) and Leiden Longevity Study (N=661; 50.5% women, median age 63.1 years), facial skin aging features (perceived age, wrinkling, pigmented spots) were assessed either manually or digitally. Associations between 25-hydroxyvitamin D and skin aging features were tested by multivariable linear regression. Mendelian randomization analyses were performed using single-nucleotide polymorphisms identified from previous genome-wide association studies. After meta-analysis of the two cohorts, we observed that higher serum 25-hydroxyvitamin D was associated with a higher perceived age (P-value=3.6×10^{-7}), more skin wrinkling (P-value=2.6×10^{-16}), but not with more pigmented spots (P-value=0.30). In contrast, a genetically determined 25-hydroxyvitamin D concentration was not associated with any skin aging feature (P-values>0.05). Furthermore, a genetically determined higher degree of pigmented spots was not associated with higher 25-hydroxyvitamin D (P-values>0.05). Our study did not indicate that associations between 25-hydroxyvitamin D and features of skin aging are causal.

INTRODUCTION

A higher perceived age – estimated age based on facial appearance – is associated with an increased risk of morbidity and mortality[1], making it a useful marker in aging research. In addition to well-described extrinsic factors, such as smoking and UV-exposure[1-3], a higher perceived age also has an intrinsic component[4-6]. It has previously been shown that high serum concentrations of glucose and cortisol were associated with a higher perceived age[7,8], whereas a high concentration of insulin-like growth factor-1 (IGF-1) was associated with a lower perceived age mainly through skin wrinkling[6,9]. Besides skin wrinkling, facial pigmented spots are also an important component of skin aging.

Although sun-exposure contributes to premature skin aging[1,2], it is essential for vitamin D synthesis in the skin[10] and vitamin D is essential for musculoskeletal health. Moreover, in clinical practice, low serum concentrations of 25-hydroxyvitamin D, or vitamin D deficiency, is a broadly accepted marker for general health status, and has been associated with multiple extraskeletal age-related diseases (e.g., type 2 diabetes mellitus and cardiovascular disease), and mortality[11-16].

Different in-vitro studies have shown that physiological concentrations of 1,25-hydroxyvitamin D, the active vitamin D metabolite, protect the skin against factors that promote skin aging, including cellular damage induced by UVB irradiation. Vitamin D has been demonstrated to influence keratinocyte proliferation[17] and differentiation[18] with the response dependent on vitamin D concentrations and culture conditions[17,19]. Although the bioavailable levels of vitamin D in human skin are unknown, a higher serum concentration of 25-hydroxyvitamin D was associated with a higher number of facial pigmented spots in the Leiden Longevity Study[20]. However, the nature of these studies is observational, and causality cannot be ascertained due to influences of, for example, residual confounding by sunlight.

Causality can be inferred between a certain exposure and outcome using Mendelian randomization studies[21,22]. With such analyses, genetic polymorphisms that are strongly related to the exposure are investigated in relation to the outcome, in the absence of confounding. Therefore, we aimed to investigate whether associations between serum 25-hydroxyvitamin D and features of skin aging are causal using a bidirectional Mendelian randomization study.

RESULTS

Characteristics of the study populations

A maximum of 3831 participants from the Rotterdam Study (median [IQR] age: 66.5 [61.0 to 71.5] years) and 661 participants from the Leiden Longevity Study (median [IQR] age: 63.1 [58.9 to 67.5] years) were included in the present study (Table 1). Compared with participants from the Leiden Longevity Study, participants from the Rotterdam Study were more frequently women (58.2% versus 50.4%), smokers (18.5% versus 13.8%), and had a lower 25-hydroxyvitamin D concentra-

tion (median: 61.0 nmol/l vs. 68.3 nmol/l). In addition, in line with the higher mean chronological age, participants from the Rotterdam Study had a higher mean perceived age (mean: 65.9 years vs. 59.4 years).

Table 1. Characteristics of the study populations

	Rotterdam Study (N=3831)	Leiden Longevity Study (N=661)
General		
Chronological age in years, median [IQR]	66.5 [61.0 – 71.5]	63.1 [58.9 – 67.5]
Females, N (%)	2229 (58.2)	334 (50.4)
Body mass index in kg/m^2, mean (SD)	27.6 (4.4)	26.6 (4.0)
Current smoking, N (%)	707 (18.5)	91 (13.8)
Skin aging features		
Perceived age in years, mean (SD)	65.9 (7.6)[a]	59.4 (7.6)
Degree of skin wrinkling, median [IQR][b]	3.9 [2.5 – 6.0]	4.5 [3.5 – 5.5]
Degree of pigmented spots, median [IQR][b]	1.3 [0.9 – 2.1][c]	4.5 [3.5 – 5.0]
Serum measurements		
25-hydroxyvitamin D in nmol/l, median [IQR]	61.0 [42.7 – 82.3]	68.3 [54.0 – 139.2]
Serum taken in winter season, N (%)	930 (24.3)	160 (24.2)
Serum taken in spring season, N (%)	1107 (28.9)	214 (32.4)
Serum taken in summer season, N (%)	863 (22.5)	135 (20.4)
Serum taken in autumn season, N (%)	850 (22.2)	152 (23.0)

Abbreviations: IQR, interquartile range; N, number of participants; SD, standard deviation.
[a]assessed in 2679 individuals; [b]for the Rotterdam Study, measured digitally as area (wrinkles or pigmented spots) as a percentage of the total facial area. For the Leiden Longevity Study, wrinkle score and pigmented spots were measured manually by 2 expert dermatologists using a photonumeric scale ranging from 1 to 9; [c]assessed in 2843 individuals.

Observational associations between 25-hydroxyvitamin D concentration and skin aging features

After meta-analyzing the results of the Rotterdam Study and the Leiden Longevity Study (Table 2), a higher 25-hydroxyvitamin D concentration was associated with a higher perceived age (β=0.149 SD per 1 ln[25-hydroxyvitamin D]; SE=0.029; P-value=3.58×10^{-7}). However, this association disappeared after additional adjustment for the degree of skin wrinkling (β=0.020 SD per 1 ln[25-hydroxyvitamin D]; SE=0.022; P-value=0.36). In line with this, a higher 25-hydroxyvitamin D concentration was associated with a higher degree of skin wrinkling (β=0.250 SD per 1 ln[25-hydroxyvitamin D]; SE=0.030; P-value 2.61×10^{-16}). In contrast, a higher 25-hydroxyvitamin D was only associated with a higher degree of pigmented spots in the Leiden Longevity Study, and not in the Rotterdam Study. After meta-analysis, a higher 25-hydroxyvitamin D was not associated with a higher degree of pigmented spots (β=-0.033 SD per 1 ln[25-hydroxyvitamin D]; SE=0.031;

P-value=0.30). These results were similar when we additionally adjusted for UV-exposure, physical activity, and dietary vitamin D and any vitamin D supplementation in the Rotterdam Study (Supplementary Table S1), despite that we observed strong associations between these factors and 25-hydroxyvitamin D level (Supplementary Table S2).

Table 2. Association between serum 25-hydroxyvitamin D and features of skin aging

	Rotterdam Study (N=3831)		Leiden Longevity Study (N=661)		Meta-analysis	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
Perceived age	0.128 (0.030)[b]	1.8×10^{-5}	0.516 (0.127)	5.4×10^{-5}	0.149 (0.029)	3.6×10^{-7}
Perceived age, adjusted[a]	0.006 (0.023)[b]	7.8×10^{-1}	0.225 (0.087)	9.8×10^{-3}	0.020 (0.022)	3.6×10^{-1}
Degree of skin wrinkling	0.241 (0.031)	2.2×10^{-14}	0.507 (0.169)	2.7×10^{-3}	0.250 (0.030)	2.6×10^{-16}
Degree of pigmented spots	-0.050 (0.032)[c]	1.2×10^{-1}	0.571 (0.189)	2.6×10^{-3}	-0.033 (0.031)	3.0×10^{-1}

Abbreviation: SE, standard error.

Effect estimates presented as the increase in standardized outcome per 1 ln-transformed unit increase in 25-hydroxyvitamin D serum concentration. Analyses adjusted for chronological age, sex, season, current smoking status and body mass index. The results of the digitally measured wrinkles and pigmented spots in the Rotterdam Study were additionally adjusted for technical variables.

[a]analyses additionally adjusted for the degree of facial skin wrinkling. Effect estimates of the meta-analysis obtained using fixed-effect models; [b]analysis based on 2679 individuals from the Rotterdam Study; [c]analysis based on 2843 individuals from the Rotterdam Study.

Mendelian randomization analyses between 25-hydroxyvitamin D concentration and skin aging features

We calculated, per participant, a weighted genetic score for 25-hydroxyvitamin D concentration based on the SNPs that were identified in a genome-wide association study (GWAS) on 25-hydroxyvitamin D concentration (notably, rs2282679 [*GC*], rs3829251 [*NADSYN1*], and rs2060793 [*CYP2R1*][23]). Based on the observational effect estimates, we had an 82% and 84% power to detect significant (α=0.05) associations between the 25-hydroxyvitamin D genetic risk score (GRS) and perceived age and degree of skin wrinkling, respectively.

After meta-analysis, all three selected 25-hydroxyvitamin D genotypes were associated with 25-hydroxyvitamin D concentration (Supplementary Table S3). In line with this, the calculated weighted genetic score for higher 25-hydroxyvitamin D concentration was associated with a higher 25-hydroxyvitamin D concentration in our study populations and meta-analysis (β=0.24 units ln[25-hydroxyvitamin D] increase per 1 unit increase in genetic score; SE=0.01; P-value=2.23×10^{-64}).

After meta-analyzing the observed estimates of the Rotterdam Study and the Leiden Longevity Study (Table 3), a higher genetically determined 25-hydroxyvitamin D concentration was not associated with (i) a higher perceived age (β=0.030 SD per 1 genetically-determined ln[25-hydroxyvitamin D]; SE=0.023; P-value=0.18); (ii) a higher perceived age additionally adjusted for skin wrinkling (β=0.017 SD per 1 genetically determined ln[25-hydroxyvitamin D]; SE=0.016; P-

value=0.28); (iii) a higher degree of skin wrinkling (β=0.000 SD per 1 genetically determined ln[25-hydroxyvitamin D]; SE=0.028; P-value=1.00); (iv) a higher degree of pigmented spots (β=0.055 SD per 1 genetically-determined ln[25-hydroxyvitamin D]; SE=0.030; P-value=0.07).

Table 3. Association between 25-hydroxyvitamin D genetic risk score and skin aging features

	Rotterdam Study (N=3831)		Leiden Longevity Study (N=661)		Meta-analysis	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
Perceived age	-0.017 (0.045)[b]	0.69	0.046 (0.026)	0.08	0.030 (0.023)	0.18
Perceived age, adjusted[a]	-0.003 (0.034)[b]	0.93	0.023 (0.018)	0.19	0.017 (0.016)	0.28
Degree of skin wrinkling	-0.064 (0.048)	0.18	0.034 (0.035)	0.32	0.000 (0.028)	1.00
Degree of pigmented spots	0.004 (0.051)[c]	0.93	0.084 (0.038)	0.03	0.055 (0.030)	0.07

Abbreviation: SE, standard error.
Effect estimates presented as the increase in the standardized outcomes per 1 unit increase in the genetic risk score. Analyses adjusted for age and sex. Effect estimates of the meta-analysis obtained using fixed-effect models.
[a]analyses additionally adjusted for the degree of facial skin wrinkling; [b]analysis based on 2679 individuals from the Rotterdam Study; [c]analysis based on 2843 individuals from the Rotterdam Study.

Mendelian randomization analyses between pigmented spots and 25-hydroxyvitamin D concentration

We found no evidence after meta-analyzing the results of the Rotterdam Study and Leiden Longevity Study that any of the genotypes for pigmented spots or perceived age (*MC1R* gene only) or the genetic risk score for pigmented spots was associated with a higher 25-hydroxyvitamin D concentration (Table 4; e.g., β=0.146 ln[25-hydroxyvitamin D in nmol/l] per 1 unit increase in pigmented spots GRS; SE=0.089; P-value=0.10).

Table 4. Mendelian randomization analyses for pigmented spots and 25-hydroxyvitamin D concentration

	Rotterdam Study (N=2843)		Leiden Longevity Study (N=661)		Meta-analysis	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
IRF4 gene, rs12203592	0.017 (0.014)	0.20	0.056 (0.023)	0.01	0.028 (0.012)	0.02
MC1R gene, rs35063026	-0.007 (0.017)	0.67	-0.014 (0.023)	0.54	-0.009 (0.014)	0.49
ASIP gene, rs6059655	0.000 (0.015)	1.00	0.020 (0.020)	0.30	0.007 (0.012)	0.55
Pigmented spots GRS	0.079 (0.106)	0.45	0.306 (0.163)	0.06	0.146 (0.089)	0.10

Abbreviations: *ASIP*, agouti signaling protein; GRS, genetic risk score; *IRF4*, interferon regulatory factor 4; *MC1R*, melanocortin 1 receptor; SE, standard error.
Effect estimates presented as the increase in the standardized outcomes per one unit increase in the genetic risk score. Analyses adjusted for age and sex. Effect estimates of the meta-analysis obtained using fixed-effect models.

DISCUSSION

We found evidence that a higher serum 25-hydroxyvitamin D concentration was associated with a higher perceived age and a higher degree of skin wrinkling. However, we found no evidence that a higher genetically determined 25-hydroxyvitamin D was associated with any of the studied skin aging features, nor was there evidence that a higher genetically determined degree of pigmented spots was associated with a higher 25-hydroxyvitamin D concentration. These results suggest that the association between 25-hydroxyvitamin D and skin aging features is not likely causal.

In several observational studies, a low 25-hydroxyvitamin D[11-15] and a higher perceived age[1] are associated with an increased risk of morbidity and mortality[16]. Therefore, low 25-hydroxyvitamin D might associate with a higher perceived age. However, participants with high 25-hydroxyvitamin D concentrations likely have a higher frequency of outdoor activities (e.g., physical activity, sun bathing), better dietary quality, and lower fat mass[24]. As UVB exposure by sunlight is a predominant factor of 25-hydroxyvitamin D[25] production and contributes to skin aging, a higher 25-hydroxyvitamin D concentration might be associated with a higher perceived age. Indeed, a higher 25-hydroxyvitamin D concentration was associated with a higher perceived age in our study populations. However, the attenuation of this association by the adjustment for skin wrinkling suggests that 25-hydroxyvitamin D only associates with certain aspects of skin aging. In addition, although the Leiden Longevity Study described an association between high 25-hydroxyvitamin D and the degree of pigmented spots in an earlier publication[20], this association was not observed in the Rotterdam Study. There is no clear reason for this difference, as different methodologies (image analysis vs. photonumeric grading) show large similarities[26].

We did not find evidence of an association between higher genetically determined 25-hydroxyvitamin D levels and features of facial skin aging. Our findings suggest that the observations in the previously published in-vitro experiments[17-19] might not have in-vivo relevance. This could be because most in-vitro studies demonstrate beneficial effects of the most potent vitamin D metabolite (1,25-hydroxyvitamin D) at very high physiological levels (≥100nmol/l) compared with no vitamin D[17-19]. In contrast, most participants in the present study had a 25-hydroxyvitamin D concentration between 40 and 140 nmol/l; hence, the biological effects in this range will likely be lower. However, bioavailable levels of 25-hydroxyvitamin D and 1,25-hydroxyvitamin D in skin need to be ascertained to determine the relevance of the in-vitro studies to in-vivo conditions.

There was no significant association between the genetic score for pigmented spots and vitamin D levels. However, there was a borderline significant association between a SNP in the *IRF4* gene and 25-hydroxyvitamin D concentration, replicating a similar finding in a different cohort[27]. This finding warrants follow-up particularly because many of the pigmented spot genes are also linked to melanin levels in skin, which protects skin from UVB radiation effects, the key determinant of vitamin D production in skin.

The observational associations between 25-hydroxyvitamin D concentration and a higher perceived age and a higher degree of skin wrinkling could be the result of residual confounding

or reverse causality. However, SNPs in the *MC1R* gene associate with a higher perceived age, but were unrelated to 25-hydroxyvitamin D concentration in our study population. This suggests that reverse causality is not at play here. We believe that the most likely explanation for the association between 25-hydroxyvitamin D concentration and features of skin aging is residual confounding, probably due to UVB radiation exposure.

The present study has a number of limitations. First, the assessment of the degree of skin wrinkling and pigmented spots was different in the Rotterdam Study and the Leiden Longevity Study, which might have caused increased disparity in the data. The differences in perceived age between the two cohorts (despite having a similar chronological age) might originate from slight methodological differences as well as differences in lifestyle factors and medical history. However, we used the data on comparable scales (Z-scores) and there is large agreement between digital and manual assessment of skin wrinkling and pigmented spots[26]. The present study populations only comprised individuals from European ancestry, and our study findings might therefore not necessarily be generalizable to populations of different ancestry backgrounds. In addition, regarding the observational associations found between 25-hydroxyvitamin D, the available UV variables used might not have captured cumulative sun exposure accurately. However, this would not affect the Mendelian randomization analyses. Furthermore, although we have validated the GRS for 25-hydrxoyvitamin D against 25-hydroxyvitamin D levels in our study populations, we cannot completely rule out that the lack of evidence for an association between the GRS and features of skin aging is the result of a lack of power for the GRS to detect 25-hydroxyvitamin D effects in skin. Lastly, the facial photographs of the Rotterdam Study and the Leiden Longevity Study were taken at a later moment in time than the blood drawing for 25-hydroxyvitamin D assessment, which could have weakened any observational links.

In summary, we did not find evidence that the previously described beneficial in-vitro effects of vitamin D on cellular processes are detectable at a population level. The observational associations in our study between 25-hydroxyvitamin D and features of skin aging are, most likely, predominately due to residual confounding.

MATERIALS AND METHODS

Study setting

The present study was conducted using data from the population-based Rotterdam Study and the Leiden Longevity Study. The Rotterdam Study is an ongoing prospective population-based cohort study following 14,926 inhabitants aged ≥45 years in Ommoord, a suburb of Rotterdam in the Netherlands since 1990. Participants were examined at baseline at the study center and invited every 4-5 years for follow-up visits at the study center. Details of the study design and objectives have been described elsewhere[28]. The Leiden Longevity Study recruited a total of 421 families containing long-lived Caucasian siblings[29]. Families were only included when at least two long-

lived siblings were still alive and met the age criteria upon study inclusion (≥ 89 years for men; ≥ 91 years for women). Here, the study was conducted in the offspring of the long-lived individuals with the partners of the offspring as controls. A more detailed description of the recruitment strategy of the study participants has been published elsewhere[30].

Both studies were approved by local Medical Ethics Committees and all included participants provided written informed consent.

Serum measurements

In the Rotterdam Study, fasted blood samples were collected between 1997 and 1999, 2000 and 2001, and 2006 and 2009, for each participant only once. Serum 25-hydroxyvitamin D concentrations were measured using electrochemiluminescence immunoassay (COBAS; Roche Diagnostics, Mannheim, Germany).

In the Leiden Longevity Study, nonfasted blood samples were collected between 2002 and 2006. Plasma 25-hydroxyvitamin D levels were measured with monoclonal antibodies using a standardized protocol with electrochemiluminescence immunoassays on a fully automated Cobas e411 analyzer (Roche Diagnostics, Almere, the Netherlands). As part of the standard protocol, standardization was performed to make the measures comparable to assays using polyclonal antibodies.

Skin aging features

In the Rotterdam Study, standardized high-resolution digital three-dimensional (3D) facial photographs (Premier 3dMDface3-plus UHD, Atlanta, GA, USA) are being collected since 2010. Enface and side 2D photographs were exported from the 3D images. The current study included 3831 participants of northwestern European ancestry, who have been photographed and examined at the research center from September 2010 until June 2014. Perceived age was assessed from front and side facial exported 2D images by on average 27 assessors per image using a previously established[31] and validated[32] method. Pigmented spots and wrinkles were measured quantitatively from frontal 2D images using image analysis algorithms (Matlab 2013b) as previously described and validated[26]. Visual inspection of the image analyses measurements[33] highlighted that the measurement mainly detected solar lentigines and very few nevi. Individuals with freckles (N=23), facial contusion (N=1), facial scars with hyperpigmentation (N=1), and postinflammatory hyperpigmentation (N=1) were excluded.

In the Leiden Longevity Study, the method to determine a person's perceived age has been described and validated previously[1,5,32]. From all participants, without make-up or hairstyling product, we took one facial photograph from the front and one at 45˚. Photographs, with hair and clothing concealed, were assessed to determine the average perceived age by 60 independent assessors. Skin wrinkling grade was determined on a nine-point scale by visual assessment of front-on, whole-face photographs[2]. Pigmented spots were graded by visual assessment of light, patchy, mottled hyperpigmentation, actinic lentigines, seborrheic keratosis, and solar freckling[5];

nevi were excluded from the grading. The grade on a nine-point photographic scale was determined using quantitative and qualitative criteria such as the area/density of pigmentation, color intensity, and uniformity of distribution[5,34].

Covariables

Chronological age was determined on the day the facial photographs were taken. Weight and height were determined by research nurses at the study center. Body mass index was calculated by dividing weight (in kilograms) by the squared height (in meters). Smoking status was determined using a home questionnaire. Season was determined at the moment blood was drawn for measuring 25-hydroxyvitamin D. For the digital measurements in the Rotterdam Study, analyses were additionally adjusted for two technical variables. For both wrinkles and pigmented spots, flashlight variance was taken into account: the within-person difference between skin lightness in the images and that assessed by a spectrophotometer (CM-600d; Konica-Minolta, Osaka, Japan) on the cheek. In addition, for wrinkles, a difference in resolution between two sets of the images was taken into account using a variable described as batch[26]. In a random subpopulation of the Rotterdam Study, six variables were available as proxy for UV-exposure based on interview data: tendency to develop sunburn (low vs. high), history of working or being outdoors ≥ 4 hours daily during at least 25 years (yes vs. no), having wintered in a sunny country between September and May for at least one month during the past 5 years (yes vs. no), having lived in a sunny country for more than 1 year (yes vs. no), sun protective behavior (i.e. wearing sunglasses and/or a brimmed hat in the sun categorized into never/almost never vs. often/almost always/always), and frequency of tanning bed visits including facial solarium (fewer vs. more than 10 times in the past 5 years). Vitamin D from dietary intake (measured in ug/day) was calculated using data collected by a food-frequency questionnaire (FFQ)[35]. The Dutch Food Composition Table of 2006 and 2011[36] was used to transform the data into daily macronutrient intake and total energy intake (kcal/day). Physical activity (measured in METhours/week) was assessed using the LASA Physical Activity Questionnaire (LAPAQ)[37]. Participants were categorized as vitamin D supplement users if they used vitamin D or multivitamin supplements at least once a week.

Genotyping

For the Rotterdam Study, DNA from whole blood was extracted and genotyped following standard protocols[28]. In brief, genotyping was carried out using the Infinium II HumanHap 550K Genotyping BeadChip version 3 (Illumina, San Diego, CA, USA) for the largest part of the cohort and Illumina Human 610 Quad Arrays for the rest of the cohort. Genome-wide genotype data was imputed using 1000-Genomes (GIANT Phase I version 3) as the reference panel[38], using a two-step procedure imputation algorithm implemented in the program MACH-Minimac with default parameters[39]. For the Leiden Longevity Study, genotyping was conducted with the Illumina Human 660W-Quad and OmniExpress BeadChips (Illumina, San Diego, CA, USA). Individuals were excluded from

further investigation if they had a mismatch in sex or familial relatedness based on genotype and phenotype.

We extracted three genetic variants as instrumental variables for 25-hydroxyvitamin D: rs2282679 (*GC*), rs3829251 (*NADSYN1*), and rs2060793 (*CYP2R1*)[23], and extracted three genetic variants as instrumental variables for pigmented spots: rs12203592 (*IRF4*), rs35063026 (*MC1R*), rs6059655 (*ASIP*)[33]. The *MC1R* gene has also been associated with perceived age[40].

Based on the effect sizes observed in the genome-wide association studies, we calculated a weighted GRS for the abovementioned determinants. GRS for 25-hydroxyvitamin D: rs2282679-C*0.38 + rs3829251-C*0.18 + rs2060793-A*0.25 [23]. GRS for facial pigmented spots: rs12203592-T*0.097 + rs35063026-T*0.080 + rs6059655-A*0.059 [33].

Statistical analyses

Characteristics of the study populations are presented as means (standard deviations) for normally distributed determinants, medians (interquartile range) for non-normally distributed determinants and frequencies (percentages) for categorical determinants, separately for the Rotterdam Study and Leiden Longevity Study.

As methodologies for determining the skin aging features differed between the Rotterdam Study and the Leiden Longevity, study outcomes were standardized to obtain a standard normal distribution. Analyses were done separately for the two cohorts, and subsequently meta-analyzed using fixed-effect meta-analysis, as part of the rmeta package in R (http://www.R-project.org). For the analyses in the Rotterdam Study, multiple imputation was performed, using the Multiple Imputation by Chained Equations (MICE) package in R, with an iteration of 20 (maximum missing data per variable was 6%). We used linear regression analyses, adjusted for age, sex, BMI, current smoking, and season to obtain the observational effect estimates for the association between 25-hydroxyvitamin D concentrations and the skin aging features. On the basis of the observational effect estimates in our total study population and considering an α of 0.05, we calculated the statistical power for the Mendelian randomization analysis on the skin aging features using a publically available power calculator (http:/cnsgenomics.com/shiny/mRnd/). Associations between 25-hydroxyvitamin D genotypes and the GRS for 25-hydroxyvitamin D were adjusted for age and sex. We also performed the Mendelian randomization analyses for the genetic instruments for pigmented spots and perceived age (*MC1R* only) with linear regression analyses, adjusted for age and sex.

All analyses for wrinkles and pigmented spots in the Rotterdam Study were additionally adjusted for the two technical variables mentioned above. Two-sided P-values below 0.05 were considered statistically significant.

ACKNOWLEDGMENTS

Rotterdam Study: The generation and management of GWAS genotype data for the Rotterdam Study (RS I, RS II, RS III) was executed by the Human Genotyping Facility of the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands. We thank Pascal Arp, Mila Jhamai, Marijn Verkerk, Lizbeth Herrera and Marjolein Peters, MSc, and Carolina Medina-Gomez, MSc, for their help in creating the GWAS database, and Karol Estrada, PhD, Yurii Aulchenko, PhD, and Carolina Medina-Gomez, MSc, for the creation and analysis of imputed data. The authors are grateful to the study participants, the staff from the Rotterdam Study and the participating general practitioners and pharmacists. We thank Emmilia Dowlatshahi, Sophie Flohil, Leonie Jacobs, Robert van der Leest, Simone van der Velden, Joris Verkouteren and Ella van der Voort for collecting the phenotypes. Additionally, we thank Andreas Wollstein for converting all photographs and Sophie van den Berg for masking and reviewing them. We acknowledge Jaspal Lall for masking the photographs and creating the digital wrinkle measurements.

Leiden Longevity Study: We would like to thank all participants, the secretary staff, Meriam H.G.F. van der Star and Ellen H.M. Bemer-Oorschot for their contribution to this study. We would also like to thank Christopher Griffiths and Tamara Griffiths for the pigmented spots and wrinkle score measurements.

REFERENCES

1. Christensen K, Thinggaard M, McGue M, Rexbye H, Hjelmborg JV, Aviv A, et al. Perceived age as clinically useful biomarker of ageing: cohort study. *BMJ*. 2009;339:b5262.
2. Griffiths CE. The clinical identification and quantification of photodamage. *Br J Dermatol*. 1992;127 Suppl 41:37-42.
3. Rexbye H, Petersen I, Johansens M, Klitkou L, Jeune B, Christensen K. Influence of environmental factors on facial ageing. *Age Ageing*. 2006;35(2):110-5.
4. Shekar SN, Luciano M, Duffy DL, Martin NG. Genetic and environmental influences on skin pattern deterioration. *The Journal of investigative dermatology*. 2005;125(6):1119-29.
5. Gunn DA, Rexbye H, Griffiths CE, Murray PG, Fereday A, Catt SD, et al. Why some women look young for their age. *PLoS One*. 2009;4(12):e8021.
6. van Drielen K, Gunn DA, Noordam R, Griffiths CE, Westendorp RG, de Craen AJ, et al. Disentangling the effects of circulating IGF-1, glucose, and cortisol on features of perceived age. *Age*. 2015;37(3):9771.
7. Noordam R, Gunn DA, Tomlin CC, Maier AB, Mooijaart SP, Slagboom PE, et al. High serum glucose levels are associated with a higher perceived age. *Age*. 2013;35(1):189-95.
8. Noordam R, Gunn DA, Tomlin CC, Rozing MP, Maier AB, Slagboom PE, et al. Cortisol serum levels in familial longevity and perceived age: the Leiden longevity study. *Psychoneuroendocrinology*. 2012;37(10):1669-75.
9. Noordam R, Gunn DA, Tomlin CC, Maier AB, Griffiths T, Catt SD, et al. Serum insulin-like growth factor 1 and facial ageing: high levels associate with reduced skin wrinkling in a cross-sectional study. *The British journal of dermatology*. 2013;168(3):533-8.
10. Holick MF. Vitamin D deficiency. *N Engl J Med*. 2007;357(3):266-81.
11. Mathieu C, Gysemans C, Giulietti A, Bouillon R. Vitamin D and diabetes. *Diabetologia*. 2005;48(7):1247-57.
12. Pilz S, Dobnig H, Nijpels G, Heine RJ, Stehouwer CD, Snijder MB, et al. Vitamin D and mortality in older men and women. *Clin Endocrinol (Oxf)*. 2009;71(5):666-72.
13. Lee JH, Gadi R, Spertus JA, Tang F, O'Keefe JH. Prevalence of vitamin D deficiency in patients with acute myocardial infarction. *Am J Cardiol*. 2011;107(11):1636-8.
14. Pilz S, Tomaschitz A, Marz W, Drechsler C, Ritz E, Zittermann A, et al. Vitamin D, cardiovascular disease and mortality. *Clin Endocrinol (Oxf)*. 2011;75(5):575-84.
15. Andrukhova O, Slavic S, Zeitz U, Riesen SC, Heppelmann MS, Ambrisko TD, et al. Vitamin D is a regulator of endothelial nitric oxide synthase and arterial stiffness in mice. *Mol Endocrinol*. 2014;28(1):53-64.
16. Chowdhury R, Kunutsor S, Vitezova A, Oliver-Williams C, Chowdhury S, Kiefte-de-Jong JC, et al. Vitamin D and risk of cause specific death: systematic review and meta-analysis of observational cohort and randomised intervention studies. *Bmj*. 2014;348:g1903.
17. Gniadecki R. Stimulation versus inhibition of keratinocyte growth by 1,25-Dihydroxyvitamin D3: dependence on cell culture conditions. *J Invest Dermatol*. 1996;106(3):510-6.
18. Manggau M, Kim DS, Ruwisch L, Vogler R, Korting HC, Schafer-Korting M, et al. 1Alpha,25-dihydroxyvitamin D3 protects human keratinocytes from apoptosis by the formation of sphingosine-1-phosphate. *J Invest Dermatol*. 2001;117(5):1241-9.
19. Bollag WB, Ducote J, Harmon CS. Biphasic effect of 1,25-dihydroxyvitamin D3 on primary mouse epidermal keratinocyte proliferation. *J Cell Physiol*. 1995;163(2):248-56.
20. van Drielen K, Gunn DA, Griffiths CE, Griffiths TW, Ogden S, Noordam R, et al. Markers of health and disease and pigmented spots in a middle-aged population. *Br J Dermatol*. 2015;173(6):1550-2.

21. Lawlor DA, Harbord RM, Sterne JA, Timpson N, Davey Smith G. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. *Statistics in medicine*. 2008;27(8):1133-63.
22. Smith GD, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? *International journal of epidemiology*. 2003;32(1):1-22.
23. Ahn J, Yu K, Stolzenberg-Solomon R, Simon KC, McCullough ML, Gallicchio L, et al. Genome-wide association study of circulating vitamin D levels. *Human molecular genetics*. 2010;19(13):2739-45.
24. Vitezova A, Muka T, Zillikens MC, Voortman T, Uitterlinden AG, Hofman A, et al. Vitamin D and body composition in the elderly. *Clinical nutrition*. 2017;36(2):585-92.
25. Sallander E, Wester U, Bengtsson E, Wiegleb Edstrom D. Vitamin D levels after UVB radiation: effects by UVA additions in a randomized controlled trial. *Photodermatology, photoimmunology & photomedicine*. 2013;29(6):323-9.
26. Hamer MA, Jacobs LC, Lall JS, Wollstein A, Hollestein LM, Rae AR, et al. Validation of image analysis techniques to measure skin aging features from facial photographs. *Skin Res Technol*. 2015;21(4):392-402.
27. Saternus R, Pilz S, Graber S, Kleber M, Marz W, Vogt T, et al. A closer look at evolution: Variants (SNPs) of genes involved in skin pigmentation, including EXOC2, TYR, TYRP1, and DCT, are associated with 25(OH)D serum concentration. *Endocrinology*. 2015;156(1):39-47.
28. Hofman A, Brusselle GG, Darwish Murad S, van Duijn CM, Franco OH, Goedegebure A, et al. The Rotterdam Study: 2016 objectives and design update. *European journal of epidemiology*. 2015;30(8):661-708.
29. Westendorp RG, van Heemst D, Rozing MP, Frolich M, Mooijaart SP, Blauw GJ, et al. Nonagenarian siblings and their offspring display lower risk of mortality and morbidity than sporadic nonagenarians: The Leiden Longevity Study. *Journal of the American Geriatrics Society*. 2009;57(9):1634-7.
30. Schoenmaker M, de Craen AJ, de Meijer PH, Beekman M, Blauw GJ, Slagboom PE, et al. Evidence of genetic enrichment for exceptional survival using a family approach: the Leiden Longevity Study. *European journal of human genetics : EJHG*. 2006;14(1):79-84.
31. Gunn DA, Dick JL, van Heemst D, Griffiths CE, Tomlin CC, Murray PG, et al. Lifestyle and youthful looks. *Br J Dermatol*. 2015;172(5):1338-45.
32. Gunn DA, Murray PG, Tomlin CC, Rexbye H, Christensen K, Mayes AE. Perceived age as a biomarker of ageing: a clinical methodology. *Biogerontology*. 2008;9(5):357-64.
33. Jacobs LC, Hamer MA, Gunn DA, Deelen J, Lall JS, van Heemst D, et al. A Genome-Wide Association Study Identifies the Skin Color Genes IRF4, MC1R, ASIP, and BNC2 Influencing Facial Pigmented Spots. *The Journal of investigative dermatology*. 2015;135(7):1735-42.
34. Griffiths CE, Wang TS, Hamilton TA, Voorhees JJ, Ellis CN. A photonumeric scale for the assessment of cutaneous photodamage. *Arch Dermatol*. 1992;128(3):347-51.
35. Goldbohm RA, van den Brandt PA, Brants HA, van't Veer P, Al M, Sturmans F, et al. Validation of a dietary questionnaire used in a large-scale prospective cohort study on diet and cancer. *European journal of clinical nutrition*. 1994;48(4):253-65.
36. RIVM. Dutch Food Composition Table (NEVO). *Bilthoven*. 2006/2011.
37. Stel VS, Smit JH, Pluijm SM, Visser M, Deeg DJ, Lips P. Comparison of the LASA Physical Activity Questionnaire with a 7-day diary and pedometer. *Journal of clinical epidemiology*. 2004;57(3):252-8.
38. Genomes Project C, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, et al. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012;491(7422):56-65.
39. Howie B, Fuchsberger C, Stephens M, Marchini J, Abecasis GR. Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. *Nat Genet*. 2012;44(8):955-9.
40. Liu F, Hamer MA, Deelen J, Lall JS, Jacobs L, van Heemst D, et al. The MC1R Gene and Youthful Looks. *Curr Biol*. 2016;26(9):1213-20.

SUPPLEMENTARY TABLES

Supplementary Table S1. Sensitivity analyses in the Rotterdam Study, using only complete cases for all variables

	Model 1		Model 2	
	β (SE)	P-value	β (SE)	P-value
Perceived age (N = 1153)	0.15 (0.04)	5.8×10^{-4}	0.15 (0.05)	9.6×10^{-4}
Perceived age, adjusted (N = 1153)[a]	0.01 (0.03)	0.75	0.02 (0.04)	0.49
Degree of skin wrinkling (N = 1487)	0.29 (0.05)	1.6×10^{-8}	0.24 (0.05)	3.8×10^{-6}
Degree of pigmented spots (N = 1003)	-0.08 (0.06)	0.18	-0.03 (0.06)	0.63

Abbreviation: SE, standard error.
Effect estimates presented as the increase in standardized outcome per 1 ln-transformed unit increase in 25-hydroxyvitamin D serum concentration.
Model 1: Analyses adjusted for age and sex, smoking status, season, body mass index and technical variables.
Model 2: Analyses additionally adjusted for UV variables, physical activity, energy intake, vitamin D derived from diet and from supplement use.
[a]adjusted for wrinkles.

Supplementary Table S2. Association between UV- and vitamin D-related covariables and 25-hydroxyvitamin D in the Rotterdam Study, using only complete cases for all variables

		Rotterdam Study (N=1487)	
		β (SE)	P-value
UV-related variables	Tendency to develop sunburn[a]	-0.09 (0.02)	7.4×10^{-5}
	Lived in a sunny country[b]	-0.07 (0.05)	0.20
	Sun-protective behavior[c]	0.03 (0.02)	0.25
	Spending winter in a sunny country[d]	0.15 (0.05)	3.6×10^{-3}
	Outdoor work history[e]	4.8×10^{-3} (0.03)	0.86
	Tanning bed use[f]	0.24 (0.03)	7.3×10^{-14}
	Physical activity[g]	1.3×10^{-3} (2.4×10^{-4})	2.3×10^{-7}
Vitamin D-related variables	Vitamin D from diet[h]	0.02 (5.9×10^{-3})	3.4×10^{-3}
	Vitamin D from supplement use[i]	0.06 (0.02)	7.9×10^{-3}

Abbreviation: SE, standard error.
Effect estimates presented as the increase in ln-transformed 25-hydroxyvitamin D serum concentration, e.g. effect estimate of -0.09 meaning the ln-transformed 25-hydroxyvitamin D serum concentration is decreased by 0.09 when tendency to develop sunburn is high. Analyses additionally adjusted for age, sex, smoking status, season, body mass index and energy intake.
[a]tendency to develop sunburn (high vs. low); [b]history of living in a sunny country >1 year (yes vs. no); [c]wearing sunglasses and/or a brimmed hat in the sunshine (often/always vs. never/almost never); [d]having wintered in a sunny country between September and May for at least one month during the past 5 years (yes vs. no); [e]worked or been outdoors ≥4 hours daily during at least 25 years (yes vs. no); [f]frequency of tanning bed visits in the past 5 years, including facial solarium (more than 10x vs. never or less than 10x); [g]physical activity measured in Metabolic Equivalent of Task (MET)hours/week; [h]vitamin D from dietary intake (ug/day); [i]vitamin D or multivitamin supplement use at least once a week (yes vs. no).

Supplementary Table S3. Association between genetic variants for 25-hydroxyvitamin D and 25-hydroxyvitamin D

	Rotterdam Study		Leiden Longevity Study		Meta-analysis	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
GC gene, rs2282679	0.135 (0.008)	8.2×10^{-57}	0.073 (0.013)	2.8×10^{-8}	0.118 (0.007)	4.0×10^{-67}
NADSYN1 gene, rs3829251	0.037 (0.010)	3.4×10^{-4}	0.015 (0.016)	0.33	0.031 (0.008)	1.4×10^{-4}
CYP2R1, rs2060793	0.039 (0.008)	6.7×10^{-7}	0.025 (0.011)	2.7×10^{-2}	0.034 (0.006)	1.3×10^{-7}
25-hydroxyvitamin D GRS	0.279 (0.017)	1.1×10^{-58}	0.152 (0.026)	5.7×10^{-9}	0.241 (0.014)	2.3×10^{-64}

Abbreviations: *CYP2R1*, cytochrome P450 family 2 subfamily R member 1; *GC*, GC vitamin D binding protein; GRS, genetic risk score; *NADSYN1*, nicotinamide adenine dinucleotide synthetase 1; SE, standard error.
Analyses adjusted for age, sex, and season. Effect estimates of the meta-analysis obtained using fixed-effect models. Effect estimates of the individual genetic instruments presented as the additive effect of the effect allele on log(25-hydroxyvitamin D) serum concentration. Effect estimates of the genetic risk score presented as the increase in ln(25-hydroxyvitamin D) serum concentration per 1 point increase in the weighted score.