



# No Causal Association between 25-Hydroxyvitamin D and Features of Skin Aging: Evidence from a Bidirectional Mendelian Randomization Study

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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 = 3,831; 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 =  $3.6 \times 10^{-7}$ ), more skin wrinkling ( $P$ -value =  $2.6 \times 10^{-16}$ ), 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.

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## INTRODUCTION

A higher perceived age—estimated age based on facial appearance—is associated with an increased risk of morbidity and mortality (Christensen et al., 2009), making it a useful marker in aging research. In addition to well-described extrinsic factors, such as smoking and UV exposure (Christensen et al., 2009; Griffiths, 1992; Rexbye et al., 2006), a higher perceived age also has an intrinsic component (Gunn et al., 2009; Shekar et al.,

2005; van Drielen et al., 2015b). It has previously been shown that high serum concentrations of glucose and cortisol were associated with a higher perceived age (Noordam et al., 2012, 2013b), whereas a high concentration of insulin-like growth factor-1 was associated with a lower perceived age mainly through skin wrinkling (Noordam et al., 2013a; van Drielen et al., 2015b). Besides skin wrinkling, facial pigmented spots are also an important component of skin aging.

Although sun exposure contributes to premature skin aging (Christensen et al., 2009; Griffiths, 1992), it is essential for vitamin D synthesis in the skin (Holick, 2007) 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 (Andrukhova et al., 2014; Chowdhury et al., 2014; Lee et al., 2011; Mathieu et al., 2005; Pilz et al., 2009, 2011).

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 (Gniadecki, 1996) and differentiation (Manggau et al., 2001) with the response dependent on vitamin D concentrations and culture conditions (Bollag et al., 1995; Gniadecki, 1996). Although the bioavailable levels of vitamin

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Abbreviations: GRS, genetic risk score; SD, standard deviation; SE, standard error

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**Table 1. Characteristics of the study population**

	Rotterdam Study (N = 3,831)	Leiden Longevity Study (N = 661)
General		
Chronological age (y), median (IQR)	66.5 (61.0, 71.5)	63.1 (58.9, 67.5)
Females, N (%)	2,229 (58.2)	334 (50.4)
Body mass index (kg/m <sup>2</sup> ), mean (SD)	27.6 (4.4)	26.6 (4.0)
Current smoking, N (%)	707 (18.5)	91 (13.8)
Skin aging features		
Perceived age (y), mean (SD)	65.9 (7.6) <sup>1</sup>	59.4 (7.6)
Degree of skin wrinkling, median (IQR) <sup>2</sup>	3.9 (2.5, 6.0)	4.5 (3.5, 5.5)
Degree of pigmented spots, median (IQR) <sup>2</sup>	1.3 (0.9, 2.1) <sup>3</sup>	4.5 (3.5, 5.0)
Serum measurements		
25-Hydroxyvitamin D (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 (%)	1,107 (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.

<sup>1</sup>Assessed in 2,679 individuals.

<sup>2</sup>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 two expert dermatologists using a photonumeric scale ranging from 1 to 9.

<sup>3</sup>Assessed in 2,843 individuals.

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 (van Drielen et al., 2015a). 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 (Lawlor et al., 2008; Smith and Ebrahim, 2003). 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 3,831 participants from the Rotterdam Study (median [interquartile range] age: 66.5 [61.0, 71.5] years) and 661 participants from the Leiden Longevity Study (median [interquartile range] age: 63.1 [58.9, 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% vs. 50.4%), smokers (18.5% vs. 13.8%), and had a lower 25-hydroxyvitamin D concentration (median: 61.0 nmol/l vs. 68.3 nmol/l). Also, 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).

### Observational associations between a 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 ( $\beta = 0.149$  standard deviation [SD] per one ln[25-

hydroxyvitamin D]; standard error [SE] = 0.029;  $P$ -value =  $3.58 \times 10^{-7}$ ). However, this association disappeared after additional adjustment for the degree of skin wrinkling ( $\beta = 0.020$  SD per one 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 ( $\beta = 0.250$  SD per one ln[25-hydroxyvitamin D]; SE = 0.030;  $P$ -value =  $2.61 \times 10^{-16}$ ). 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 ( $\beta = -0.033$  SD per one 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 online), despite that we observed strong associations between these factors and the 25-hydroxyvitamin D level (Supplementary Table S2 online).

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

We calculated, per participant, a weighted genetic score for a 25-hydroxyvitamin D concentration based on the single nucleotide polymorphisms that were identified in a genome-wide association study on a 25-hydroxyvitamin D concentration (notably, rs2282679 [GC], rs3829251 [NADSYN1], and rs2060793 [CYP2R1]; Ahn et al., 2010). On the basis of the observational effect estimates, we had an 82% and 84% power to detect significant ( $\alpha = 0.05$ ) 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 a 25-hydroxyvitamin D concentration (Supplementary Table S3 online). In line with

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

	Rotterdam Study (N = 3,831)		Leiden Longevity Study (N = 661)		Meta-analysis	
	$\beta$ (SE)	P-value	$\beta$ (SE)	P-value	$\beta$ (SE)	P-value
Perceived age	0.128 (0.030) <sup>2</sup>	$1.77 \times 10^{-5}$	0.516 (0.127)	$5.36 \times 10^{-5}$	0.149 (0.029)	$3.58 \times 10^{-7}$
Perceived age, adjusted <sup>1</sup>	0.006 (0.023) <sup>2</sup>	$7.82 \times 10^{-1}$	0.225 (0.087)	$9.84 \times 10^{-3}$	0.020 (0.022)	$3.61 \times 10^{-1}$
Degree of skin wrinkling	0.241 (0.031)	$2.17 \times 10^{-14}$	0.507 (0.169)	$2.73 \times 10^{-3}$	0.250 (0.030)	$2.61 \times 10^{-16}$
Degree of pigmented spots	-0.050 (0.032) <sup>3</sup>	$1.16 \times 10^{-1}$	0.571 (0.189)	$2.63 \times 10^{-3}$	-0.033 (0.031)	$3.00 \times 10^{-1}$

Effect estimates presented as the increase in standardized outcome per one ln-transformed unit increase in 25-hydroxyvitamin D serum concentration.

Abbreviation: SE, standard error.

<sup>1</sup>Analyses additionally adjusted for the degree of facial skin wrinkling. Effect estimates of the meta-analysis obtained using fixed-effect models.

<sup>2</sup>Analysis based on 2,679 individuals from the Rotterdam Study.

<sup>3</sup>Analysis based on 2,843 individuals from the Rotterdam Study. 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.

this, the calculated weighted genetic score for a higher 25-hydroxyvitamin D concentration was associated with a higher 25-hydroxyvitamin D concentration in our study populations and meta-analysis ( $\beta = 0.24$  units ln[25-hydroxyvitamin D] increase per one unit increase in genetic score; SE = 0.01; P-value =  $2.23 \times 10^{-64}$ ).

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 ( $\beta = 0.030$  SD per one genetically determined ln[25-hydroxyvitamin D]; SE = 0.023; P-value = 0.18); (ii) a higher perceived age additionally adjusted for skin wrinkling ( $\beta = 0.017$  SD per one genetically determined ln[25-hydroxyvitamin D]; SE = 0.016; P-value = 0.28); (iii) a higher degree of skin wrinkling ( $\beta = 0.000$  SD per one genetically determined ln[25-hydroxyvitamin D]; SE = 0.028; P-value = 1.00); (iv) a higher degree of pigmented spots ( $\beta = 0.055$  SD per one genetically determined ln[25-hydroxyvitamin D]; SE = 0.030; P-value = 0.07).

#### Mendelian randomization analyses between pigmented spots and a 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.,  $\beta = 0.146$  ln[25-hydroxyvitamin D in nmol/l] per one unit increases in pigmented spots GRS; SE = 0.089; P-value = 0.10).

#### 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 (Andrukhova et al., 2014; Lee et al., 2011; Mathieu et al., 2005; Pilz et al., 2009, 2011) and a higher perceived age (Christensen et al., 2009) are associated with an increased risk of morbidity and mortality (Chowdhury et al., 2014). Therefore, a 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 (Vitezova et al., 2017). As UVB exposure by sunlight is a predominant factor of 25-hydroxyvitamin D (Sallander et al., 2013) 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 (van Drielen et al., 2015a), this association was not observed in the Rotterdam Study. There is no clear reason for this difference, as different methodologies (image analysis vs. photometric grading) show large similarities (Hamer et al., 2015).

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 (Bollag et al., 1995; Gniadecki, 1996; Manggau et al., 2001) 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 ( $\geq 100$  nmol/l) compared with no vitamin D (Bollag et al., 1995; Gniadecki, 1996; Manggau et al., 2001). 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.

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

	Rotterdam Study (N = 3,831)		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)	0.69	0.046 (0.026)	0.08	0.030 (0.023)	0.18
Perceived age, adjusted <sup>1</sup>	-0.003 (0.034)	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 <sup>2</sup>	0.004 (0.051)	0.93	0.084 (0.038)	0.03	0.055 (0.030)	0.07

Effect estimates presented as the increase in the standardized outcomes per one unit increase in the genetic risk score.

Abbreviation: SE, standard error.

<sup>1</sup>Analyses additionally adjusted for the degree of facial skin wrinkling.

<sup>2</sup>Analysis based on 2,843 individuals from the Rotterdam Study. Analyses adjusted for age and sex. Effect estimates of the meta-analysis obtained using fixed-effect models.

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 single nucleotide polymorphism in the *IRF4* gene and 25-hydroxyvitamin D concentration, replicating a similar finding in a different cohort (Saternus et al., 2015). 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, single nucleotide polymorphisms in the *MC1R* gene associate with a higher perceived age, but were unrelated to 25-hydroxyvitamin D concentration in our study population suggesting 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 (Hamer et al., 2015). 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-hydroxyvitamin 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.

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

	Rotterdam Study (N = 2,843)		Leiden Longevity Study (N = 661)		Meta-analysis	
	β (SE)	P-value	β (SE)	P-value	β (SE)	P-value
<i>IRF4</i> gene, rs12203592	0.017 (0.014)	0.20	0.056 (0.023)	0.01	0.028 (0.012)	0.02
<i>MC1R</i> gene, rs35063026	-0.007 (0.017)	0.67	-0.014 (0.023)	0.54	-0.009 (0.014)	0.49
<i>ASIP</i> 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

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.

Abbreviations: *ASIP*, agouti signaling protein; GRS, genetic risk score; *IRF4*, interferon regulatory factor 4; *MC1R*, melanocortin 1 receptor; SE, standard error.



## 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  $\geq 45$  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 (Hofman et al., 2015). The Leiden Longevity Study recruited a total of 421 families containing long-lived Caucasian siblings (Westendorp et al., 2009). Families were only included when at least two long-lived siblings were still alive and met the age criteria upon study inclusion ( $\geq 89$  years for men;  $\geq 91$  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 (Schoenmaker et al., 2006).

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) are being collected since 2010. Enface and side 2D photographs were exported from the 3D images. The current study included 3,831 participants of north-western 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 (Gunn et al., 2015) and validated (Gunn et al., 2008) method. Pigmented spots and wrinkles were measured quantitatively from frontal 2D images using image analysis algorithms (MATLAB 2013b) as previously described and validated (Hamer et al., 2015). Visual inspection of the image analyses measurements (Jacobs et al., 2015) 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 (Christensen et al., 2009; Gunn et al., 2008, 2009). 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 (Griffiths, 1992). Pigmented spots were graded by visual assessment of light, patchy, mottled hyperpigmentation, actinic lentigines, seborrheic keratosis, and solar freckling (Gunn et al., 2009); 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 (Griffiths et al., 1992; Gunn et al., 2009).

### 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 height (in meters squared). 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 (Hamer et al., 2015). 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  $\geq 4$  hours daily during at least 25 years (yes vs. no), having wintered in a sunny country between September and May for at least 1 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  $\mu\text{g}/\text{day}$ ) was calculated using data collected by a food-frequency questionnaire (Goldbohm et al., 1994). The Dutch Food Composition Table of 2006 and 2011 (RIVM, 2006/2011) 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 (Stel et al., 2004). 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 (Hofman et al., 2015). In brief, genotyping was carried out using the Infinium II HumanHap 550K Genotyping BeadChip version 3 (Illumina, San Diego, CA) 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 (1000 Genomes Project Consortium et al., 2012), using a two-step procedure imputation algorithm implemented in the program MACH-Minimac with default parameters (Howie et al., 2012). For the Leiden Longevity Study, genotyping was conducted with the Illumina Human 660W-Quad and OmniExpress BeadChips

(Illumina). 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*) (Ahn et al., 2010), and extracted three genetic variants as instrumental variables for pigmented spots: rs12203592 (*IRF4*), rs35063026 (*MC1R*), rs6059655 (*ASIP*) (Jacobs et al., 2015). The *MC1R* gene has also been associated with perceived age (Liu et al., 2016).

On the basis of the effect sizes observed in the genome-wide association studies, we calculated a weighted GRS for the above-mentioned determinants. GRS for 25-hydroxyvitamin D:  $rs2282679-C*0.38 + rs3829251-C*0.18 + rs2060793-A*0.25$  (Ahn et al., 2010). GRS for facial pigmented spots:  $rs12203592-T*0.097 + rs35063026-T*0.080 + rs6059655-A*0.059$  (Jacobs et al., 2015).

### Statistical analyses

Characteristics of the study populations are presented as means (SD) 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, body mass index, 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  $\alpha$  of 0.05, we calculated the statistical power for the Mendelian randomization analysis on the skin aging features using a publicly 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.

### CONFLICT OF INTEREST

TN and MAH received a restricted research grant for skin aging research from Unilever. DAG is a Unilever employee. Although no products were tested, it is possible this manuscript could promote antiaging products that could lead to financial gain for Unilever.

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### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at [www.jidonline.org](http://www.jidonline.org), and at <http://dx.doi.org/10.1016/j.jid.2017.07.817>.

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