Report

Current Biology

The *MC1R* Gene and Youthful Looks

Highlights

- We present the first genetic associations with how old people look (perceived age)
- Variants in *MC1R*, a pigmentation gene, significantly associated with perceived age
- The *MC1R* association was independent of wrinkling, skin color, and sun exposure
- The *MC1R* genetic effect resulted in looking up to 2 years older for one's age

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In Brief

The biological basis of why some people look younger and others older for their age remains poorly understood. Of over eight million tested, Liu et al. find DNA variants in *MC1R*, a pigmentation and skin cancer gene, as the most significantly associated with perceived facial age, providing new molecular leads to the understanding of youthful looks.

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Report

The *MC1R* Gene and Youthful Looks

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SUMMARY

Looking young for one's age has been a desire since time immemorial. This desire is attributable to the belief that appearance reflects health and fecundity. Indeed, perceived age predicts survival [1] and associates with molecular markers of aging such as telomere length [2]. Understanding the underlying molecular biology of perceived age is vital for identifying new aging therapies among other purposes, but studies are lacking thus far. As a first attempt, we performed genome-wide association studies (GWASs) of perceived facial age and wrinkling estimated from digital facial images by analyzing over eight million SNPs in 2,693 elderly Dutch Europeans from the Rotterdam Study. The strongest genetic associations with perceived facial age were found for multiple SNPs in the *MC1R* gene ($p < 1 \times 10^{-7}$). This effect was enhanced for a compound heterozygosity marker constructed from four pre-selected functional *MC1R* SNPs ($p = 2.69 \times 10^{-12}$), which was replicated in 599 Dutch Europeans from the Leiden Longevity Study ($p = 0.042$) and in 1,173 Europeans of the TwinsUK Study ($p = 3 \times 10^{-3}$). Individuals carrying the homozygote *MC1R* risk haplotype looked on average up to 2 years older than non-carriers. This association was independent of age, sex, skin color, and sun damage (wrinkling, pigmented spots) and persisted through different sun-exposure levels. Hence, a role for *MC1R* in youthful looks independent of its known melanin synthesis function is suggested. Our study uncovers the first genetic evidence explaining why some people look older for their age and provides new leads for further investigating the biological basis of how old or young people look.

RESULTS

The discovery cohort included 2,693 Dutch European subjects from the Rotterdam Study [3] (Table S1). As expected, perceived facial age (termed perceived age from now on) was strongly correlated with chronological age of the subjects ($R^2 = 44\%$, $p < 10^{-300}$). However, women tended to look slightly older (by 1.53 years on average) and men slightly younger (by −1.49 years on average) for their respective chronological age (Figure S1A). On average, the percentage of facial skin covered by wrinkling was estimated as 1.27% (SD 0.66%; Table S1). Facial wrinkling was strongly correlated with perceived age, as measured by the residuals of regressing perceived age on chronological age, in women ($R^2 = 35\%$, $p = 9.5 \times 10^{-138}$) as well as in men ($R^2 = 21\%$, $p = 3.1 \times 10^{-65}$) (Figure S1B). The effect of wrinkling and non-wrinkling components on facial aging is illustrated using averaged faces of women who, although being of the same chronological age, looked younger or older either influenced by (Figures 1A and 1B) or irrespective of (Figures 1C and 1D) facial wrinkling. Facial pigmented spots showed a weaker correlation with perceived age in women ($R^2 = 1.0\%$, $p = 0.001$) and in men ($R^2 = 0.8\%$, $p = 0.002$) (Figure S1C). Most subjects were not sunbed users and had white as opposed to pale skin color or white to olive skin color (Table S1).

Genome-wide Association Studies on Perceived Age and Wrinkles in the Rotterdam Study

In the discovery genome-wide association studies (GWASs) using 2,693 samples from the Rotterdam Study, we searched for

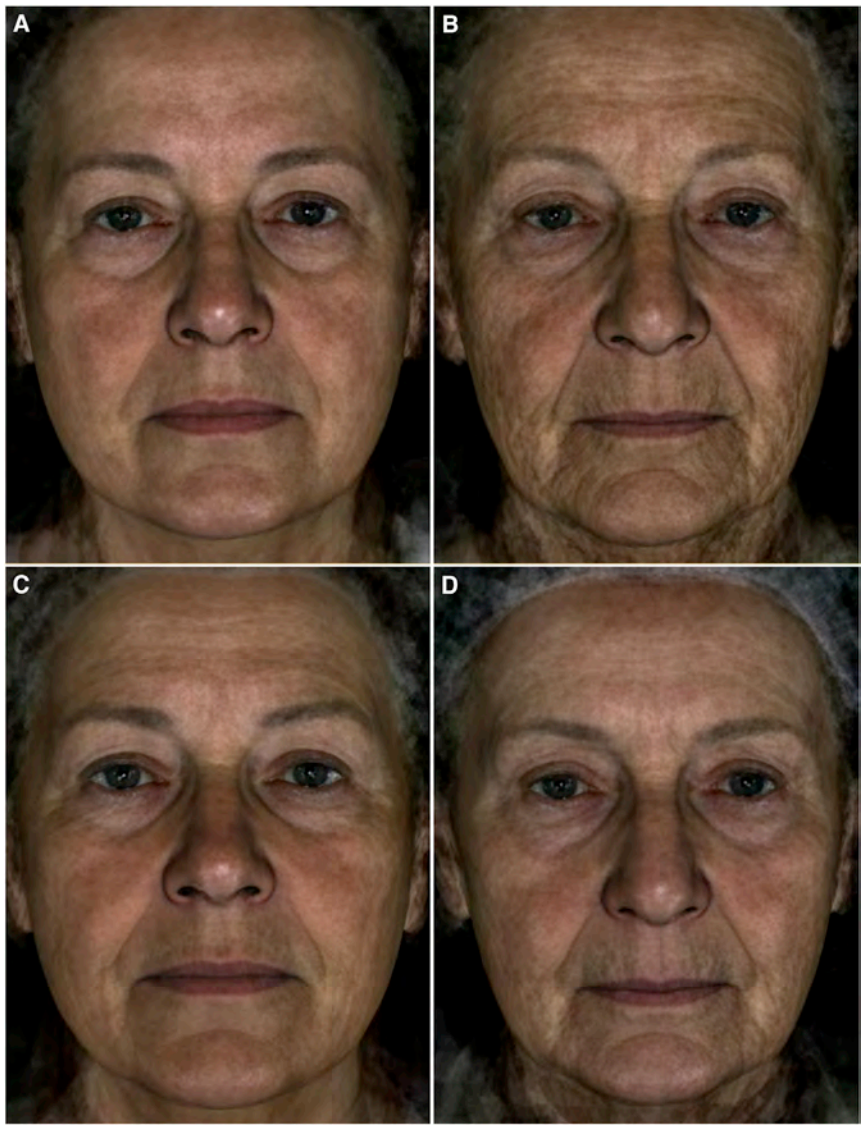

Figure 1. Illustration of the Effect of Wrinkling and Non-wrinkling Components on Perceived Facial Age

(A–D) Facial averages of Dutch European women who looked young or old for their chronological age without (A and B) and with (C and D) adjustment for the effect of wrinkles. Enface average image of 22 women (mean chronological age 70) who looked young for their chronological age (mean perceived age 59) (A) and 22 women (mean chronological age 70) who look old for their chronological age (mean perceived age 80) (B); differences in face shape changes (e.g., lip size, jawline sag, nasolabial fold) and wrinkles (average percent of skin covered by wrinkles was 2% for A and 10% for B) are evident. Enface average image of 20 women (mean chronological age 69) who looked young for their chronological age (average perceived age after adjusting for wrinkles was 60) (C) and 20 women (mean chronological age 69) who looked old for their chronological age (mean perceived age after adjusting for wrinkles was 78) (D); differences in face shape changes and skin color are evident. The mean total skin area covered by wrinkles for (C) and (D) was the same (5%). See Figure S1 for correlations of perceived age with chronological age and age-related sub-phenotypes such as wrinkles and pigmented spots in the Rotterdam Study discovery cohort. See also Table S3.

SNPs that associated with perceived age, wrinkling, and the non-wrinkling component of perceived age (i.e., adjusted for wrinkles). Although genome-wide significant associations for perceived age (Table S2) and wrinkling were not observed (Table S3), multiple SNPs at the *MC1R* gene locus on chromosome 16 showed borderline genome-wide significant association with perceived age after adjustment for age, sex, and wrinkles (Tables 1 and S2; Figures 2, S2A, and S2B).

We then constructed a collapsed compound heterozygosity marker (herein termed *MC1R* compound marker) based on a haplotype analysis of four *MC1R* DNA variants, rs1805005 (V60L), rs1805007 (R151C), rs1805008 (R160W), and rs1805009 (D294H), which were selected a priori because of previous knowledge that they (1) are missense loss-of-function variants [4], (2) are causing phenotypes such as red hair color and pale skin in a compound heterozygote manner [4, 5], and (3) are involved in age-related skin phenotypes such as pigmented spots [6]. These four missense *MC1R* DNA variants were collapsed into three possible haplotypes, WT/WT, WT/R, and R/R, where R is the risk haplotype consisting of at least one risk allele from any of the four *MC1R* variants and the WT is the wild-type haplotype consisting of none of the risk alleles (Supplemental Information). This *MC1R* compound marker demonstrated a genome-wide significant association with perceived age after adjustment for age, sex, and wrinkles ($p = 2.69 \times 10^{-12}$; Table 1; Figure 2). On average, the homozygote *MC1R* risk haplotype carriers (R/R) looked almost 2 years older (1.81 years) and the heterozygote carriers (R/WT) almost 1 year older (0.94 years) than the non-carriers (WT/WT) (Table 2), with a slightly larger effect size in men compared to women (Figure S2C).

Replication Analyses in the Leiden Longevity Study and the TwinsUK Study

To replicate our findings, we used the Leiden Longevity Study [7] with perceived age and wrinkle grading from facial photographs and genetic data of 599 Dutch European subjects (Table S1 and Supplemental Information). This analysis successfully confirmed the perceived age association (also after adjusting for age, sex, and wrinkles) of SNPs within or close to *MC1R* (e.g., rs1805007(T), $\beta = 0.80$, $p = 0.046$) but no other loci (Table 1). One of the *MC1R* variants (chr16:89913406:D) became genome-wide significant ($p = 3.85 \times 10^{-8}$) when combining the test statistics from both cohorts using a meta-analysis (Table 1). The *MC1R* compound marker in the Leiden Longevity

Table 1. SNPs Associated with Perceived Facial Age from a GWAS in the Discovery Cohort, Their Association in the First Replication Cohort, and in a Meta-analysis

					Discovery Cohort (n = 2,693)				First Replication Cohort (n = 599)				Meta-analysis (n = 3,292)		
Gene	CHR	MBP	SNP	EA	EAF	β	SE	p Value	EAF	β	SE	p Value	β	SE	p Value
CALN1	7	71.4	rs10259553	C	0.26	−0.64	0.13	9.36E−07	0.25	−0.06	0.22	0.796	−0.49	0.11	1.18E−05
CORO2A	9	100.9	rs35480968	G	0.33	−0.61	0.12	3.87E−07	0.33	0.09	0.22	0.668	−0.44	0.10	2.24E−05
MC1R	16	89.8	rs34265416	A	0.09	0.98	0.19	5.11E−07	0.10	0.43	0.35	0.214	0.85	0.17	5.52E−07
MC1R	16	89.8	rs4785704	G	0.10	1.00	0.19	2.64E−07	0.10	0.46	0.36	0.200	0.88	0.17	2.55E−07
MC1R	16	89.8	rs34714188	A	0.07	1.10	0.22	5.10E−07	0.08	0.63	0.38	0.098	0.98	0.19	2.02E−07
MC1R	16	89.8	rs12924124	T	0.07	1.10	0.22	5.10E−07	0.07	0.66	0.38	0.084	0.99	0.19	1.66E−07
MC1R	16	89.8	rs35026726	T	0.07	1.10	0.22	5.10E−07	0.07	0.66	0.38	0.084	0.99	0.19	1.66E−07
MC1R	16	89.8	rs12931267	G	0.07	1.09	0.22	5.74E−07	0.07	0.66	0.38	0.084	0.98	0.19	1.96E−07
MC1R	16	89.8	rs75570604	C	0.07	1.11	0.22	3.46E−07	0.07	0.68	0.39	0.079	1.01	0.19	1.05E−07
MC1R	16	89.9	MERGED_ DEL_2_86235	D	0.07	1.14	0.22	1.92E−07	0.07	0.69	0.39	0.077	1.03	0.19	5.82E−08
MC1R	16	89.9	16:89913406:D	D	0.07	1.15	0.23	3.78E−07	0.06	0.96	0.46	0.036	1.11	0.20	3.85E−08
MC1R	16	90.0	Compound	R	0.26	0.93	0.13	2.69E−12	0.28	0.61	0.30	0.042	0.88	0.12	1.68E−13
MC1R	16	90.0	rs1805007	T	0.07	1.09	0.22	9.23E−07	0.07	0.80	0.40	0.046	1.02	0.19	1.33E−07
MC1R	16	90.1	rs112556696	G	0.06	1.18	0.24	9.49E−07	0.05	0.55	0.56	0.321	1.08	0.22	9.14E−07

The Rotterdam Study was used as discovery cohort and the Leiden Longevity Study as first replication cohort. All SNPs with perceived age association $p < 1 \times 10^{-6}$ in the Rotterdam Study GWAS are shown. CHR, chromosome; MBP, mega base pair position of the SNPs according to GRCh37.p13; EA, effect allele; EAF, effect allele frequency; β, increase in perceived age per increase in effect allele; SE, standard error of the β; Compound, a collapsed compound heterozygosity marker based on a haplotype analysis of four pre-selected *MC1R*-coding DNA variants rs1805005 (V60L), rs1805007 (R151C), rs1805008 (R160W), and rs1805009 (D294H). All analyses were adjusted for age, sex, and wrinkling. See also Figures 2 and S2 as well as Tables S1 and S2.

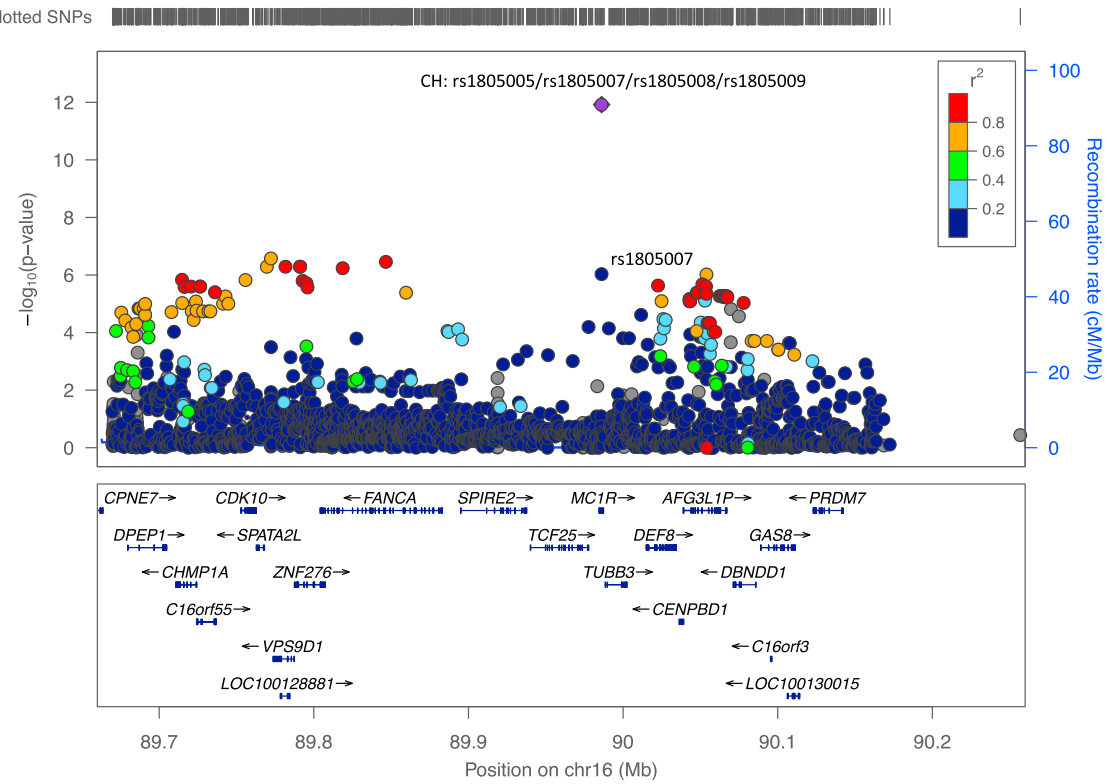

Figure 2. Regional Manhattan Plot of the *MC1R* Gene Locus with Perceived Facial Age in the Rotterdam Study Discovery Cohort

The physical positions of the SNPs used in the GWAS (using hg19) are plotted against the $-\log_{10}$ p values (left-hand axis) for their association with perceived age after adjustment for age, sex, and wrinkling in the Rotterdam Study (n = 2,693). The genomic region from 89.66 to 90.26 Mb on chromosome 16 is displayed along the x-axis. The association signal for the *MC1R* compound marker was superimposed onto the plot using the same physical position as rs1805007. Linkage disequilibrium (LD) r^2 values between all SNPs and rs1805007 are scaled by redness, and known genes are aligned below. See Figure S2 for genome-wide Manhattan and Q-Q plots and for the perceived age effect of the *MC1R* compound marker in the Rotterdam Study discovery cohort.

Study (Table 2) also replicated with nominal significance in this sample (p = 0.042; Table 1) and demonstrated a genome-wide significant association with perceived age in the combined analysis ($p = 1.69 \times 10^{-13}$).

To further confirm that the *MC1R* compound marker association with perceived age in the Rotterdam Study was genuine and the replication in the Leiden Longevity Study was not a false-positive finding, e.g., due to multiple testing, we performed a second replication analysis of the *MC1R* compound marker in 1,173 European subjects (99% female) of the TwinsUK Study [8]. Although the two rarest of the four *MC1R* SNPs (rs1805005 and rs1805009) were unavailable in the TwinsUK dataset used (Table 2; Supplemental Information), the *MC1R* compound marker constructed from the two available and more common SNPs (rs1805007 and rs1805008) demonstrated statistically significant association with perceived age after adjusting for age, sex, and wrinkles ($p = 3.6 \times 10^{-3}$). Moreover, the effect size seen in the TwinsUK Study ($\beta = 0.60$ per risk haplotype) was almost identical to that found in the Leiden Longevity Study ($\beta = 0.61$).

Testing the Genetic Effects of Additional Sub-phenotypes of Perceived Age

MC1R SNPs have previously been associated with variation in skin color [9, 10] and pigmented spots [6]. In a skin color stratified analysis, the *MC1R* compound marker association with perceived age persisted though different skin color groups with weakening effect sizes ($\beta = 0.95$ in pale, $\beta = 0.81$ in white, $\beta = 0.80$ in white to olive; Table S4). Furthermore, a candidate gene analysis of eight SNPs from eight pigmentation genes selected from a recent skin color GWAS [10] revealed nominally significant association ($p < 0.05$) with perceived age in the Rotterdam Study for SNPs in four genes, i.e., *IRF4*, *RALY/ASIP*, *SLC45A2*, and *TYR*, in addition to the *MC1R* compound marker (Table S5). The significance levels all remained when skin color was additionally adjusted for (Table S5), and *TYR* rs1393350 remained nominally significant (p = 0.04) after Bonferroni correction.

A multivariable regression analysis of perceived age was performed to test the independent effects of genetic factors and sub-phenotypes on perceived age (Table S6). In this analysis, the *MC1R* compound marker association with perceived age remained genome-wide significant, and *TYR* rs1393350 ($p = 6.8 \times 10^{-3}$) and *SLC45A2* rs183671 (p = 0.02) showed nominally significant association with perceived age (Table S6). Including sunbed usage as a covariate in the multivariable analysis had little impact on the effect of *MC1R* in the model (β remained the same at 0.74, and p value slightly changed from 2.1×10^{-8} to 2.3×10^{-8}), as also shown in a sunbed-use stratified analysis,

Table 2. Frequencies of the *MC1R* Compound Marker Haplotypes and Their Associated Mean Perceived Facial Ages in the Discovery Cohort, the First Replication Cohort, and the Second Replication Cohort

	Discovery Cohort (n = 2,693)				First Replication Cohort (n = 599)				Second Replication Cohort (n = 1,173)			
MC1R Haplotype	n	%	Perceived Age*	SE	n	%	Perceived Age*	SE	n	%	Perceived Age*	SE
WT/WT	1,426	52.95	65.29	0.08	317	52.92	62.99	0.01	674	65.76	59.54	0.10
WT/R	1,119	41.55	66.16	0.09	240	40.06	63.41	0.01	310	30.24	60.01	0.15
R/R	148	5.5	67.10	0.25	42	7.01	63.99	0.09	41	4.00	61.07	0.43

The Rotterdam Study was used as discovery cohort, the Leiden Longevity Study as first replication cohort, and the TwinsUK Study as second replication cohort. The *MC1R* compound marker haplotypes were constructed from four pre-selected *MC1R*-coding DNA variants rs1805005 (V60L), rs1805007 (R151C), rs1805008 (R160W), and rs1805009 (D294H), except in the second replication cohort TwinsUK Study where only rs1805007 and rs1805008 were available (see Supplemental Information). Asterisk (*) indicates mean perceived age in years after adjusting for age, sex, and wrinkles. SE, standard error of the perceived age estimate in years; R, risk haplotype; WT, wild-type haplotype. See also Figure S2 and Tables S4–S6.

where the *MC1R* effect was slightly attenuated in frequent sunbed users (Table S4). Adjusting for sun exposure in the Leiden Longevity Study (i.e., mainly, often, or rarely in the sun in the summer) had little effect on the *MC1R* association (β changed from 0.61 to 0.66, and p value decreased from 0.042 to 0.031), and in the stratified analysis, *MC1R* SNPs also showed an attenuated effect in the high exposure group (Table S4).

DISCUSSION

There have been no studies to date investigating the genetic basis of perceived age, despite its links to health (e.g., [1]) and the evidence of a large additive genetic component to perceived age variation [11]. In the present study, we detected in Dutch Europeans a significant association between DNA variants in the *MC1R* gene and perceived age, after removing the influence of age, sex, and wrinkles, which successfully replicated in two independent European samples from the Netherlands and the UK. The observed *MC1R* perceived age associations were independent of skin color and pigmented spots, indicating other facial features were responsible for the associations. In addition, we found little evidence that sun exposure was the main route through which *MC1R* gene variants were associating with perceived age.

The *MC1R* gene encodes the melanocortin 1 receptor, which is a key regulator of melanogenesis, and controls the ratio of pheomelanin to eumelanin synthesis. A diminished MC1R activity, as caused by multiple loss-of-function polymorphisms in *MC1R*, produces the yellow to reddish pheomelanin, which has a weaker UV shielding capacity than that of the brown to black eumelanin [12]. However, multiple studies have shown loss-of-function *MC1R* variants significantly associate with age spots, actinic keratosis, and various types of skin cancers in a skin-color-independent and/or UV-exposure-independent manner [6, 13–18], and in the present study, we showed that *MC1R* variants associated with perceived age after skin color and sun exposure adjustments. These observations are in line with previous findings from functional studies suggesting a pleotropic role for *MC1R* in inflammation [19] and nucleotide excision repair [20], as well as in fibroblasts during wound healing and tissue repair [21], and are consistent with the previously demonstrated UV-independent carcinogenesis mechanism of *MC1R* via oxidative damage [22].

Small-scale GWASs on photoaging [23] and a skin age score [24] have been performed previously; these two studies each identified different genes, and none were *MC1R*. A direct comparison with the present study is difficult, as both previous studies used very different skin aging phenotypes compared to perceived age used here as well as smaller sample sizes (<503 subjects). The *MC1R* association with perceived age we found here replicated in two independent cohorts, and these DNA variants have been significantly associated with other skin-aging-related phenotypes in recent studies (e.g., pigmented spots [6]) also independently of skin color, which together provide confidence that our findings are non-spurious. In addition, a previous candidate gene study in 530 middle-aged French women reported associations between variants in *MC1R* and severe facial photoaging [25]. However, a key feature of the photoaging measure was facial wrinkles, whereas we found that the *MC1R* variants mainly explained the non-wrinkling components of perceived age. Our data therefore highlight that further studies are needed to identify the specific cellular pathway (e.g., DNA repair) and facial feature (e.g., skin sag) responsible for the link between *MC1R* variants and facial aging.

The discovery set of this study uses a relatively small sample size compared to current GWAS standards, which minimized the statistical power to detect genetic effects smaller in size than the observed *MC1R* compound marker effect of almost 2 years. The GWAS quantile-quantile (Q-Q) plot (Figure S2B) indeed shows many SNPs with a lower p value than expected, albeit to only a small degree. This is in line with many SNPs having small effects on perceived age, which is not surprising given the wide variety of facial features that influence age perception, i.e., it is a very complex phenotype. Much larger sample sizes are now required to reveal additional gene variant effects on perceived age as well as their effects in younger and non-European populations.

Appearance and age prediction from DNA with the aim to find unknown perpetrators, who in principle cannot be identified via conventional DNA profiling, has gained enormous interest in the forensic genetics field over the last few years [26, 27]. Given that the *MC1R* compound marker explained only a small proportion of the perceived age variation, a more complete list of genetic loci involved in perceived age is required to accurately predict perceived age (given chronological age is available or can be reliably estimated from molecular biomarkers thereof), such as in forensic applications. In support of this, we found SNPs in several other skin color genes associated in the expected direction with perceived age in a multivariable model.

Finally, as *MC1R* correlates with advanced facial aging, it provides clues to mechanisms of biological aging beyond cosmetic and forensic interests. Indeed, it is notable that the 2-year effect

of the *MC1R* DNA variants on perceived age observed here is similar to the effect of smoking reported previously in the Leiden Longevity Study [28], indicating that *MC1R* variants can have a considerable impact on facial appearance over many years.

In conclusion, this study is the first to identify genetic variants significantly associated with perceived age. We provide evidence that, of eight million tested, DNA variants in the *MC1R* gene had the strongest association with perceived age in subjects of European ancestry, and a *MC1R* compound marker was genome-wide significant independently of age, sex, skin color, sun exposure, wrinkles, and pigmented spots. Follow-up work on how the MC1R protein is affecting facial aging, for example, through non-UV pro-oxidant phenomelanin effects [22] or fibroblast function [21], is now required. Moreover, as this study demonstrates that a GWAS of perceived facial age is indeed feasible, future studies using large consortia GWASs should be performed to identify additional genetic loci that associate with perceived facial age. Expectedly, this will provide further insights into the biological pathways that underlie variation in facial aging and eventually on the utility of genotype-based prediction of perceived age alongside chronological age estimation from molecular biomarkers.

EXPERIMENTAL PROCEDURES

Each study was approved by the research ethics committees of the contributing institutions, and all participants provided written informed consent.

Rotterdam Study

The Rotterdam Study is a prospective cohort study ongoing since 1990 in the city of Rotterdam in the Netherlands [3]. Perceived age, i.e., how old the subjects looked, was assessed from front and side facial images from the 3dMD system by on average 27 assessors per image (totaling ~73,000 assessments) using a previously used [28] and validated method ([29] and Supplemental Information). Pigmented spots and wrinkles were measured quantitatively from the frontal images using image analysis algorithms (MATLAB 2013b) as previously described and validated ([30] and Supplemental Information). Sunbed use (i.e., never, <10 times, 10–50 times, >50 times) was assessed through questionnaires. Skin color was graded as pale, white, or white to olive skinned based on a full body examination while subjects were in a state of undress [31]. To merge photographs together for comparisons of facial appearance, facial images were combined together as previously detailed [11, 32] using face shape, color, and texture information. Genotyping, imputation, and quality control procedures are described in detail elsewhere ([3] and Supplemental Information).

Leiden Longevity Study

The Leiden Longevity Study has been described in detail elsewhere [7, 33, 34]. Perceived age was assessed from front and side facial images by on average 60 assessors (totaling ~36,000 assessments) and wrinkles graded into nine photo-numeric grades, both as previously reported [35]. Summer sun exposure (mainly in the sun, often in the sun, and rarely in the sun) was captured through questionnaires [28]. Genotyping was performed using Illumina Human660W-Quad and OmniExpress BeadChips as described elsewhere [34]. Association testing was conducted using QTassoc [36].

TwinsUK Study

The UK Adult Twin Registry (TwinsUK Study) is described elsewhere [8]. Perceived age was graded from 3dMD photos by four assessors per image, and wrinkles were graded according to the above described photo-numeric grading by five assessors (Supplemental Information). *MC1R* SNP data from TwinsUK were ascertained from the imputed genome-wide SNP dataset described elsewhere [8].

Statistical Analyses

Genetic association was tested per SNP in the GWAS using a linear model assuming an additive allele effect, always including sex, chronological age, and the top four genetic principal components as covariates using PLINK [37]. Wrinkles, skin color, sunbed use, and summer sun exposure were adjusted for where appropriate. The *MC1R* compound marker analysis in each of the three cohorts is detailed in Supplemental Information. We conducted a stepwise multivariable regression analysis to investigate the independent effects of all phenotypes and factors as performed using R version 3.2.0 (http://www.r-project.org/); see Supplemental Information for further details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.03.008.

AUTHOR CONTRIBUTIONS

M.A.H. and J.D. contributed equally to this study. D.A.G., M.K., P.E.S., and T.N. initiated the study and together with F.L., M.A.H., J.S.L., L.J., D.v.H., P.G.M., M.B., and A.G.U. were involved in data collection. F.L., M.A.H., J.D., and D.A.G. mainly carried out the data analyses and results interpretation, supported by C.Z., A.W., and L.M.P. D.A.G., M.K., P.E.S., T.N., and A.H. provided crucial resources. F.L., M.K., and D.A.G. wrote most parts of the manuscript. All authors approved the final manuscript.

CONFLICTS OF INTEREST

D.A.G., J.S.L., and P.G.M. are Unilever employees. Although no products were tested, this work could potentially promote the use of anti-aging products and lead to financial gain for Unilever.

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Supplemental Information

The *MC1R* Gene and Youthful Looks

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Supplemental Figures

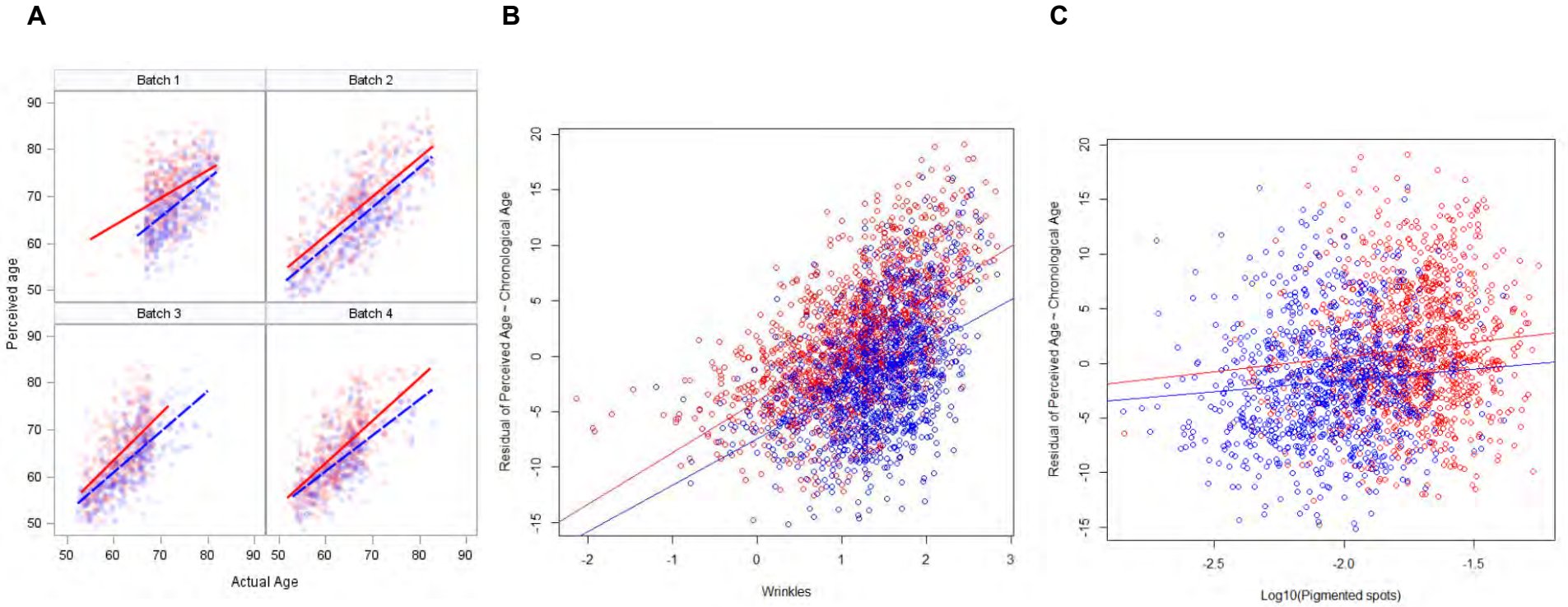

Figure S1: Correlations of perceived age with age and age-related phenotypes (wrinkles and pigmented spots) in the Rotterdam Study discovery cohort (n=2,693). A) Correlation of perceived age versus chronological age for males and females in the Rotterdam Study across the 4 batches of age estimations. On average, males (blue points) looked 1.49 years younger than their actual age whereas women (red points) looked 1.53 years older. Differences between the different batches were observed (i.e. technical variation), possibly in part due to the fact the different Rotterdam Study cohorts were of different ages. Hence, batch ID was adjusted for in all analyses. **B**) Wrinkles are strongly correlated with the residuals of regressing perceived age on chronological age in women (red, *r*=0.59) and men (blue, *r*=0.46). **C**) Pigmented spots are weakly correlated with the residuals of regressing perceived age on chronological age in women (red, *r*=0.10) and men (blue, *r*=0.09). Related to Figure 1.

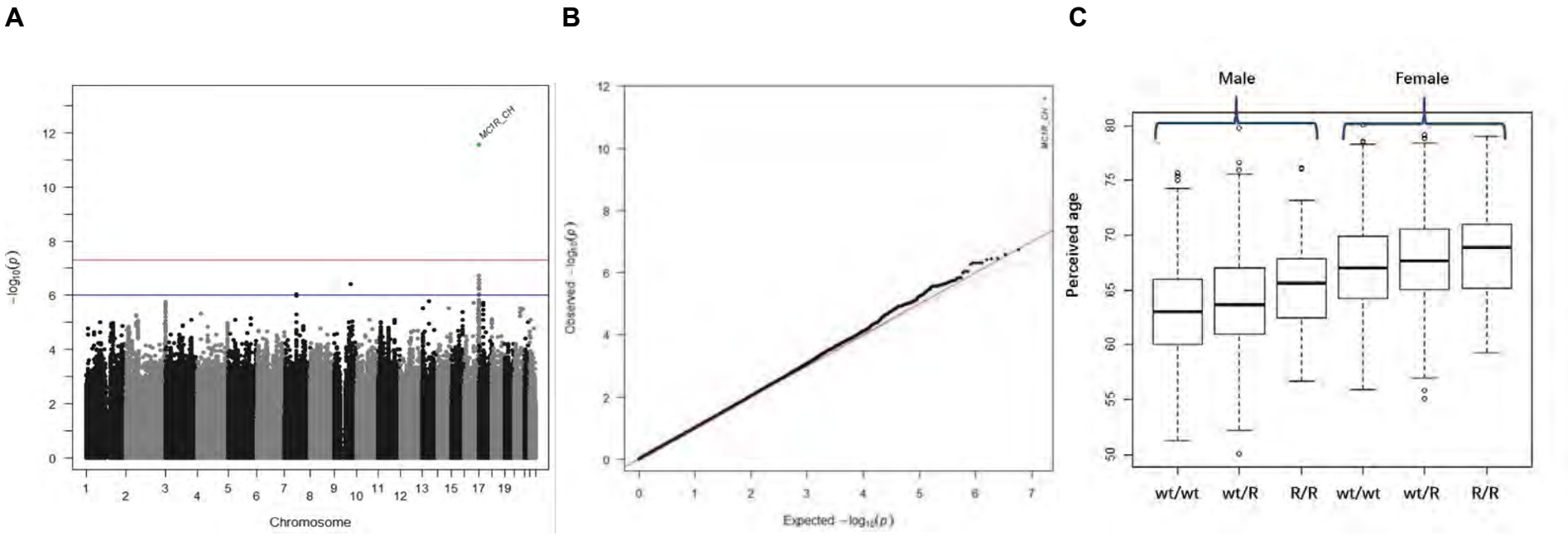

Figure S2: SNP association with perceived age after adjustment for age, sex and wrinkles as viewed by a Manhattan plot (A) and a QQ plot (B), and perceived age effect of the *MC1R* compound marker after adjustment for age, sex and wrinkles as viewed by a box plot (C) in the Rotterdam Study discovery cohort (n=2,693). Suggestive and genome-wide significance levels are delineated in (**A**) by the blue and red lines, and in **(B)** the expected p-values from the null distribution are indicated by a red line. In (**A**) and (**B**) each point represents a SNP, except for the *MC1R* compound heterozygosity marker (*MC1R*_CH) genotype, depicted as green point, which is superimposed onto the original plots and labelled. **C**) Perceived age estimates are depicted for males and females separately and for the three *MC1R* compound haplotypes separately. The black center line of the box is the median, and the inter-quartile range (25%-75%) is the box area. The values within 1.5 times from the upper or lower quartile are covered by the dashed lines extending from the box, values at a greater distance are plotted as individual points. Mean perceived age values were 63.11 years for male homozygote wild-type carriers (wt/wt), 64.03 years for male heterozygotes (wt/R), 65.34 years for male homozygotes risk allele carriers (R/R), 67.1 years for female homozygote wild-type carriers (wt/wt), 67.89 years for female heterozygotes (wt/R), and 68.59 years for female homozygote risk allele carriers (R/R). For results from the combined male-female analysis, see Table 2. Related to Figure 2, Table 1, Table 2.

Supplemental Tables

Table S1: Characteristics of the samples used for discovery (Rotterdam Study), 1st replication (Leiden Longevity Study), 2nd replication (TwinsUK Study). Related to Table 1.

Characteristics	Rotterdam Study (n=2,693)		Leiden Longevity Study (n=599)		TwinsUK Study (n=1,173)	
Females (n, %)	1449	53.81	351	58.60	1171	99.83
Age (mean, SD)	65.75	6.97	63.23	6.58	59.73	9.44
Perceived age (mean, SD)	65.77	7.49	59.67	7.66	60.74	8.59
Difference between age and perceived age (mean, SD)	0.02	5.70	-3.56	5.26	1.01	5.05
Wrinkles (mean, SD)	1.28	0.64	4.64	1.27	4.44	1.33
Pigmented Spots percent (mean, SD)*	1.01	0.80				
Skin color (n, %)						
1, Pale white	290	10.77				
2, White	2037	75.64				
3, White to olive	366	13.59				
Sunbed use in 5 years (n, %)*						
1, Never	1559	79.02				
2, < 10 times	166	8.41				
3, 10 - 50 times	181	9.17				
4, ≥ 50 times	67	3.4				
Sun exposure (n, %)						
1, Rarely in sun			78	13.18		
2, Often in sun			378	63.85		
3, Mostly in sun			136	22.97		

n – number of individuals; SD – standard deviation. *Not all Rotterdam Study subjects had scores available.

For Table S2, see separate Excel file

For Table S3, see separate Excel file

Table S4: Genetic association of the *MC1R* compound marker with perceived age within the skin color (A) and sunbed use (B) categories of the Rotterdam Study (n=2,693), and the summer sun-exposure categories (C) in the Leiden Longevity Study (n=599). Related to Table 2.

	EAF	β	SE	p-value
A, skin color category				
Pale (n=209)	0.37	0.95	0.35	0.007
White (n=2,037)	0.26	0.81	0.16	2.32E-07
White to Olive (n=366)	0.19	0.80	0.39	0.043
B, sun bed use in last 5 years				
Never (n=2,187)	0.26	0.91	0.15	7.05E-10
< 10 times (n=203)	0.29	1.38	0.48	0.005
10-50 times (n=222)	0.27	0.83	0.52	0.109
≥ 50 times (81)	0.23	0.76	0.77	0.327
C, summer sun exposure				
Rarely in sun (n=78)		1.79	0.66	0.008
Often in sun (n=378)		0.55	0.35	0.116
Mostly in sun (n=143)		0.21	0.58	0.710

EAF, frequency of the *MC1R* compound marker risk haplotype; Beta (β) is the increase in perceived age per increase in *MC1R* risk haplotype; SE, standard error of the beta.

Table S5: Association of skin color SNPs with perceived age in the Rotterdam Study. Related to Table 2.

Gene	CHR	SNP	EA	EAF	β	A) p-value	B) p-value
SLC45A2	5	rs183671	T	0.02	-0.97	1.28E-02	5.91E-02
IRF4	6	rs12203592	T	0.09	0.47	1.73E-02	3.47E-02
BNC2	9	rs10756819	G	0.32	-0.14	2.51E-01	3.01E-01
TYR	11	rs1393350	A	0.23	0.43	1.33E-03	4.64E-03*
SLC24A4	14	rs17128291	G	0.16	0.12	4.28E-01	4.96E-01
HERC2	15	rs12913832	A	0.23	-0.26	6.60E-02	3.57E-01
SLC24A5	15	rs2924567	T	0.37	0.04	7.43E-01	7.27E-01
MC1R	16	Compound	R	0.26	0.93	2.69E-12	1.27E-09*
RALY/ASIP	20	rs6059655	A	0.07	0.55	1.07E-02	4.48E-02

Except the *MC1R* compound marker, all skin color SNPs are from Table 1 of Liu et al. (2015) *Hum. Genet.* 134:823–835; CHR, chromosome; EA, effect allele, or minor allele; EAF, effect allele frequency; Beta (β) is the increase in perceived age per increase in effect allele; P-values were adjusted for age, sex and wrinkles (A) and additionally for skin color (B); Compound, a collapsed compound *MC1R* marker based on a haplotype analysis of 4 pre-selected *MC1R* coding variants rs1805005 (V60L), rs1805007 (R151C), rs1805008 (R160W), and rs1805009 (D294H); * p-value < 0.05 after Bonferroni correction of 9 tests.

Table S6: Multivariable regression of phenotypic and genetic factors explaining perceived age variation in the Rotterdam Study. Related to Table 2.

Factor	CHR	Gene	EA	EAF	β	SE	p-value	R^2 (%)
Female					2.92	0.19	3.16E-49	6.90
Age (years)					0.57	0.01	1.96E-265	18.52
Wrinkles (% of facial area)					6.18	0.14	1.33E-319	41.29
Skin Color (light to dark levels)					-0.64	0.17	1.59E-04	0.21
Pigmented Spots (% of facial area)					3.23	0.38	3.26E-17	1.52
rs183671	5	*SLC45A2*	T	0.02	-0.88	0.38	0.020	0.05
rs12203592	6	*IRF4*	T	0.09	0.17	0.19	0.391	0.01
rs10756819	9	*BNC2*	G	0.32	-0.09	0.12	0.438	0.01
rs1393350	11	*TYR*	A	0.23	0.35	0.13	6.87E-03	0.07
rs17128291	14	*SLC24A4*	G	0.16	0.12	0.15	0.435	0.01
rs12913832	15	*HERC2*	A	0.23	-0.18	0.14	0.198	0.02
rs2924567	15	*SLC24A5*	T	0.37	0.05	0.12	0.692	0.00
Compound	16	*MC1R*	R	0.26	0.74	0.13	2.10E-08	0.38
rs6059655	20	*RALY/ASIP*	A	0.07	0.29	0.21	0.167	0.02

CHR, chromosome; EA, the effect allele; EAF, effect allele frequency; Beta (β) is the increase in perceived age per increase in effect allele; SE, standard error of the beta; Compound, a collapsed compound heterozygosity marker based on a haplotype analysis of 4 pre-selected *MC1R* coding DNA variants rs1805005 (V60L), rs1805007 (R151C), rs1805008 (R160W), and rs1805009 (D294H).

Supplemental Experimental Procedure

Phenotype analyses

For perceived age, we employed a previously validated methodology [S1] which has been used in numerous studies (e.g. [S2]). In brief, standardized full-face photographs were obtained of participants not wearing make-up, face cream nor jewelry, in a room without daylight; make-up was evident for some subjects in Twins UK. Facial images for the RS and Twins UK were created using the Premier 3dMD face3-plus UHD camera (3dMD, Atlanta, Georgia, USA), whereas they were created from a Fuji digital camera in the Leiden Longevity study [S1]. All 3D images from the 3dMD system comprised separate shape (mesh grid) and surface information (bitmap data) and were aligned into the same pose. For the current analysis, we considered 2D projections from the front and side view that were generated using in house made blender scripts (V2.70 https://www.blender.org/).

For the Rotterdam Study and the Leiden Longevity Study, frontal and side facial images (left side of subject's face at an approximately 45° angle to the camera) excluding scalp hair or clothing cues were presented to assessors (Unilever employees of various ages and sex, but predominately British), each having a different randomized presentation order to minimize bias from preceding images. Assessors were unaware of the subjects' ages and age ranges. Assessors selected a five-year age range they thought the subject looked. Correlations between individual assessor perceived ages in the RS (mean r=0.65) were similar to that reported in previous studies (e.g. r=0.67 [S1] [1]) including that reported for the Leiden Longevity study [S3]. For the Rotterdam Study, perceived ages were generated from 4 different 'batches' of assessments over a two-year period (**Figure S1A**); subtle differences in perceived ages between the 4 batches were found and therefore used as a covariate in all

statistical models. A linear mixed model with subject and assessor as random effects and order of assessment as a fixed effect was used to generate the mean perceived ages.

Four assessors graded perceived age for 1,550 Twins UK subjects (two images per subject - from slightly below and of the left and right (approximately 30° angle to the camera angle) sides of the subjects' faces. Prior to the assessments, a subset of 50 subjects, randomly selected from the subjects without genetic data, was used as training set, for whom the assessors were aware of the subjects' ages for calibration purpose. After training, the four assessors independently scored the perceived ages for the remaining subjects, and were unaware of the subjects' ages. The average perceived age from the four assessors was used as the phenotype in subsequent analyses. Since perceived age in TwinsUK Study was assessed by much fewer age assessors than for the Rotterdam Study (27 per subject) and the Leiden Longevity Study (60 per subject), we calculated twin correlations for the difference between perceived facial age and chronological age within 362 pairs of monozygotic twins and 413 pairs of dizygotic twins. A much higher correlation was obtained in monozygotic twin pairs (r=0.59) than in dizygotic twin pairs (r=0.25), which indirectly supports the reproducibility of this data. In addition, high correlations between perceived and chronological ages were obtained for each of the assessors (Pearson correlation r from the four accessors: 0.76, 0.76, 0.81, and 0.82) as well as between assessors (average r=0.77, min r= 0.72, max r=0.82). Note that a larger number of subjects were available for twin correlation analysis (n=1,550) than for genetic association analysis (N=1,173) because not all subjects with facial image had genetic data. The vast majority of the Twins UK cohort is female (99%), and excluding the male subjects (N=2) had little influence on the association results. The TwinsUK Study participants (mean age=59.7 years, SD=9.4) were on average 6 years and 3 years younger than the Rotterdam Study and the Leiden Longevity Study participants, respectively (**Table S1**).

The wrinkle and pigmented spot area measures in the Rotterdam Study consisted of the cumulative number of pixels detected as wrinkles or pigmented spots (shaded lines on the face for wrinkles, round spots with a high blue to red and green contrast for pigmented spots) as a percentage of total facial skin area; described in more detail recently [S4]. Due to flash light variation over time, the difference between skin lightness in the images and that taken by a spectrophotometer (CM-600d; Konica-Minolta, Osaka, Japan) in-person was used as a confounding covariate in any statistical analyses with wrinkle area, along with a covariate used for variations in mask size. Skin wrinkle grading of the facial images in the Leiden Longevity Study and TwinsUK were carried out as previously reported [S5] using a 1–9 photonumeric scale and graded by the number and depth of facial wrinkles.

Statistical analyses

The GWAS analysis of perceived age included a total of 2,693 Dutch Europeans from the Rotterdam Study after genotype and phenotype quality controls. We considered p-values equal or smaller than 5×10^{-8} as genome-wide significance. The inflation factor lambda was close to 1.0 (λ=1.01) and not further considered. All SNPs with $<1\times10^{-5}$ were selected for replication in the Leiden Longevity Study cohort, and the *MC1R* compound marker was further selected for replication in the Twins UK cohort and $p<0.05$ considered as successful replication. For multivariate modelling, all factors were iteratively added into the regression model according to their significance level, the R^2 difference between the current and previous model was then the percentage of perceived age variance explainable by the added factor. For TwinsUK, association testing in related subjects was conducted using MERLIN [S6] adjusted for family relationship, further randomly excluding one sib from any pair of monozygotic twins (119 subjects excluded) had little influence on the *MC1R* results.

Genotyping and imputation

Quality control included the removal of SNPs with Hardy-Weinberg equilibrium deviations ($p<10^{-6}$), genotyping call rate < 97%, sex mismatch and a high mean autosomal heterozygosity. For the Rotterdam Study, duplicates were identified and excluded based on identity-by-descent estimates and outliers (three standard deviations away from the population mean) using multi-dimensional scaling analysis with four genetic principal components. Genome-wide genotype data was imputed using 1000-Genomes (GIANT Phase I version 3) as the reference panel [S7], using a two-step procedure imputation algorithm implemented in the program MACH-Minimac with default parameters [S8].

The *MC1R* collapsed compound heterozygosity marker

The *MC1R* gene (melanocortin 1 receptor; 16q24.3) has been a subject of intensive research ever since its variants were first associated with human red hair and fair skin in the mid-1990s [S9]. *MC1R* DNA variants are known to be involved in human red hair and related phenotypes in a compound heterozygote manner [S10]. Compound heterozygosity in classical genetics is the presence of two different recessive mutations at a particular gene locus, one on each homologue chromosome [S11]. To construct an *MC1R* collapsed compound heterozygosity marker (*MC1R* compound marker), we *a priori* selected 4 *MC1R* DNA variants rs1805005 (V60L), rs1805007 (R151C), rs1805008 (R160W), and rs1805009 (D294H) because they i) are missense loss-of-function variants, ii) cause human phenotypes such as red-hair and related ones in a compound heterozygote manner, and iii) are involved in age-related phenotypes such as pigmented spots. Furthermore, they passed all genetic quality controls in the Rotterdam study with sufficient minor allele frequency (>1%). These 4 missense *MC1R* DNA variants were collapsed into 3 possible genotypes wt/wt/, wt/R, and R/R, where R is the risk haplotype consisting of at least one risk allele from any of the 4

MC1R variants and the wt is the wild-type haplotype consisting of none of the risk alleles. The haplotype configuration between these 4 variants was resolved using R package haplo.stats [S12] with high confidence (posterior probability > 0.99 for all inferred haplotypes). We then treated this *MC1R* compound marker as a discrete marker in the GWAS. Note that in the selection of DNA variants for the compound marker construction we did not try multiple combinations between different SNPs variants; neither did we try multiple ways of genotype collapsing, both providing arguments that multiple test correction is not necessary here. In TwinsUK, the *MC1R* compound marker was constructed using rs1805007 and rs1805008 because rs1805005 and rs1805009 did not pass imputation quality control in the available dataset. However, the two available SNPs were more important than the missing ones as reflected by their effect allele frequencies in TwinsUK (**Table 2**) and other European populations (HapMap CEU rs1805007 T = 0.12, HapMap CEU rs1805008 T = 0.12, HapMap CEU rs1805005 T = 0.07, AF EUR rs1805009 C = 0.008).

Wrinkles statistical analysis and GWAS results

The phenotype wrinkle area showed a highly right-skewed distribution. Therefore, we ln-transformed the phenotype, resulting in an approximately normal distribution of both the phenotype and the regression residuals. All wrinkle analyses in the RS were adjusted for age, sex, mask size variability, variance between participants in flashlight illumination of the skin (represented by the residuals of the lightness of the photograph regressed on the lightness of the facial skin), and the first four genetic principal components. Association with autosomal SNPs was tested using linear regression assuming an additive allele effect. All GWAS analyses were conducted using PLINK [S13]. A $p<5\times10^{-8}$ were considered as genome-wide significant. A total of 84 SNPs with a $p<1\times10^{-5}$ from the GWAS in the RS were selected for

replication analysis in the Leiden Longevity Study. Note that a quantile normalization of wrinkles data (instead of using a ln-transform) had little influence on the GWAS results.

After genotype and phenotype quality controls, the GWAS of facial wrinkle area in the Rotterdam Study included a total of 2619 subjects from the group analyzed for perceived age. The GWAS adjusted for age, sex, mask size variability and variance in flashlight illumination showed suggestive, but no genome-wide significant hits (**Table S3**). The most significant hit was SNP rs34316087 with a $p=8.84\times10^{-8}$ (**Table S3**). Replication of the top 84 hits (p-value$<1\times10^{-5}$) showed no significant findings. Meta-analysis of the Rotterdam Study and Leiden Longevity Study data did not result in significant hits (data not shown).

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