The *MC1R* gene and youthful looks

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ABSTRACT

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 single-nucleotide polymorphisms (SNPs) in 2693 elderly northwestern Europeans from the Rotterdam Study. The strongest genetic associations with perceived facial age were found for multiple SNPs in the *MC1R* gene (P-value $<1\times10^{-7}$). This effect was enhanced for a compound heterozygosity marker constructed from four pre-selected functional *MC1R* SNPs (P-value=2.69×10^{-12}), which was replicated in 599 Dutch Europeans from the Leiden Longevity Study (P-value=0.042) and in 1173 Europeans of the TwinsUK Study (P-value=3×10^{-3}). Individuals carrying the homozygote *MC1R* risk haplotype looked on average up to two 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 2693 northwestern 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-value $<10^{-300}$). 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-value=9.5×10^{-138}) as well as in men (r^2 21%, P-value=3.1×10^{-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-value=0.001) and in men (r^2=0.8%, P-value=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 GWAS using 2693 samples from the Rotterdam Study, we searched for 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; Figure 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-value=2.69×10^{-12}, Table 1; Figure 2). On average, the homozygote *MC1R* risk haplotype carriers (R/R) looked almost two years older (1.81 years) than the non-carriers (WT/WT); the heterozygote carriers (R/WT) looked almost one year older (0.87 years) than the non-carriers (WT/WT) (Table 2), with a slightly larger effect size in men compared to women (Figure S2C).

Figure 1. Illustration of the effect of wrinkling and non-wrinkling components on perceived facial age. (**A**–**D**) Facial averages of northwestern 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.

Table 1. SNPs associated with perceived facial age from a GWAS in the discovery cohort (Rotterdam Study), their association in the first replication cohort (Leiden Longevity Study), and in a meta-analysis

					Discovery cohort (N=2693)				First replication cohort (N=599)				Meta-analysis (N=3292)		
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	9.4×10^{-7}	0.25	-0.06	0.22	0.796	-0.49	0.11	1.2×10^{-5}
CORO2A	9	100.9	rs35480968	G	0.33	-0.61	0.12	3.9×10^{-7}	0.33	0.09	0.22	0.668	-0.44	0.10	2.2×10^{-5}
MC1R	16	89.8	rs34265416	A	0.09	0.98	0.19	5.1×10^{-7}	0.10	0.43	0.35	0.214	0.85	0.17	5.5×10^{-7}
MC1R	16	89.8	rs4785704	G	0.10	1.00	0.19	2.6×10^{-7}	0.10	0.46	0.36	0.200	0.88	0.17	2.6×10^{-7}
MC1R	16	89.8	rs34714188	A	0.07	1.10	0.22	5.1×10^{-7}	0.08	0.63	0.38	0.098	0.98	0.19	2.0×10^{-7}
MC1R	16	89.8	rs12924124	T	0.07	1.10	0.22	5.1×10^{-7}	0.07	0.66	0.38	0.084	0.99	0.19	1.7×10^{-7}
MC1R	16	89.8	rs35026726	T	0.07	1.10	0.22	5.1×10^{-7}	0.07	0.66	0.38	0.084	0.99	0.19	1.7×10^{-7}
MC1R	16	89.8	rs12931267	G	0.07	1.09	0.22	5.7×10^{-7}	0.07	0.66	0.38	0.084	0.98	0.19	2.0×10^{-7}
MC1R	16	89.8	rs75570604	C	0.07	1.11	0.22	3.5×10^{-7}	0.07	0.68	0.39	0.079	1.01	0.19	1.1×10^{-7}
MC1R	16	89.9	MERGED_DEL_2_86235	D	0.07	1.14	0.22	1.9×10^{-7}	0.07	0.69	0.39	0.077	1.03	0.19	5.8×10^{-8}
MC1R	16	89.9	16:89913406:D	D	0.07	1.15	0.23	3.8×10^{-7}	0.06	0.96	0.46	0.036	1.11	0.20	3.9×10^{-8}
MC1R	16	90.0	Compound	R	0.26	0.93	0.13	2.7×10^{-12}	0.28	0.61	0.30	0.042	0.88	0.12	1.7×10^{-13}
MC1R	16	90.0	rs1805007	T	0.07	1.09	0.22	9.2×10^{-7}	0.07	0.80	0.40	0.046	1.02	0.19	1.3×10^{-7}
MC1R	16	90.1	rs112556696	G	0.06	1.18	0.24	9.5×10^{-7}	0.05	0.55	0.56	0.321	1.08	0.22	9.1×10^{-7}

Abbreviations: β (beta), increase in perceived age per increase in effect allele; CHR, chromosome; 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); EA, effect allele; EAF, effect allele frequency; MBP, mega base pair position of the SNPs according to GRCh37.p13; SE, standard error of the β.

All SNPs with perceived age association P-value $<1\times10^{-6}$ in the Rotterdam Study GWAS are shown.

All analyses were adjusted for age, sex, and wrinkles.

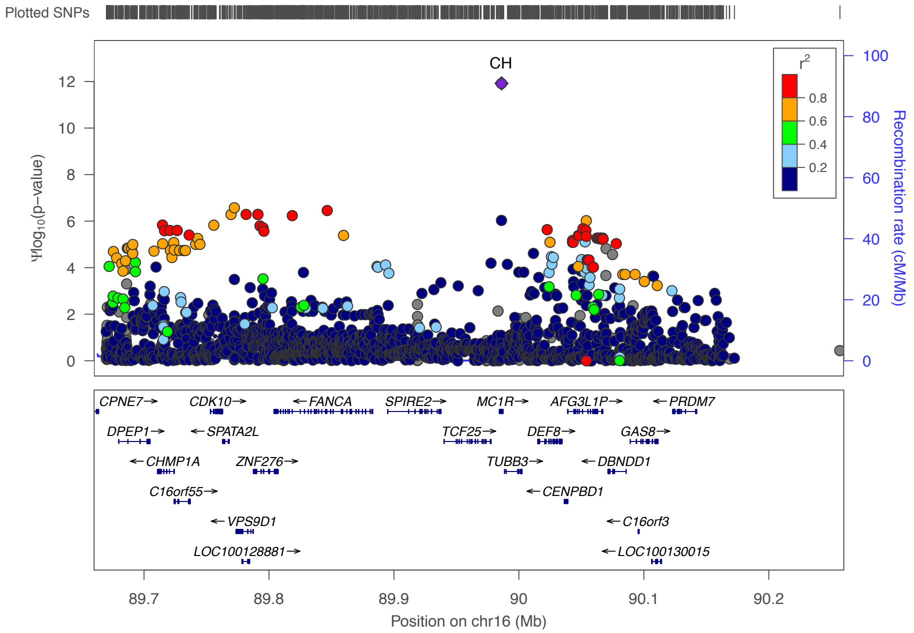

Figure 2. Regional Manhattan plot of the *MC1R* gene locus with perceived facial age in the Rotterdam Study discovery cohort. The physical positions of the SNPs used in the GWAS (using hg19) are plotted against the -$\log_{10}$P-values (left-hand axis) for their association with perceived age after adjustment for age, sex, and wrinkling in the Rotterdam Study (N=2693). 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.

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), β=0.80, P-value=0.046) but no other loci (Table 1). One of the *MC1R* variants (chr16:89913406:D) became genome-wide significant (P-value=3.85×10^{-8}) when combining the test statistics from both cohorts using a meta-analysis (Table 1). The *MC1R* compound marker in the Leiden Longevity Study (Table 2) also replicated with nominal significance in this sample (P-value=0.042, Table 1) and demonstrated a genome-wide significant association with perceived age in the combined analysis (P-value=1.68×10^{-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 1173 European subjects (99% female) of the TwinsUK Study[8].

Table 2. Frequencies of the *MC1R* compound marker haplotypes and their associated mean perceived facial ages in the discovery cohort (Rotterdam Study), the first replication cohort (Leiden Longevity Study), and the second replication cohort (TwinsUK Study)

	Discovery cohort (N=2693)				First replication cohort (N=599)				Second replication cohort (N=1173)			
***MC1R* haplotype**[a]	**N**	**%**	**Perceived age**[b]	**SE**	**N**	**%**	**Perceived age**[b]	**SE**	**N**	**%**	**Perceived age**[b]	**SE**
WT/WT	1426	52.95	65.29	0.08	317	52.92	62.99	0.01	674	65.76	59.54	0.10
WT/R	1119	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

Abbreviations: SE, standard error of the perceived age estimate in years; R, risk haplotype; WT, wild-type haplotype. [a]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 rs1805007and rs1805008 were available (see Supplemental Information); [b]mean perceived age in years after adjusting for age, sex, and wrinkles.

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-value=3.6×10^{-3}). Moreover, the effect size seen in the TwinsUK Study (β=0.60 per risk haplotype) was almost identical to that found in the Leiden Longevity Study (β =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 (β=0.95 in pale, β=0.81 in white, β=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-value<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-value=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-value=6.8×10^{-3}) and *SLC45A2* rs183671 (P-value=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, 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 from 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[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 that 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 and replicated in two independent cohorts, and these DNA variants having been significantly associated with other skin aging-related phenotypes in recent studies (e.g., pigmented spots[6]) also independently of skin color, 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 two years. The GWAS quantile-quantile (QQ) 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 giving 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 pheomelanin 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 whilst 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 photonumeric 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 photonumeric 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.

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