A genome-wide association study identifies the skin color genes *IRF4*, *MC1R*, *ASIP*, and *BNC2* influencing facial pigmented spots

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ABSTRACT

Facial pigmented spots are a common skin aging feature, but genetic predisposition has yet to be thoroughly investigated. We conducted a genome-wide association study for pigmented spots in 2844 northwestern Europeans from the Rotterdam Study (mean age: 66.9 ±8.0; 47% male). Using semi-automated image analysis of high-resolution digital facial photographs, facial pigmented spots were quantified as the percentage of affected skin area (mean women: 2.0% ±0.9, men: 0.9% ±0.6). We identified genome-wide significant association with pigmented spots at three genetic loci: *IRF4* (rs12203592, P-value 1.8×10^{-27}), *MC1R* (compound heterozygosity score, P-value 2.3×10^{-24}), and *RALY/ASIP* (rs6059655, P-value 1.9×10^{-9}). In addition, after adjustment for the other three top-associated loci, the *BNC2* locus demonstrated significant association (rs62543565, P-value 2.3×10^{-8}). The association signals observed at all four loci were successfully replicated (P-value<0.05) in an independent Dutch cohort (Leiden Longevity Study N=599). Although the four genes have previously been associated with skin color variation and skin cancer risk, all association signals remained highly significant (P-value<2×10^{-8}) when conditioning the association analyses on skin color. We conclude that genetic variations in *IRF4*, *MC1R*, *RALY/ASIP*, and *BNC2* contribute to the acquired amount of facial pigmented spots during aging, through pathways independent of the basal melanin production.

INTRODUCTION

Facial pigmented spots are regarded as a common skin aging feature[1]. In global populations, the demand for products that prevent the appearance of skin aging features has increased markedly. However, to offer substantiated advice and effective treatment, it is prerequisite to understand skin aging etiology.

Pigmented spots (solar lentigines and seborrheic keratoses) are part of the complex skin aging phenotype, which also includes wrinkling, sagging, and telangiectasia, which together have been considered as one skin aging phenotype in previous studies[2,3]. Important known risk factors for skin aging include age, cumulative UV-exposure, and light skin color[4-6]. Candidate gene studies have been performed, where gene variants in the pigmentation genes *SLC45A2* (solute carrier family 45 member 2) in Asians[7] and *MC1R* (melanocortin 1 receptor) in Europeans[8] have been found to be associated with the presence of solar lentigines. However, the genetic predisposition to facial pigmented spots has not been investigated at the genome-wide scale.

To provide insight into which other genes may be involved in the development of pigmented spots during aging, we performed a genome-wide association study (GWAS) in 2844 individuals of northwestern European ancestry from the Rotterdam Study (RS). Facial pigmented spots were quantified from high-resolution digital photographs, using semi-automated image analysis. We then replicated our findings in an independent cohort of 599 Dutch participants of the Leiden Longevity Study (LLS). To clarify whether the genetic associations with pigmented spots were independent of skin color or not, we additionally adjusted the identified associations for skin color.

RESULTS

Discovery GWAS

All 2844 individuals from the discovery RS cohort (mean age: 66.9 ±8.0, 47% men, Table 1) were of northwestern European ancestry. Women were more severely affected with on average 2.0% (±0.9%) of their facial area being covered by pigmented spots (Figure 1), compared to men (0.9% ±0.6%, Table 1). A total of 168 single-nucleotide polymorphisms (SNPs; nine genotyped SNPs, Supplementary Table S1) in three distinct loci showed genome-wide significant association with pigmented spots (P-value<5×10^{-8}, Figure 2, Supplementary Figure S1). All three loci harbor a known skin color gene, namely *IRF4* (6p25), *MC1R* (16q24) and *ASIP* (20q11). The most strongly associated SNP was rs12203592(T) in the 4th intron of *IRF4* (24.9%Δ per allele, P-value 1.9×10^{-27}, Table 2, Supplementary Figure S2A). The association at 16q24 consisted of a large number of SNPs (Supplementary Figure S2B). This locus contains many genes, and the top associating SNP was rs35063026(T) (20.29%Δ, P-value 9.4×10^{-15}) located in exon 3 of *c16orf55/SPATA33*, which is ~250 kb upstream from the skin color gene *MC1R*. The third locus was found at 20q11, where the most strongly associated SNP rs6059655(A) was located in intron 8 of the *RALY* gene (14.6%Δ, P-value

Table 1. Characteristics of 2844 northwestern European participants from the Rotterdam Study

Characteristics	Men (N=1323)	Women (N=1521)
Pigmented spots; mean (SD)	0.9% (0.6)	2.0% (0.9)
Age (years); mean (SD)	67.1 (7.9)	66.8 (8.0)
Skin color; number (%)	1323	1521
very white	100 (7.6)	141 (9.3)
white	1016 (76.8)	1196 (78.6)
white-to-olive	207 (15.6)	184 (12.1)

Abbreviation: SD, standard deviation.
Pigmented spots were measured as affected area per total measured facial area.

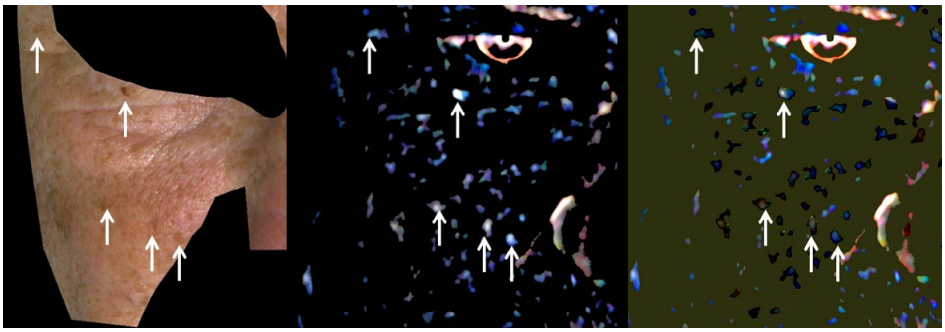

Figure 1. Example image of pigmented spots detection. (**A**) All non-skin areas were masked. The arrows are pointing at pigmented spots. (**B**) This contrasted image targets features approximate in size to pigmented spots, which appear blue to white in color (brown to black in the regular photograph). (**C**) Spots subsequently detected as pigmented spots are shaded.

2.6×10^{-9}, Table 2, Supplementary Figure S2C). *RALY* is located <200 kb upstream from the skin color gene *ASIP.* Linkage disequilibrium between the top-associated SNP in *ASIP* (rs1205312(A), P-value 1.8×10^{-6}) and rs6059655 was substantial (r^2=0.59).

We performed a second genome-wide association analysis for pigmented spots in the RS, conditional on the three most strongly associating SNPs (rs12203592 (*IRF4*), rs35063026 (*MC1R*), and rs6059655 (*RALY/ASIP*)). In this conditional analysis, none of the SNPs at the *IRF4* and *RALY* loci were associated with pigmented spots at genome-wide significance (P-value>0.005). In contrast, at the *MC1R* locus, a large number (N=31) of SNPs still showed genome-wide significant association. Interestingly, one additional locus at 9p22 was identified to be significantly associated with pigmented spots, where rs62543565(C) showed the most significant association (-6.4%Δ, P-value 2.3×10^{-8}). This SNP is located 30 kb upstream from Basonuclin 2 (*BNC2;* Supplementary Figure S2D), which was recently found to be involved in skin coloration[9]. A sex-stratified GWAS could not identify new loci or SNPs with strong sex-specific effects (Supplementary Table S2).

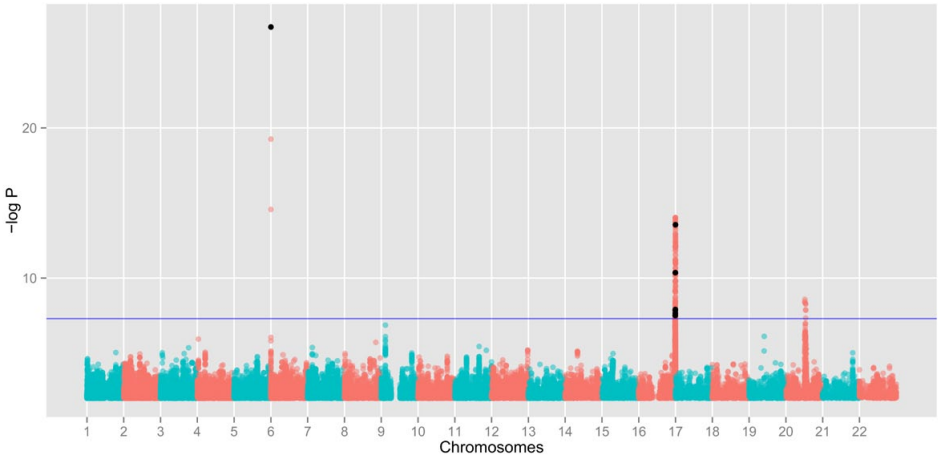

Figure 2. Manhattan plot of the GWAS in 2844 northwestern European individuals from the Rotterdam Study. The observed -$\log_{10}$ P-values (y-axis) of the association between each single-nucleotide polymorphism (SNP) and pigmented spots are shown. All SNPs are represented by dots and displayed per chromosome (x-axis). The horizontal line indicates the genome-wide significant threshold of P-value 5×10^{-8}. Genotyped SNPs passing this threshold are colored black.

Table 2. GWAS in the discovery cohort and the replication results, for pigmented spots in two independent cohorts

				Discovery cohort				Replication cohort			
				RS (N=2844)				LLS (N=599)			
Chromosome	**Gene**	**SNP**	**EA**	**EAF**	**%Δ**	**SE**	**P-value**	**EAF**	**β**	**SE**	**P-value**
6p25	*IRF4*	rs12203592	T	0.09	24.94	2.05	1.9×10^{-27}	0.08	0.44	0.12	4.4×10^{-4}
9p22	*BNC2*	rs62543565	C	0.37	-6.32	1.25	1.5×10^{-7}	0.41	-0.15	0.07	0.033
16q24	*MC1R*	rs35063026	T	0.07	20.29	2.40	9.4×10^{-15}	0.08	0.33	0.13	0.011
20q11	*RALY/ASIP*	rs6059655	A	0.08	14.58	2.31	2.6×10^{-9}	0.10	0.30	0.11	0.009

Abbreviations: %Δ, percentage change in the pigmented spots area, per increase in effect allele; β, the increase in pigmented spots severity category, per increase in effect allele; EA, effect allele, or minor allele; EAF, effect allele frequency; GWAS, genome-wide association study; LLS, Leiden Longevity Study; RS, Rotterdam Study; SE, standard error of the %Δ or β; SNP, single-nucleotide polymorphism.
The most significant signal per locus of the GWAS in the RS with a P-value<5×10^{-7} is shown; the signals were replicated in the LLS.

Replication of findings

A replication study for the 168 top-associated SNPs was conducted in an independent cohort, the LLS, of Dutch ancestry (Supplementary Table S1). This study consisted of 599 individuals (mean age: 63.1 ±6.7, 46% men), with facial pigmented spots graded in severity categories (ranging from 2 to 8, mean: 4.4 ±1.2). Although the pigmented spots phenotype of this replication cohort was assessed differently (categorical) compared with the discovery cohort (percentage of affected area), both methods reflect the severity of facial pigmented spots. The four top SNPs from the discovery GWAS were all successfully replicated in the LLS (P<0.05, Table 2). These included rs12203592(T) (*IRF4*, beta 0.44, P-value 4.4×10^{-4}), rs35063026(T) (*MC1R*, beta 0.33, P-value 0.011), rs6059655(A)

(*RALY/ASIP*, beta 0.30, P-value 0.009) and rs62543565(C) (*BNC2* (non-conditional analysis), beta -0.15, P-value 0.033).

MC1R compound heterozygosity

The association pattern at chr16q24 was consistent with previous GWAS findings of skin color[10-12], where multiple SNPs in a large region around *MC1R* demonstrate independent association (Supplementary Figure S2B). There are six SNPs within *MC1R* that are frequent in Europeans (MAF>1%) and associate with skin color together in a compound heterozygous manner[13]. Compound heterozygosity implies that if both homologous chromosomes carry one effect allele but in different SNPs, the effect is similar to that of a homozygous allele. In the RS, a compound heterozygosity score (CHS) was calculated from the haplotypes of the six independent skin color-associated *MC1R* SNPs (Supplementary Table S3). The CHS was more significantly associated with pigmented spots (14.0%Δ, P-value 1.6×10^{-24}) than the top-associated SNP in this region, demonstrating that compound heterozygosity also has a role in pigmented spot development. In addition, when the GWAS was adjusted for the CHS, no more genome-wide significant SNPs on chr16q24 could be detected; this implies that these six *MC1R* skin color SNPs together explained a large part of the *MC1R* association with pigmented spots.

Skin color-adjusted analyses

Because all four identified loci are known to be involved in skin color, we performed additional skin color-adjusted and -stratified analyses in the RS. Adjustment for skin color showed that *IRF4* and *BNC2* SNPs hardly reduced in association effect size. *MC1R* (CHS) and *RALY/ASIP* (rs6059655) slightly reduced in effect size (~8.5% lower %Δ), but were still genome-wide significant (Table 3).

Stratification for the three skin color categories showed that the effect sizes (%Δ) in the very white and the white skin color subgroups were similar for all four top SNPs (Table 3). The SNPs in *RALY/ASIP* (rs6059655) and *BNC2* (rs62543565) did not reach significance in the very white subgroup, likely because of the small sample size (N=241). The effect sizes were stronger in the white-to-olive skin color subgroup, although only rs12203592 (*IRF4*) showed a significant interaction with skin color (P-value 0.04).

In addition, we investigated whether other well-known pigmentation genes associated with pigmented spots, which we might not have picked up with the GWAS, due to smaller effect sizes (Table 4). However, none of the eight additional pigmentation genes we selected showed significant association with pigmented spots (P>0.005). The total variance of the pigmented spots phenotype explained by age, sex, skin color, and the pigmentation genes combined was very high (r^2=40.3%, Table 4), with sex (r^2=30.4%) and age (r^2=3.5%) being the strongest predictors. The *IRF4* SNP rs12203592 explained the largest proportion of the phenotypic variance (r^2=2.3%) of the four top SNPs combined (r^2=5.6%).

The four genes that associated with pigmented spots risk here, also showed association with perceived skin color in a previous investigation in the RS[9]. The most striking difference between

the association with skin color and pigmented spots, in terms of significance, was observed for *HERC2*, where rs12913832 showed a highly significant association with skin color (P-value 1.5×10^{-109})[9], but not with pigmented spots (P-value 0.49, Table 4).

Table 3. Skin color-adjusted and -stratified analysis in 2844 northwestern European individuals from the Rotterdam Study

		IRF4 rs12203592		***MC1R*** CHS		***RALY/ASIP*** rs6059655		***BNC2*** rs62543565	
Analysis	**N**	**%Δ**	**P-value**	**%Δ**	**P-value**	**%Δ**	**P-value**	**%Δ**	**P-value**
SC-adjusted	2844	24.12	2.3×10^{-26}	12.85	3.5×10^{-21}	13.32	3.7×10^{-8}	-6.20	2.0×10^{-7}
SC-stratified									
very white	241	21.85	2.8×10^{-5}	11.88	2.9×10^{-3}	8.91	0.179	-5.43	0.141
white	2212	21.54	4.5×10^{-17}	11.70	3.7×10^{-15}	13.25	7.7×10^{-7}	-5.38	6.7×10^{-5}
white-to-olive	391	46.37	6.3×10^{-10}	19.80	9.5×10^{-7}	19.10	0.028	-12.09	3.1×10^{-4}

Abbreviations: %Δ, percentage change in the pigmented spots area, per increase in effect allele; CHS, compound heterozygosity score; SC, skin color; SC adjusted, regression analysis additionally adjusted for skin color; SC stratified, regression analysis per skin color stratum.

Table 4. Multivariable analysis of pigmentation genes and pigmented spots in 2844 northwestern European individuals from the Rotterdam Study

Factor	**SNP**	**EA**	**EAF**	**%Δ**	**SE**	**P-value**	**r^2 (%)**
Age (years)				1.50	0.11	1.1×10^{-42}	3.54
Female sex				86.91	1.69	1.0×10^{-245}	30.39
Light skin color				9.39	1.87	1.2×10^{-7}	0.57
SLC45A2	rs16891982	C	0.03	-6.83	3.78	0.056	0.06
IRF4	rs12203592	T	0.09	24.43	2.03	5.4×10^{-27}	2.26
TYRP1	rs1408799	T	0.31	1.74	1.28	0.175	0.03
BNC2	rs62543565	C	0.37	-6.54	1.21	2.3×10^{-8}	0.58
TPCN2	rs35264875	T	0.17	-1.58	1.57	0.308	0.02
TYR	rs1393350	A	0.23	2.71	1.41	0.057	0.06
KITLG	rs12821256	C	0.13	0.83	1.76	0.637	0.004
SLC24A4	rs12896399	G	0.49	-0.15	1.19	0.900	0.0003
OCA2	rs1800407	T	0.05	-4.50	3.04	0.125	0.04
HERC2	rs12913832	A	0.22	1.07	1.57	0.494	0.01
MC1R	CHS	-	-	13.42	1.27	6.6×10^{-23}	2.02
RALY/ASIP	rs6059655	A	0.08	13.45	2.26	1.9×10^{-8}	0.74
Total							**40.32**

Abbreviations: %Δ, percentage change in the pigmented spots area, per increase in effect allele; EA, effect allele; EAF, effect allele frequency; r^2, percentage variance in pigmented spots area, explained by the predictor; SE, standard error of the %Δ; SNP, single-nucleotide polymorphism.
Multivariable linear regression analysis. Age, sex, skin color, the four top SNPs from the GWAS, and the top SNPs of eight known pigmentation genes were tested. For *MC1R*, the CHS was used (compound heterozygosity score).

DISCUSSION

We detected SNPs in and around the genes *IRF4*, *MC1R*, *ASIP*, and *BNC2* that demonstrated genome-wide significant associations with facial pigmented spots, and all were successfully replicated in a second independent cohort. Furthermore, our data demonstrate that the associations of *IRF4*, *MC1R*, *ASIP*, and *BNC2* with facial pigmented spots were at least partially independent of skin color.

The four identified genes are known to be associated with visible skin traits in Europeans, including pigmentation variation (eye, hair, and skin color)[9,10,12], freckling[12,14], tanning response[15], and different types of skin cancer (basal cell carcinoma, squamous cell carcinoma, and melanoma)[16-19]. However, not all skin color-associated genes have an additional effect on the development of pigmented spots, such as *HERC2*. Previously, GWAS on skin sagging and global photoaging did not identify any skin color genes being involved[20,21], but we now demonstrate that skin color genes clearly have a role in the appearance of a specific feature of skin aging.

The SNP rs12203592 in *IRF4* (interferon regulatory factor 4) showed the strongest association with pigmented spots, explaining more than two percent of the phenotypic variance. Gene variants in *IRF4* are also associated with related phenotypes, namely skin color, freckling, and all skin cancer types[10,14,22]. Similarly, the compound *MC1R* haplotype was strongly associated with pigmented spots, explaining two percent of the variation. Many SNPs located close to *MC1R* showed association and, after adjusting for the *MC1R* CHS, no more SNPs were genome-wide significantly associated. All common variants in *MC1R* are associated with hair and skin color, freckling, and skin cancer types[10,11,23,24], showing that *MC1R* is pleiotropic in nature. The rs6059655 SNP in *RALY* (heterogeneous nuclear ribonucleoprotein) is located close to the skin color gene *ASIP* (agouti signaling protein). In previous studies, many variants around *ASIP* showed association with skin color-related phenotypes such as freckling, sun sensitivity and skin cancer[11,25]. Hence, the *RALY* SNP could affect *ASIP* expression via a long-range regulation, or it is in LD with another SNP closer to *ASIP*, which is affecting *ASIP* expression[26]. Finally, rs62543565 close to *BNC2* (basonuclin 2) was genome-wide significantly associated with pigmented spots after adjusting for the other three top-associated SNPs. Variants in *BNC2* are associated with skin color[9], and with freckling[14], but not yet found to be associated with skin cancer. This is a relatively new skin color gene and the function of *BNC2* in pigmentation needs to be further investigated in future studies. The four pigmentation genes together explain a non-trivial portion of 5.6% of the phenotypic variance, which is large compared with typical human complex traits – e.g., for adult body height ~2000 SNPs together could explain about 21% of the phenotypic variance[27].

The gene variant associations with pigmented spots were found to be independent of skin color, similar to what is found for gene variants that are associated with different types of skin cancer[24,28,29]. Pigmented spots and skin cancer share cumulative UV-exposure as a major risk factor, and facial pigmented spots have also shown to be a risk factor for skin cancer[30,31]. In addition, in the rare recessively inherited disease xeroderma pigmentosum, all affected individuals suffer

from many solar lentigines and skin (pre-)malignancies from a young age onward because of a defect in DNA repair mechanisms[23]. Therefore, it could be hypothesized that a less effective repair of UV-induced DNA damage explains the skin color independent effects of skin color genes in pigmented spots and skin cancer. In support of this, *MC1R* loss of function alleles have been associated with a higher level of UV-induced DNA damage in melanocytes[32,33], which is independent of the total melanin content[34]. Possibly, the melanocytes react to DNA damage by locally boosting melanin production to provide a subsequent UV protection. However, the specific role of these genes in the development of pigmented spots histology remains elusive.

DNA variants at all four loci, in particular *IRF4*, showed a stronger effect in darker colored individuals compared with white skinned individuals in a skin color-stratified analysis. Such an effect has been shown before for *MC1R* and melanoma[35]. Possibly, individuals with a darker skin color are less likely to avoid the sun as they will burn less easily, which aggravates the effect by cumulative UV-exposure. A second hypothesis is that the gene variant effects in the lighter skin color groups are ameliorated by other gene variants prevalent in these groups. This is supported by the observation that light skin color is still significantly associated with pigmented spots after adjustment for the top SNPs found here.

In women, we found a much higher prevalence of facial pigmented spots with 30% of the pigmented spot variance explained by sex, which could not be explained by genetic differences in our study. Previous studies are inconclusive about sex differences; some found a higher risk in women[4] and others in men[36]. Although we cannot rule out that our computer-aided phenotyping method used here was biased for pigmented spot detection in female compared to male skin, the same sex difference was also present in the LLS expert grading data, which were manually graded by experts[37]. Possible explanations are that higher levels of estrogen and progesterone may increase the risk of developing pigmented spots[5], women may exhibit a different lifestyle, or epigenetic regulation mechanisms may differ among the sexes.

To our knowledge, digitally quantified pigmented spots to identify risk factors are previously unreported. Photonumeric scales have been used to assess pigmented spot severity[36,38], but the advantage of digital quantification is a more objective and a more sensitive approach. However, a possible disadvantage is the inability to differentiate between the different facial pigmented lesions. We aimed to measure solar lentigines as a skin aging characteristic but simultaneously measured seborrheic keratosis (brown warty lesions in elderly). It is unlikely that other types of pigmented facial spots (melanocytic nevi, freckles, and melasma[5,39,40]) have biased our measure because they are more common in young individuals, and we additionally excluded all heavily freckled individuals. Therefore, our pigmented spots phenotype consists of solar lentigines and (a minority of) seborrheic keratosis. Because these two are often assumed to reflect the same phenotype (histologically they show clear overlapping features[41]), the elucidated genes likely influence both, but this should be confirmed in future research.

Conclusion

DNA variants in *IRF4*, *MC1R*, *ASIP*, and *BNC2* are significantly associated with facial pigmented spots independently of age, sex, and skin color. Future studies should investigate the biological function of these genes in the skin and, in particular, how they could be influencing pigmented spot development independently of basal melanin production.

MATERIALS & METHODS

Study populations

Rotterdam Study

The RS is a population-based prospective study of unrelated elderly subjects (>45 years of age) consisting of an initial cohort and two extensions[42]. The present study includes 2844 participants of northwestern European ancestry, for whom facial photographs and genotype data were available, after quality control. During routine visits at the research center, a full-body skin examination was performed by trained physicians and high-resolution standardized full-face photographs were obtained of participants not wearing make-up, cream, or jewelry, using a premier 3dMD face3-plus UHD (3dMD, Atlanta, GA, USA). The photos used in this study were collected from September 2010 to July 2013. The medical ethics committee of the Erasmus MC University Medical Center approved the study protocol, and all participants provided written informed consent.

Leiden Longevity Study

The LLS has been described in detail previously[43]. This family-based study consists of 1671 offspring of 421 nonagenarian sibling pairs of Dutch descent and their 744 partners. The current study includes 599 participants with facial pigmented spot grades and genotype data available after quality control. During routine visits at the Leiden research center, high-resolution standardized full-face photographs were obtained of participants not wearing make-up, cream, or jewelry, using a Fuji S2 (Tokyo, Japan) camera system. The photos used in this study were collected from November 2006 to April 2008. The study protocol was approved by the medical ethics committee of the Leiden University Medical Center, and all participants gave written informed consent.

Phenotyping

In the RS, pigmented spot presence was digitally quantified using semi-automated image analysis of high-resolution facial frontal photographs. The algorithms, digital rendering, measurement, and validation of the outcome measure have been described in detail using a randomly selected subset of images of 100 participants[44]. In short, the analysis detects areas that are dark brown, i.e., hyperpigmented relative to the surrounding skin with a roundish shape, present on the forehead, cheeks, and nose (Figure 1). It subsequently calculates the percentage of skin area detected as hyperpigmented spots. To test the image analysis accuracy, two independent physi-

cians manually graded the 100 photographs using a 5-point photonumeric scale. There was a high correlation between the average of the two manual grades and the values from the image analysis (Spearman's rho correlation coefficient 0.69)[44]. Furthermore, all 2844 photos were visually controlled for the type of hyperpigmentation, which should be solar lentigines or seborrheic keratoses. Therefore, individuals with freckles (N=23), facial contusion (N=1), facial scars with hyperpigmentation (N=1), and post inflammatory hyperpigmentation (N=1) were excluded. During the full-body skin examination, constitutional skin color was assessed at sun-protected skin sites (trunk, upper legs)[45]. The skin color was graded into three levels: very white (9%), white (78%), and white-to-olive (14%) (Table 1).

In the LLS, severity of pigmented spots was manually graded using a 9-point photonumeric scale, taking area, intensity of color, and uniformity of distribution into account[46]. Grading was performed independently by two skin aging experts using frontal digital photographs, as described previously[37,47].

Genotyping

In the RS, genotyping was carried out separately in the initial cohort and the two extension cohorts using the Infinium II HumanHap 550K and 660K Genotyping BeadChip version 3 (Illumina, San Diego, CA, USA). Collection and purification of DNA have been described previously[48]. All genotyped SNPs (N=537,405) were imputed using the MACH software[49] based on the 1000G Phase I Integrated Release Version 3 (released in March 2012) reference panel[50] separately for the three cohorts. Genotyping and quality control have been described in detail previously[51]. After quality control, the current study included a total of 6,846,125 autosomal SNPs (MAF>0.03, imputation Rsq>0.3, SNP call rate>0.97, HWE>1×10^{-4}) and 2844 individuals (individual call rate >0.95, pairwise identity by descent (IBD) sharing <0.25 (--genome option in PLINK), excluding x-mismatches and outliers from MDS analysis). We additionally conducted a GWAS using a more stringent IBD sharing threshold (IBD<0.1, N=2501). The results are identical in terms of the loci showing significant association with pigmented spots and the effect sizes (Supplementary Table S2, Supplementary Figure S3), which shows that including individuals with an IBD sharing <0.25 does not affect the reliability of GWAS results.

The LLS offspring and partners were genotyped using Illumina Infinium HD Human660W-Quad BeadChips and Illumina OmniExpress and imputation was performed using IMPUTE with the 1000G Phase I Integrated Release Version 3 (released in March 2012) reference panel. Family relations and imputation uncertainty were taken into account in the analysis by specialized software, QT-assoc[52].

Statistical analysis

In the RS, the phenotype (area of pigmented spots) showed a highly right-skewed distribution. We thus log transformed the phenotype, resulting in an approximately normal distribution of both the phenotype and the regression residuals. Because effect estimates (regression betas) of log

transformed outcome variables are not directly interpretable, we represent all regression betas as the percentage change (%Δ) – i.e., the percentage increase of the mean value of the dependent variable (in our case pigmented spots area) per unit increase of the independent variables (such as one year of age or carrying one additional minor allele), calculated as (exp (beta) - 1) * 100.

All analyses in the RS were adjusted for age, sex, the first four genetic principal components, and for variance between participants in flashlight illumination of the skin (Supplementary Methods). In the discovery GWAS (RS), association with autosomal SNPs was tested using linear regression assuming an additive allele effect. The inflation factor lambda was close to 1.0 (λ=1.02) and not further considered. A conditional GWAS, adjusted for the top SNP per locus, was performed. We also conducted GWAS separately in men (N=1323) and in women (N=1521). All GWAS analyses were conducted using PLINK [53].

A total of 167 SNPs in three loci with P-values<5×10^{-8} from the GWAS in the RS, plus the top SNP (at *BNC2*) from the conditional GWAS, were selected for replication analysis in the LLS. SNPs selected for replication were analyzed using linear regression, adjusting for age, sex and familial relations using the software package QT-assoc[52], which is based on a modified version of the score test. P-values<0.05 were considered as a significant replication.

The CHS of *MC1R* was calculated based on the haplotypes of six known and independent skin color SNPs in *MC1R* (rs1805005, rs2228479, rs1805007, rs1805008, rs885479, and rs1805009)[26], which were present in the RS. The haplotypes were calculated with statistical software R (www.R-project.org), package "haplo.stats". To calculate the *MC1R* CHS, we added up the number of variant type haplotypes per individual (Supplementary Table S3). A variant type haplotype carries at least one effect allele. The CHS is therefore coded as 0, 1, or 2 and comparable to a SNP in linear regression analysis.

Additional skin color-adjusted analyses were conducted in the RS. A skin color-adjusted and a skin color-stratified analysis were conducted for the top SNPs per locus in relation to pigmented spots. Furthermore, known pigmentation genes were tested for association with pigmented spots. Selection of the pigmentation genes was based on significant association with hair, eye, or skin color in previous GWAS studies[10-12,14,15,19] and included the reported top-associated SNP at each of the gene loci: *MC1R*, *HERC2*, *OCA2*, *ASIP*, *TYR*, *TYRP1*, *IRF4*, *SLC45A2*, *SLC24A4*, *TPCN2*, *KITLG*, and *BNC2*, unless a different SNP was associated with pigmented spots in this study. Association was tested in a multivariable analysis, including these 12 pigmentation SNPs, age, sex, and skin color (to test their independent effects, significance threshold P-value<0.005) and calculated the explained variance of pigmented spots (r^2). All statistical analyses were conducted using statistical software R.

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