



Genome-Wide Association Shows that Pigmentation Genes Play a Role in Skin Aging

Matthew H. Law¹, Sarah E. Medland¹, Gu Zhu¹, Seyhan Yazar², Ana Viñuela³, Leanne Wallace⁴, Sri Niranjana Shekar^{1,5}, David L. Duffy¹, Veronique Bataille^{3,6}, Dan Glass^{3,7}, Tim D. Spector³, Diane Wood⁸, the MuTHER Consortium*, Scott D. Gordon¹, Julie M. Barbour⁹, Anjali K. Henders¹, Alex W. Hewitt^{10,11}, Grant W. Montgomery^{12,1}, Richard A. Sturm¹³, David A. Mackey², Adèle C. Green^{1,14}, Nicholas G. Martin¹ and Stuart MacGregor¹

Loss of fine skin patterning is a sign of both aging and photoaging. Studies investigating the genetic contribution to skin patterning offer an opportunity to better understand a trait that influences both physical appearance and risk of keratinocyte skin cancer. We undertook a meta-analysis of genome-wide association studies of a measure of skin pattern (microtopography score) damage in 1,671 twin pairs and 1,745 singletons ($N = 5,087$) drawn from three independent cohorts. We identified that rs185146 near *SLC45A2* is associated with a skin aging trait at genome-wide significance ($P = 4.1 \times 10^{-9}$); to our knowledge this is previously unreported. We also confirm previously identified loci, rs12203592 near *IRF4* ($P = 8.8 \times 10^{-13}$) and rs4268748 near *MC1R* ($P = 1.2 \times 10^{-15}$). At all three loci we highlight putative functionally relevant SNPs. There are a number of red hair/low pigmentation alleles of *MC1R*; we found that together these *MC1R* alleles explained 4.1% of variance in skin pattern damage. We also show that skin aging and reported experience of sunburns was proportional to the degree of penetrance for red hair of alleles of *MC1R*. Our work has uncovered genetic contributions to skin aging and confirmed previous findings, showing that pigmentation is a critical determinant of skin aging.

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INTRODUCTION

Skin aging, characterized by epidermal thinning and reduced DNA repair, results from both external and internal influences. Major extrinsic factors include exposure to UVR, causing photoaging, and smoking. Specifically, photoaging is typified by elastosis and damage to the complex extracellular support matrix of the dermis (for review, see Bilac et al., 2014). This photodamage alters mechanical properties in the skin, leading to deterioration and loss of regularity of the fine reticular patterning of the skin surface (Lavker et al., 1980), as well as wrinkles and sagging. Because UVR also induces DNA damage, the degree of photoaging correlates with skin carcinogenesis (Gandini et al., 2005; Green, 1991; Holman et al., 1984). Thus, heritable factors that modify skin

aging, particularly photoaging, have implications for both personal appearance and risk of skin cancer.

To date, a number of genome-wide association studies (GWASs) have analyzed various measures of photoaging or overall skin aging. These include perceived facial age (Liu et al., 2016), facial photoaging (Le Clerc et al., 2013), and the combined impact of intrinsic and extrinsic factors on facial aging (Chang et al., 2014). Additional GWASs have been conducted on traits related to photoaging such as actinic keratosis and facial pigmented spots (Jacobs et al., 2015a, 2015b; Laville et al., 2016). Together, these have identified genetic variants on chromosomes 3, 6, 16, 20, and X.

Here, we report on a meta-analysis of five GWASs drawn from three independent cohorts (Methods) analyzed using the

¹QIMR Berghofer Medical Research Institute, Brisbane, Australia; ²Centre for Ophthalmology and Visual Science, Lions Eye Institute, University of Western Australia, Perth, Western Australia, Australia; ³Department of Twin Research and Genetic Epidemiology, King's College London, London, UK; ⁴Institute for Molecular Biosciences, Program in Complex Trait Genomics, University of Queensland, Brisbane, Australia; ⁵LSE Health, London School of Economics and Political Science, London, UK; ⁶Dermatology, West Herts NHS Trust, Hemel Hempstead, Herts, UK; ⁷Department of Dermatology, Northwick Park Hospital, London, UK; ⁸School of Population Health, University of Western Australia, Perth, Western Australia, Australia; ⁹The Launceston Eye Institute, Launceston, Tasmania, Australia; ¹⁰Menzies Institute for Medical Research, School of Medicine, University of Tasmania, Hobart, Australia; ¹¹Centre for Eye Research Australia, University of Melbourne, Royal Victorian Eye and Ear Hospital, Melbourne, Victoria, Australia; ¹²Molecular Biology, the University of Queensland, Brisbane, Australia; ¹³Dermatology Research Centre, The University of Queensland, Diamantina Institute, Translational Research Institute, Brisbane, Australia;

and ¹⁴CRUK Manchester Institute and Institute of Inflammation and Repair, University of Manchester, Manchester, UK

*Collaborators from the MuTHER Consortium are listed in the Supplementary Materials online

Correspondence: Matthew H. Law, Statistical Genetics Laboratory, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, 4006, QLD, Australia. E-mail: matthew.law@qimrberghofer.edu.au

Abbreviations: BG6, Beagley-Gibson six-point rating system; eQTL, expression quantitative trait locus; GWAS, genome-wide association study; LD, linkage disequilibrium; QC, quality control; SL, solar lentiginosis; SNP, single-nucleotide polymorphism; TEST, Twins Eyes Study in Tasmania

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six-point Beagley and Gibson (BG6) microtopography scoring system of skin patterning regularity and complexity (Beagley and Gibson, 1980; Holman et al., 1984). BG6 score strongly correlates with amounts of dermal solar elastosis (Battistutta et al., 2006; Fritschi et al., 1995; Seddon et al., 1992), more so at younger ages (Hughes et al., 2012). It is correlated with self-reported sun exposure history and skin phototype, and sunscreen can protect against the age-related increase in BG6 score (Hughes et al., 2013). At age 12 years, BG6 is highly heritable (86%), with heritability decreasing to 62% in adults (Shekar et al., 2005), suggesting that BG6 is a useful measure to explore the genetics of photoaging. Here, we aim to confirm previous GWAS findings, identify additional loci, and characterize the interaction of *MC1R* red hair alleles with BG6 and various sun exposure behaviors.

RESULTS

Skin patterning GWAS meta-analysis

We performed a meta-analysis of five BG6 GWASs drawn from three independent cohorts, representing 1,671 twin pairs and 1,745 singletons (N = 5,087) (see Methods and Table 1). To account for differences in age, genotyping array, and phenotype, a total of five BG6 GWASs (Table 1) were conducted using imputed genotype dosage scores in Merlin-offline (Abecasis et al., 2002), a method that appropriately models the family structure in related samples, including twins (see Methods and Figures 1 and 2). GWAS quantile-quantile plots indicated that there was no evidence for population stratification (see Supplementary Figure S1 online). Single-nucleotide polymorphisms (SNPs) at or near *SLC45A2* reached genome-wide significance with a measurement of skin aging, as did SNPs near *IRF4* and *MC1R* (Table 2; Supplementary Figures S2, S3, and S4 online). Full results for all SNPs with *P* less than 1×10^{-5} are reported in Supplementary Table S1 online. Conditioning on the top SNPs did not show additional associations (see Supplementary Methods, Supplementary Table S2, and Supplementary Figure S5 online).

In addition to BG6, a number of other skin aging-related traits have been analyzed by GWAS (Chang et al., 2014; Jacobs et al., 2015a, 2015b; Laville et al., 2016; Le Clerc et al., 2013; Liu et al., 2016). Supplementary Table S3 online

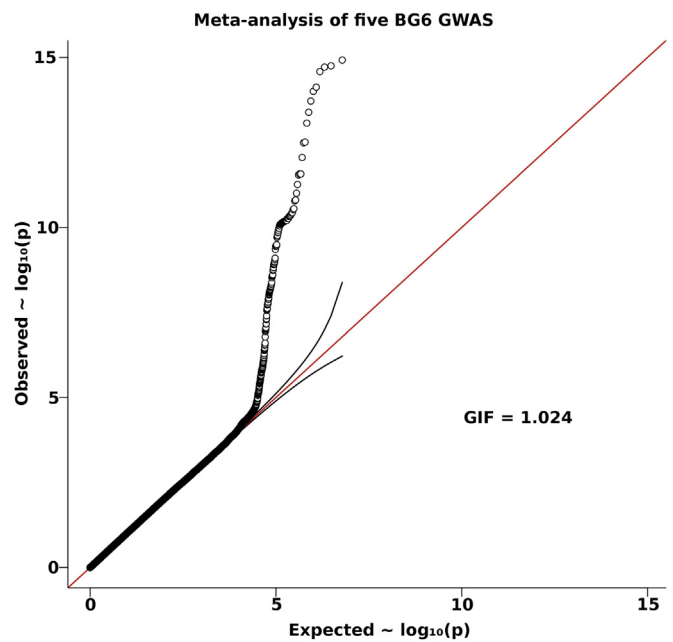


Figure 1. Quantile-quantile plot of $-\log_{10}$ of fixed-effects *P*-values derived from the meta-analysis of BG6 GWASs. The 95% confidence interval for the expected $-\log_{10}(P)$ is indicated by black lines. BG6, Beagley-Gibson six-point rating system; GIF, genomic inflation factor; GWAS, genome-wide association study.

summarizes the BG6 association for those SNPs previously reported as reaching genome-wide significance with other skin aging-related traits. Of note, the top SNP from the chromosome 3 region reported by Le Clerc et al. (2013) as associated with facial photoaging; rs322458 has a *P*-value of 0.07 for BG6. More promising is the nearby SNP rs470647, noted as a possible functional SNP tagged by rs3322458 (BG6 $P = 3.8 \times 10^{-3}$).

Gene-based approach

We used the gene-based association test VEGAS2 to test for multiple independent signals within a gene that are individually not genome-wide significant (Mishra and Macgregor, 2015) (see Supplementary Methods). All results with a *P*-value less than 5×10^{-5} are reported in Supplementary Table S4 online. Although many genes at the 16q24.3 locus have significant gene-based *P*-values ($P < 2.084 \times 10^{-6}$), because of extensive linkage disequilibrium (LD) across this region, this is driven by red hair alleles in *MC1R*.

Functional annotation

Because the top SNP at each locus may not be the functional one, meta-analysis results were filtered to *P*-values less than 1×10^{-7} and homogenous effects sizes across contributing studies ($r^2 < 31\%$), leaving 102 SNPs mapping to the three genome-wide significant regions on chromosomes 5, 6, and 16 (see Supplementary Methods and Supplementary Table S5 online). Our annotation work did not identify any additional regulatory or functional SNPs at 5p13.2/*SLC45A2* beyond that our peak SNPs are in LD ($r^2 = 0.73$) with a known missense rs16891982 (F374L) variant. The L374 allele, rare in European-derived populations, is associated with darker skin (Graf et al., 2005) and here with reduced BG6 score,

Table 1. Population Description

Dataset	Adolescent				
	Phase 1	Phase 2	Adult	TEST	Raine
MZ, pairs	384	194	60	19	0
DZ, pairs	634	292	46	42	0
Singleton twins, n	110	55	170	8	0
Siblings, n	409	167	0	6	0
Unrelated, n	0	0	0	0	820
Total, n	2,555	1,194	382	136	820
Total families, n	1,061	504	197	71	820
Mean age in years (SD)	15.3 (1.9)	14.4 (2.0)	44.6 (9.8)	9.6 (3.0)	22.2 (0.6)
Female:male	1,304:1,251	649:545	228:154	69:67	412:408

Abbreviations: DZ, dizygotic twins; SD, standard deviation; MZ, monozygotic twins; TEST, Twin Eyes Study in Tasmania.

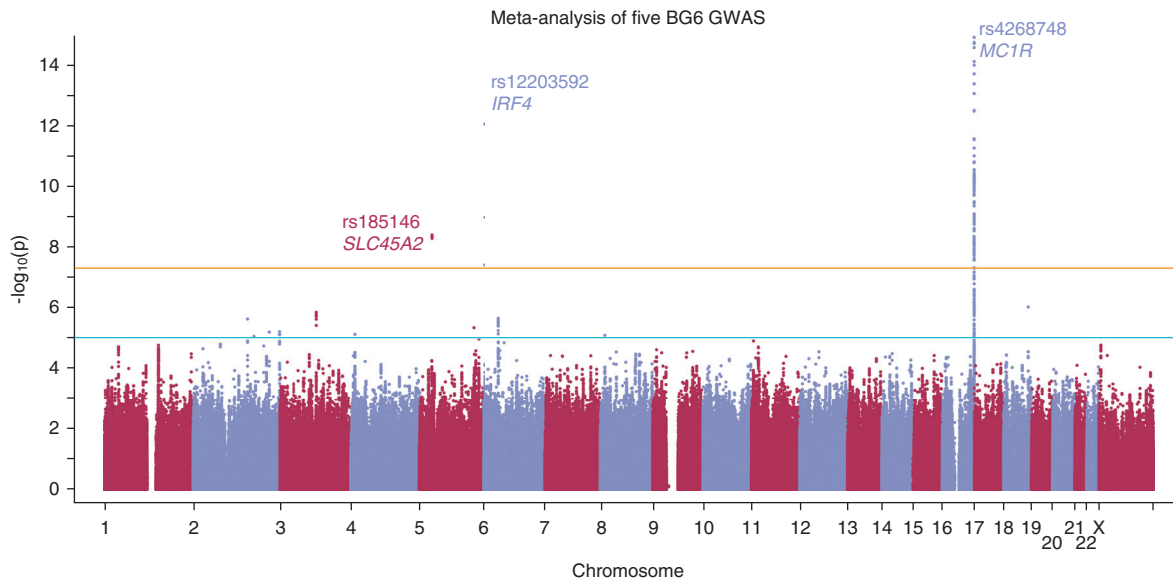


Figure 2. Manhattan plot of $-\log_{10}$ fixed-effects P -values derived from the meta-analysis of BG6 GWASs. The negative \log_{10} of meta-analysis P -values are plotted by chromosome. The orange line indicates genome-wide significance ($P = 5 \times 10^{-8}$) while the light blue line is $P = 5 \times 10^{-5}$. The top SNP and gene are indicated for loci reaching genome-wide significance. BG6, Beagley-Gibson six-point rating system; GWAS, genome-wide association study; SNP, single nucleotide polymorphism.

indicating less photoaging (Table 2). The association at 6p25.3, near *IRF4*, is strongest at rs12203592, which lies in a melanocyte enhancer and is a weak *IRF4* expression quantitative trait locus (eQTL) in whole blood and lymphocytes (Genotype-Tissue Expression [GTEx] project [GTEx Consortium, 2013] $P = 6.0 \times 10^{-7}$ and $P = 5.0 \times 10^{-7}$, respectively). rs12203592 has been shown to modulate *IRF4* regulation (Praetorius et al., 2013). In the MuTHER dataset (Nica et al., 2011; Grundberg et al., 2012), 17 BG6-associated SNPs at 16q24.3 are eQTLs for seven genes (see Supplementary Methods and Supplementary Table S6 online), and an overlapping list of 16 SNPs are eQTLs for 10 genes in the Westra data (Westra et al., 2013) (see Supplementary Table S7 online). As reported by Grundberg et al. (2012) these include the *MC1R* red hair SNP rs1805007 as an eQTL for *DBNDD1*. Together, these show direct impacts on pigmentation via nonsynonymous changes to *SLC45A2* and *MC1R*, as well as expression modulation of *IRF4*. Annotation also highlights that *MC1R* red hair alleles may also be associated with altered

expression in additional genes, which may further influence skin patterning and photoaging.

Impact of *MC1R* alleles on sun exposure effects

Our GWAS meta-analysis provides evidence that red hair alleles of *MC1R* are associated with skin patterning. 1,246 of the adolescent twins used in the adolescent phase 1 GWAS (Table 1) have been previously genotyped for nine low-frequency-coding *MC1R* alleles (Duffy et al., 2004). To improve our statistical power, we grouped these *MC1R* alleles by their penetrance for red hair and tested them for association with a range of sun exposure behaviors; a summary of these measures and their correlation can be found in the Supplementary Methods and Supplementary Tables S8 and S9 online. Male and female participants were analyzed separately given the influence of sex on skin patterning (see Supplementary Methods).

There were significant differences in BG6 for *MC1R* compound genotypes (having any combination of two red hair

Table 2. Loci with SNPs with P -values less than 5×10^{-8} after the fixed effects meta-analysis¹

Hg19 Position	SNP	Gene	A1 Effect Allele	A2	A1 Frequency	min \hat{r}^2	Fixed P	Fixed β	I^2
5:33952106	rs185146	<i>SLC45A2</i>	C	T	0.06	0.52	4.1×10^{-9}	-0.25	0
6:396321	rs12203592	<i>IRF4</i>	A	G	0.21	0.90	8.8×10^{-13}	0.13	0 ²
16:90026512	rs4268748	<i>MC1R</i>	C	T	0.29	0.95	1.2×10^{-15}	0.13	1.8
16:89986117	rs1805007	<i>MC1R</i>	T	C	0.09	0.83	1.2×10^{-10}	0.18	0
16:89986144	rs1805008	<i>MC1R</i>	T	C	0.08	0.98	1.1×10^{-5}	0.12	24.6

Abbreviations: A, adenine; C, cytosine; G, guanine; min, minimum; SNP, single-nucleotide polymorphism; T, thymine.

¹We have reported the top SNP, and if known, additional functional SNPs at the same loci. The full results, including random effects estimates and individual genome-wide association study data, for all SNPs with P -values less than 1×10^{-5} can be found in Supplementary Table S1. Note that Table 1 results are given for the minor allele for ease of interpretation; as indicated in Supplementary Table S1, effect sizes may be reported for the other allele. SNP positions are Hg19. A1 Frequency is the frequency of allele 1 in the combined dataset, weighted by sample number. min \hat{r}^2 is the minimum imputation quality score across contributing genome-wide association studies. Beta is the change in BG6 score for each copy of the A1 allele. A negative effect size indicates that allele 1 is associated with reduced BG6 scores and reduced skin aging. I^2 is the measure of heterogeneity of effect sizes across the included studies, and a low I^2 score indicates that the signals are homogenous.

²rs12203592 did not pass quality control in the Raine study; all other SNPs in Table 1 are reported for all contributing studies.

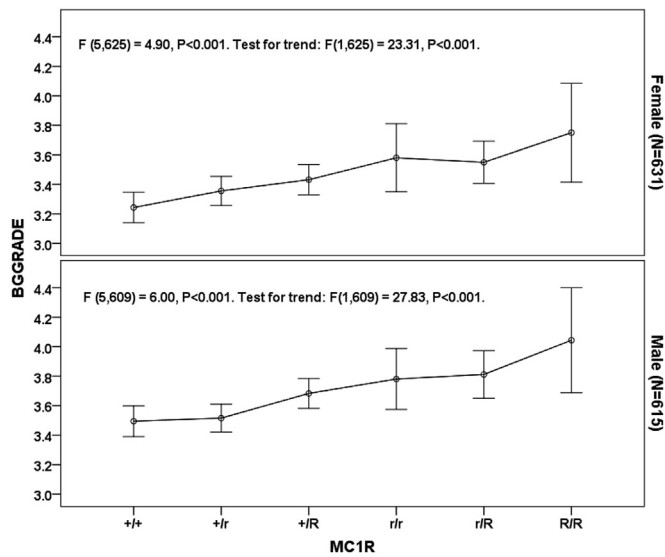


Figure 3. Adolescent BG6 scores by MC1R compound genotype. Error bars are mean ± standard error of the mean. The y-axis is BG6 score and x-axis is MC1R compound genotype. R indicates strongly penetrant red hair alleles (D84E, R151C, R160W, D294H), r weakly penetrant (V60L, V92M, R163Q), and + is the consensus haplotype. For each sex, the first *F* test result represents the test for significant difference in mean BG6 score across the compound genotype categories. A second *F* statistic and *P*-value is provided for the test for trend across all seven compound genotypes. BG6, Beagle-Gibson six-point rating system; r, weakly penetrant red hair alleles; R, strongly penetrant red hair alleles.

alleles at *MC1R*) in both sexes ($P < 0.001$, Figure 3), with a significant trend for increasing BG6 score and greater red hair penetrance ($P < 0.001$). Together *MC1R* red hair alleles explain at least 4.12% of BG6 variation. rs12203592 in *IRF4* and rs16891982 in *SLC45A2* explain a further 2.95% and 0.26% of BG6 variation respectively. Sunburn risk increased for compound genotypes in line with the degree of penetrance for red hair (Figure 4). Additionally, we explored the impact of the *MC1R* genotype and thus vulnerability to skin damage on sun exposure behavior and use of sun protection (see Supplementary Methods). We concluded that there were no differences in sun-exposed hours (UV index justified, see Supplementary Figure S6 online) or sun protection behavior between the *MC1R* genotype groups (see Supplementary Figure S7 online). The genome-wide significant SNP rs12203592 in *IRF4* did not exhibit any association with sun exposure or protection traits (data not shown).

DISCUSSION

We show that *SLC45A2* SNPs in LD with the nonsynonymous rs16891982 (F374L) (Graf et al., 2005) are genome-wide significantly associated with a measure of skin aging, BG6; to our knowledge, this is previously unreported. We also replicate previous associations at *IRF4* and *MC1R* (see Supplementary Table S2) (Jacobs et al., 2015a, 2015b; Liu et al., 2016). The associations include well-known alleles affecting pigmentation (Duffy et al., 2004). For each variant, the allele correlated with greater skin pigmentation was associated with reduced photoaging. A functional SNP rs470647 previously reported to be associated with facial photoaging (Le Clerc et al., 2013) was associated ($P = 3.8 \times$

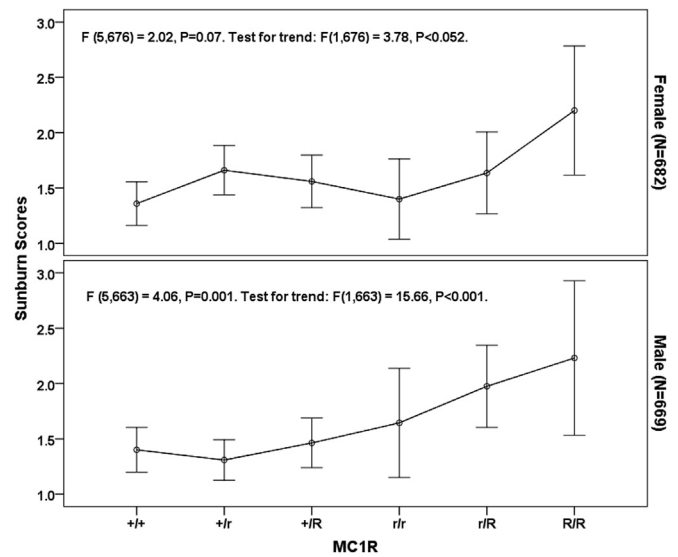


Figure 4. Self-report sunburn scores by compound MC1R genotype. Error bars are mean ± standard error of the mean. The y-axis is Sunburn Score (Supplementary Methods) and x-axis is MC1R compound genotype. R indicates strongly penetrant red hair alleles (D84E, R151C, R160W, D294H), r weakly penetrant (V60L, V92M, R163Q), and + is the consensus haplotype. For each sex, the first *F* test result represents the test for significant difference in mean BG6 score across the compound genotype categories. A second *F* statistic and *P*-value is provided for the test for trend across all seven compound genotypes. r, weakly penetrant red hair alleles; R, strongly penetrant red hair alleles.

10^{-3}) with BG6. The detection of overlapping SNPs using a range of different measures of skin aging is promising (see Supplementary Table S3) and confirms that there are germline effects on multiple skin aging components. The combined effects of the seven *MC1R* red hair alleles polymorphic in our population explained at least 4.12% of BG6 variation, representing a gene of large effect compared with most quantitative trait loci.

Replication across independent datasets is critical for confidence in GWAS findings. Data contributing to this meta-analysis were drawn from three different Australian cohorts and analyzed as five discrete GWASs to account for differences in genotyping array. BG6-associated SNPs at *SLC45A2* and *MC1R* had *P*-values of less than 0.05 in three or more GWAS sets drawn from two independent cohorts; however, SNPs at *IRF4* did not pass the imputation threshold in the Raine study (Straker et al., 2015) (see Supplementary Table S1 online). Nevertheless, the fact that we replicated skin aging association at *MC1R* and *IRF4* suggests that our observations are robust. Although conditional analysis did not show additional genome-wide significant associations (see Supplementary Figure S5), a second red hair allele, rs1805008 (R160W) was also associated with BG6 at $P = 1.1 \times 10^{-5}$ (see Supplementary Table S1), suggesting that, like many other complex traits, additional variation remains to be discovered with larger sample sizes.

The three loci associated with BG6 are well characterized, giving direct insights into how they influence fine skin patterning. Animal models suggest that *SLC45A2* low-activity alleles impair the processing and transportation of melanogenic proteins to melanosomes

(Costin et al., 2003), possibly via altered ability to maintain melanosome pH (Dooley et al., 2013). As well as regulating pigmentation together with SLC45A2, MC1R signaling enhances melanocyte UVR resistance by rapidly up-regulating antioxidant defenses (Song et al., 2009) independent of melanin synthesis (Maresca et al., 2010). Melanocytes can act as a sink for reactive oxygen species generated by keratinocytes in vitro (Pelle et al., 2005), and MC1R red hair alleles have a reduced capacity to mediate reactive oxygen species protection (Kadekaro et al., 2010). MC1R-null “red hair” mice are at higher risk of melanoma and oxidative DNA damage in the absence of UVR, suggesting that the increased synthesis of pheomelanin is itself pro-oxidant (Mitra et al., 2012). In parallel to animal models, there is some evidence that MC1R red hair alleles are associated with higher risk for keratinocyte skin cancers in a high pigmentation background (Bastiaens et al., 2001; Box et al., 2001). Skin cancer risk is likely influenced by the fact that individuals carrying MC1R variant genotypes experience more sunburns (Figure 4). Liu et al. (2016) reported that any influence of MC1R on perceived aging is independent of wrinkling; here we show that MC1R SNPs are associated with higher BG6 scores, which correlates with greater photoaging, dermal solar elastosis, and wrinkling. This suggests that these genetic variants are not acting independently of wrinkling.

We have used VEGAS2 to identify multiple genes near MC1R as potentially associated with BG6 (see Supplementary Table S4). MC1R red hair alleles, and SNPs in LD, are eQTLs for genes other than MC1R (Grundberg et al., 2012; Westra et al., 2013). However, it is also possible that the observation of additional genes via VEGAS2, and MC1R red hair alleles acting as eQTLs, are simply a product of the strong selection on the MC1R locus in Europeans and resulting long-range LD.

Three SNPs at 6p25.3 are genome-wide significantly associated with BG6, with the lowest *P*-value at rs12203592 ($P = 1.0 \times 10^{-13}$) in intron four of *IRF4* (Table 2). Variants in *IRF4* including the T allele of rs12203592 (associated with higher BG6 or more severe skin aging) have been associated with lower pigmentation (but not red hair), poorer tanning ability, greater freckling, and in adults lower mole nevus counts and a higher risk of melanoma and keratinocyte skin cancers (Duffy et al., 2010; Han et al., 2008, 2011; Sulem et al., 2007; Zhang et al., 2013). The same high photoaging T allele has been shown to reduce activity of an *IRF4* enhancer in melanocytes (Praetorius et al., 2013). The direction of effect for the T allele of rs12203592 on nevus count and melanoma risk may be reversed in adolescents, with the T allele associated with lower rates of melanoma and higher mole counts (Duffy et al., 2010; Kvakoff et al., 2011). We do not see evidence of a reversal in the BG6 association, the direction of effect of rs12203592 is consistent in adolescents and adults.

Of particular interest is the overlap in genes associated with two different phenotypes, skin patterning and pigmented spots/lesions (Jacobs et al., 2015a). Both studies report the same functional SNP in *IRF4*, rs12203592. The top BG6 SNP at MC1R is rs4268748, whereas for pigmented lesions it is rs35063026, and rs35063026 is also associated with BG6 ($P = 3.5 \times 10^{-10}$; see Supplementary Table S1). Although the LD between MC1R SNPs

rs4268748 and rs35063026 is modest ($r^2 = 0.22$; 1000 Genomes phase 1 version 3 [1000 Genomes Project Consortium et al., 2010]), rs35063026 is in LD with the red hair allele rs1805007 ($r^2 = 0.83$), and conditioning on red hair alleles at MC1R abolished the association of rs35063026 with pigmented lesions (Jacobs et al., 2015a). The minor allele of rs4268748 is modestly in LD with the minor red hair allele of both rs1805007 ($r^2 = 0.24$) and rs1805008 ($r^2 = 0.17$), likely explaining why it shows the strongest association with BG6. Conditioning on rs4268748 essentially abolishes the BG6 association of rs1805007 (see Supplementary Table S2; $P = 0.03$) and rs1805008 ($P = 0.4$). In summary, in this study the low skin pigmentation minor alleles of functional SNPs in *IRF4* and *MC1R* are associated with higher BG6 scores (i.e., more severe photoaging). The same alleles are associated with a greater percentage of the face covered with pigmented lesions (Jacobs et al., 2015a).

In Jacobs et al. (2015a), the pigmented spots/lesions measured included solar lentigines (SLs) and seborrheic keratoses. SLs are pigmented lesions that contain increased numbers of melanocytes and increased pigmentation of basal keratinocytes, and they are a consequence of chronic UVR damage to the epidermis (Cario-Andre et al., 2004; Praetorius et al., 2014). Seborrheic keratoses are common benign skin tumors appearing with age, usually pigmented, and the evidence that they are associated with sun exposure and UVR damage is less conclusive (Hafner and Vogt, 2008). Furthermore, seborrheic keratoses are seen in all skin types, whereas SLs are more common in fairer skin. Skin patterning, as measured by BG6, and epidermal dyspigmentation including SLs, are influenced by both chronological aging and photo-damage. Although the sites of the tests in the two studies differ, both are assessing an outcome of sun damage (and natural aging) on a highly sun exposed site, and thus we find overlapping genes that mediate UVR protection (*MC1R*, *IRF4*). It is worth noting that, in the case of pigmented lesions, this association is at least partly independent of skin color (Jacobs et al., 2015a), and the relationship between skin color and SL is complex (Praetorius et al., 2014), so nonpigmentary roles of these genes may also be involved (e.g. Mitra et al., 2012).

In conclusion, we have identified a (to our knowledge previously unreported) genome-wide significant association with *SLC45A2* and confirmed that functional SNPs in *IRF4* and *MC1R* are associated with BG6, a measure of wrinkling and photoaging. At each locus it is the lower-pigmentation alleles that are associated with more severe photoaging. Low-pigmentation alleles of *SLC45A2* and *MC1R* have been extensively associated with increased risk for melanoma and keratinocyte skin cancers (Bastiaens et al., 2001; Chatzinasiou et al., 2011; Han et al., 2006). MC1R red hair alleles, even in the heterozygous state, are associated with a higher burden of somatic mutations in melanoma (Robles-Espinoza et al., 2016). Photoaging is itself associated with keratinocyte skin cancers. Thus, although photoaging and general changes in appearance as we age are of interest from a quality-of-life and cosmetic point of view, the genes discovered through BG6, which can be measured easily and quantitatively in large-scale populations, may improve our understanding of cancer pathogenesis.

MATERIALS AND METHODS

Samples

Three independent cohorts were used for this analysis. For the first, adolescent twins and their siblings were recruited in Brisbane, Australia, as a part of the Brisbane Twin Nevus Study (McGregor et al., 1999; Zhu et al., 1999, 2007), totaling 3,759 adolescent twins and their siblings from 1,565 families; these were genotyped in two phases of 2,555 and 1,194 (Table 1). An unrelated set of adult twins comprising a total of 382 people from 197 families was drawn from an alcohol use study (Heath et al., 1997; Whitfield et al., 2000) collected as a part of the same cohort. The second cohort, the Twins Eyes Study in Tasmania (TEST) (Mackey et al., 2009), contributed phenotype and genotype data from 136 twins. For the third cohort, 820 Raine participants were drawn from the 22-year follow-up (the Raine Eye Health Study, conducted between March 2012 and July 2014) of the ongoing prospective Western Australian Pregnancy Cohort Study funded by the Raine Medical Research Foundation (Straker et al., 2015).

Phenotype collection

For all participants in all cohorts, imprints of the back of the left hand were made using Affinis light-body silicone elastomer (Coltène AG, Altstätten, Switzerland) (Barnes, 1973; Sarkany, 1962; Sarkany and Caron, 1965). Imprints for adolescent twins were made close to their 12th, 14th, and 16th birthdays; BG6 scores at age 12 were used preferentially, with missing data supplemented with data from age 14 years over age 16 years, because trait heritability decreases with age. Coincident with the skin measurements, a sun exposure questionnaire was administered for 2,204 of the phase 1 adolescent twins (see Supplementary Methods) (Shekar et al., 2005; Zhu et al., 1999). Imprints for nontwin siblings were collected during their twin sibling's 12th birthday visit. Imprints for the adult sample were collected at a single visit (Shekar et al., 2006, Shekaret al., 2005), as were the Raine and TEST sets.

A single rater (T. Luong) used a low-power dissection microscope to rate all skin imprints according to the six categories of the Beagley-Gibson rating system, with higher scores reflecting greater disruption of the normal pattern of the lines on the epidermis (Holman et al., 1984). The test-retest correlation for 50 BG6 measurements rescored a second time after 9 months was 0.87 (95% confidence interval = 0.66–0.97) (Shekar et al., 2006).

Ethical statement

Approval to undertake this study was obtained from the Human Research Ethics Committee of the QIMR Berghofer Medical Research Institute. The Raine Study and TEST protocols were approved by the Human Research Ethics Committee of the University of Western Australia and the University of Tasmania, respectively. All studies conformed to the Declaration of Helsinki protocols, with participants giving their written informed consent.

Genotyping and imputation

Adolescent samples were genotyped in two phases. Adolescent phase 1 and TEST samples were genotyped using Human610-Quadv1_B arrays (Illumina, San Diego, CA) at deCODE Genetics (Reykjavik, Iceland). For imputation they were merged with a larger set of individuals genotyped on Human610-Quadv1_B, and Human660W-Quad_v1_C arrays (Illumina), and filtered to a common set of 482,586 markers after quality control (QC). Adolescent phase 2 samples were genotyped on HumanOmniExpress-12v1-1_A (Illumina), HumanOmni25M-8v1-

1_B (Illumina), and HumanCoreExome 12v1-0_C arrays (Illumina) at the Diamantina Institute, University of Queensland (Queensland, Australia) and filtered to a common set of 233,546 SNPs that passed QC in all sets.

Adult samples were genotyped on 317K, HumanCNV370-Quadv3_C (Illumina), Human610-Quadv1_B, and Human660W-Quad_v1_C arrays by the Center for Inherited Disease Research (Johns Hopkins University, Baltimore, MD), the University of Helsinki (Helsinki, Finland), and deCODE. For imputation they were merged with a large set of individuals genotyped on the same chips and filtered to a common set of 276,755 SNPs after QC.

Adolescent, adult, and TEST SNP data were excluded if they failed standard SNP filters in GenomeStudio (Illumina) as recommended by Illumina, and further cleaned out if BeadStudio GenCall (Illumina) was less than 0.7, minor allele frequency was less than 1%, Hardy-Weinberg equilibrium P -value was less than 10^{-6} , or call rate was less than 95%. X chromosome SNPs were filtered if their heterozygosity was greater than 1% in males. Samples with missingness of 5% or greater were dropped, and principal component analysis in Eigenstrat (Price et al., 2006) was used to exclude samples with a principal component 1/principal component 2 value of more than ± 6 standard deviations from 1000 Genomes Project phase 1 European populations (1000 Genomes Project Consortium et al., 2010). Identity by descent estimates in PLINK version 1.07 (Purcell et al., 2007) were used to confirm pedigree structure. Because the analyses for these four sets were done in Merlin-offline, which explicitly models familial relationships, samples were not filtered for relatedness.

Adolescent, adult, and TEST samples were then imputed to the November 23, 2010 release of the 1000 Genomes Phase 1 version 3 (1000 Genomes Project Consortium et al., 2010) using minimac (July 17, 2013 version) (Fuchsberger et al., 2015; Howie et al., 2012) with markers with ≤ 1 copy of the minor alleles removed. Imputation was performed in two phases based on chip overlap: (i) adolescent phase 1 and TEST; (ii) adult and adolescent phase 2. After imputation the four sets were analyzed separately. For genotyped SNPs original genotype calls were used; for imputed SNPs imputation dosage scores were used.

Raine participants were genotyped on the 660 Quad Array (Illumina) at the Centre for Applied Genomics (Toronto, Ontario, Canada). As part of QC, individuals were excluded if they had identity by descent of π greater than 0.1875 with another participant or greater than 3% missingness. SNPs were filtered out at Hardy-Weinberg equilibrium P -value greater than 5.7×10^{-7} , missingness greater than 5%, or a minor allele frequency less than 0.01. Potential population stratification was controlled for via a principal component analysis using Eigenstrat (Price et al., 2006). Imputation of 22 autosomes was performed using MaCH, version 2.3.0 software (Li et al., 2010) and the November 23, 2010, version of the 1000 Genome Project phase 1 European reference panel (1000 Genomes Project Consortium et al., 2010).

Genome-wide association scans

For the adolescent phases 1 and 2, adult, and TEST samples, GWASs were performed separately using 6-point BG6 scores as a continuous variable and imputed dosage genotypes using the Merlin-offline software (<http://www.sph.umich.edu/csg/abecasis/Merlin/>) under an additive model that explicitly models relatedness and produces unbiased estimates with standard errors corrected for the sample size inflation due to familial relationship (Chen and Abecasis, 2007). Sex, age, age², sex \times age, and sex \times age² were included as covariates.

For Raine, a linear regression analysis was performed in R (R Core Team, 2014) using the ProbABEL package (Aulchenko et al., 2010). The model was adjusted for age, sex, age², age × sex, age² × sex, and the five principal components that accounted for the population stratification.

Postimputation QC and meta-analysis

Imputation quality was assessed by the ratio of imputed genotype dosage variance to the variance of the allele frequency under Hardy-Weinberg equilibrium (MaCH \hat{r}^2) (Li et al., 2010). For adolescent phases 1 and 2, TEST, and adult sets, SNPs with a \hat{r}^2 less than 0.5 or minor allele frequency less than 0.05 in any set were excluded; for Raine, SNPs with imputation quality less than 0.5 were excluded; retained SNPs were meta-analyzed using PLINK version 1.9 (<https://www.cog-genomics.org/plink2>) (Chang et al., 2015), with variant β coefficients weighted by their inverse variance. Between-study heterogeneity was assessed using the I^2 value (DerSimonian and Laird, 1986).

CONFLICT OF INTEREST

The authors state no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2017.04.026>.

REFERENCES

- 1000 Genomes Project Consortium, Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, et al. A map of human genome variation from population-scale sequencing. *Nature* 2010;467(7319):1061–73.
- Abecasis GR, Cherny SS, Cookson WO, Cardon LR. Merlin—rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 2002;30:97–101.
- Aulchenko YS, Struchalin MV, van Duijn CM. ProbABEL package for genome-wide association analysis of imputed data. *BMC Bioinformatics* 2010;11:134.
- Barnes IE. Techniques for the replication of skin surfaces. A new method. *Br J Dermatol* 1973;89:277–83.
- Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ, et al. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. *Am J Hum Genet* 2001;68:884–94.
- Battistutta D, Pandeya N, Strutton GM, Fourtanier A, Tison S, Green AC. Skin surface topography grading is a valid measure of skin photoaging. *Photodermatol Photoimmunol Photomed* 2006;22:39–45.
- Beagley J, Gibson IM. Changes in skin condition in relation to degree of exposure to ultraviolet light. Perth: Western Australia Institute of Technology School of Biology; 1980.
- Bilac C, Sahin MT, Ozturkcan S. Chronic actinic damage of facial skin. *Clin Dermatol* 2014;32:752–62.
- Box NF, Duffy DL, Irving RE, Russell A, Chen W, Griffyths LR, et al. Melanocortin-1 receptor genotype is a risk factor for basal and squamous cell carcinoma. *J Invest Dermatol* 2001;116:224–9.
- Cario-Andre M, Lepreux S, Pain C, Nizard C, Noblesse E, Taieb A. Perilesional vs. lesional skin changes in senile lentigo. *J Cutan Pathol* 2004;31:441–7.
- Chang AL, Atzmon G, Bergman A, Brugmann S, Atwood SX, Chang HY, et al. Identification of genes promoting skin youthfulness by genome-wide association study. *J Invest Dermatol* 2014;134:651–7.
- Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. Second-generation PLINK: rising to the challenge of larger and richer datasets. *GigaScience* 2015;4:7.
- Chatzinasiou F, Lill CM, Kypreou K, Stefanaki I, Nicolaou V, Spyrou G, et al. Comprehensive field synopsis and systematic meta-analyses of genetic association studies in cutaneous melanoma. *J Natl Cancer Inst* 2011;103:1227–35.
- Chen WM, Abecasis GR. Family-based association tests for genomewide association scans. *Am J Hum Genet* 2007;81:913–26.
- Costin GE, Valencia JC, Vieira WD, Lamoreux ML, Hearing VJ. Tyrosinase processing and intracellular trafficking is disrupted in mouse primary melanocytes carrying the underwhite (uw) mutation. A model for oculocutaneous albinism (OCA) type 4. *J Cell Sci* 2003;116(Pt. 15):3203–12.
- DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986;7:177–88.
- Dooley CM, Schwarz H, Mueller KP, Mongera A, Konantz M, Neuhaus SC, et al. Slc45a2 and V-ATPase are regulators of melanosomal pH homeostasis in zebrafish, providing a mechanism for human pigment evolution and disease. *Pigment Cell Melanoma Res* 2013;26:205–17.
- Duffy DL, Box NF, Chen W, Palmer JS, Montgomery GW, James MR, et al. Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. *Hum Mol Genet* 2004;13:447–61.
- Duffy DL, Iles MM, Glass D, Zhu G, Barrett JH, Hoiom V, et al. IRF4 variants have age-specific effects on nevus count and predispose to melanoma. *Am J Hum Genet* 2010;87:6–16.
- Fritschi L, Battistutta D, Strutton GM, Green A. A non-invasive measure of photoageing. *Int J Epidemiol* 1995;24:150–4.
- Fuchsberger C, Abecasis GR, Hinds DA. minimac2: faster genotype imputation. *Bioinformatics* 2015;31:782–4.
- Gandini S, Sera F, Cattaruzza MS, Pasquini P, Zanetti R, Masini C, et al. Meta-analysis of risk factors for cutaneous melanoma: III. Family history, actinic damage and phenotypic factors. *Eur J Cancer* 2005;41:2040–59.
- Graf J, Hodgson R, van Daal A. Single nucleotide polymorphisms in the MTP gene are associated with normal human pigmentation variation. *Hum Mutat* 2005;25:278–84.
- Green AC. Premature ageing of the skin in a Queensland population. *Med J Aust* 1991;155:473–4. 7–8.
- Grundberg E, Small KS, Hedman AK, Nica AC, Buil A, Keildson S, et al. Mapping cis- and trans-regulatory effects across multiple tissues in twins. *Nat Genet* 2012;44:1084–9.
- GTEX Consortium. The Genotype-Tissue Expression (GTEx) project. *Nat Genet* 2013;45:580–5.
- Hafner C, Vogt T. Seborrheic keratosis. *J Dtsch Dermatol Ges* 2008;6:664–77.
- Han J, Kraft P, Colditz GA, Wong J, Hunter DJ. Melanocortin 1 receptor variants and skin cancer risk. *Int J Cancer* 2006;119:1976–84.
- Han J, Kraft P, Nan H, Guo Q, Chen C, Qureshi A, et al. A genome-wide association study identifies novel alleles associated with hair color and skin pigmentation. *PLoS Genet* 2008;4(5):e1000074.
- Han J, Qureshi AA, Nan H, Zhang J, Song Y, Guo Q, et al. A germline variant in the interferon regulatory factor 4 gene as a novel skin cancer risk locus. *Cancer Res* 2011;71:1533–9.
- Heath AC, Buchholz KK, Madden PA, Dinwiddie SH, Slutske WS, Bierut LJ, et al. Genetic and environmental contributions to alcohol dependence risk in a national twin sample: consistency of findings in women and men. *Psychol Med* 1997;27:1381–96.
- Holman CD, Armstrong BK, Evans PR, Lumsden GJ, Dallimore KJ, Meehan CJ, et al. Relationship of solar keratosis and history of skin cancer to objective measures of actinic skin damage. *Br J Dermatol* 1984;110:129–38.
- Howie B, Fuchsberger C, Stephens M, Marchini J, Abecasis GR. Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. *Nat Genet* 2012;44:955–9.
- Hughes MC, Strutton GM, Fourtanier A, Green AC. Validation of skin surface microtopography as a measure of skin photoaging in a subtropical population aged 40 and over. *Photodermatol Photoimmunol Photomed* 2012;28:153–8.
- Hughes MC, Williams GM, Baker P, Green AC. Sunscreen and prevention of skin aging: a randomized trial. *Ann Intern Med* 2013;158:781–90.
- Jacobs LC, Hamer MA, Gunn DA, Deelen J, Lall JS, van Heemst D, et al. A genome-wide association study identifies the skin color genes IRF4, MC1R, ASIP, and BNC2 influencing facial pigmented spots. *J Invest Dermatol* 2015a;135:1735–42.
- Jacobs LC, Liu F, Pardo LM, Hofman A, Uitterlinden AG, Kayser M, et al. IRF4, MC1R and TYR genes are risk factors for actinic keratosis independent of skin color. *Hum Mol Genet* 2015b;24:3296–303.
- Kadekaro AL, Leachman S, Kavanagh RJ, Swope V, Cassidy P, Supp D, et al. Melanocortin 1 receptor genotype: an important determinant of the damage response of melanocytes to ultraviolet radiation. *FASEB J* 2010;24:3850–60.
- Kvaskoff M, Whiteman DC, Zhao ZZ, Montgomery GW, Martin NG, Hayward NK, et al. Polymorphisms in nevus-associated genes MTAP, PLA2G6, and IRF4 and the risk of invasive cutaneous melanoma. *Twin Res Hum Genet* 2011;14:422–32.

- Laville V, Le Clerc S, Ezzedine K, Jdid R, Taing L, Labib T, et al. A genome-wide association study in Caucasian women suggests the involvement of HLA genes in the severity of facial solar lentigines. *Pigment Cell Melanoma Res* 2016;29:550–8.
- Lavker RM, Kwong F, Klighman AM. Changes in skin surface patterns with age. *J Gerontol* 1980;35:348–54.
- Le Clerc S, Taing L, Ezzedine K, Latreille J, Delaneau O, Labib T, et al. A genome-wide association study in Caucasian women points out a putative role of the STXBP5L gene in facial photoaging. *J Invest Dermatol* 2013;133:929–35.
- Li Y, Willer CJ, Ding J, Scheet P, Abecasis GR. MaCH: using sequence and genotype data to estimate haplotypes and unobserved genotypes. *Genet Epidemiol* 2010;34:816–34.
- Liu F, Hamer Merel A, Deelen J, Lall Japal S, Jacobs L, van Heemst D, et al. The MC1R gene and youthful looks. *Curr Biol* 2016;26(May 9):1–8.
- Mackey DA, Mackinnon JR, Brown SA, Kearns LS, Ruddle JB, Sanfilippo PG, et al. Twins eye study in Tasmania (TEST): rationale and methodology to recruit and examine twins. *Twin Res Hum Genet* 2009;12:441–54.
- Maresca V, Flori E, Bellei B, Aspite N, Kovacs D, Picardo M. MC1R stimulation by alpha-MSH induces catalase and promotes its re-distribution to the cell periphery and dendrites. *Pigment Cell Melanoma Res* 2010;23:263–75.
- McGregor B, Pfitzner J, Zhu G, Grace M, Eldridge A, Pearson J, et al. Genetic and environmental contributions to size, color, shape, and other characteristics of melanocytic naevi in a sample of adolescent twins. *Genet Epidemiol* 1999;16:40–53.
- Mishra A, Macgregor S. VEGAS2: software for more flexible gene-based testing. *Twin Res Hum Genet* 2015;18:86–91.
- Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. *Nature* 2012;491(7424):449–53.
- Nica AC, Parts L, Glass D, Nisbet J, Barrett A, Sekowska M, et al. The architecture of gene regulatory variation across multiple human tissues: the MuTHER study. *PLoS Genet* 2011;7:e1002003.
- Pelle E, Mammone T, Maes D, Frenkel K. Keratinocytes act as a source of reactive oxygen species by transferring hydrogen peroxide to melanocytes. *J Invest Dermatol* 2005;124:793–7.
- Praetorius C, Grill C, Stacey SN, Metcalf AM, Gorkin DU, Robinson KC, et al. A polymorphism in IRF4 affects human pigmentation through a tyrosinase-dependent MITF/TFAP2A pathway. *Cell* 2013;155:1022–33.
- Praetorius C, Sturm RA, Steingrimsson E. Sun-induced freckling: ephelides and solar lentigines. *Pigment Cell Melanoma Res* 2014;27:339–50.
- Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet* 2006;38:904–9.
- Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analysis. *Am J Hum Genet* 2007;81:559–75.
- R Core Team. R: a language and environment for statistical computing. Vienna, Austria; 2014.
- Robles-Espinoza CD, Roberts ND, Chen S, Leacy FP, Alexandrov LB, Pornputtapong N, et al. Germline MC1R status influences somatic mutation burden in melanoma. *Nat Comm* 2016;7:12064.
- Sarkany I. A method for studying the microtopography of the skin. *Br J Dermatol* 1962;74:254–9.
- Sarkany I, Caron GA. Microtopography of the human skin. Studies with metal-shadowed replicas from plastic impressions. *J Anat* 1965;99:359–64.
- Seddon JM, Egan KM, Zhang Y, Gelles EJ, Glynn RJ, Tucker CA, et al. Evaluation of skin microtopography as a measure of ultraviolet exposure. *Invest Ophthalmol Vis Sci* 1992;33:1903–8.
- Shekar SN, Duffy DL, Montgomery GW, Martin NG. A genome scan for epidermal skin pattern in adolescent twins reveals suggestive linkage on 12p13.31. *J Invest Dermatol* 2006;126:277–82.
- Shekar SN, Luciano M, Duffy DL, Martin NG. Genetic and environmental influences on skin pattern deterioration. *J Invest Dermatol* 2005;125:1119–29.
- Song X, Mosby N, Yang J, Xu A, Abdel-Malek Z, Kadakara AL. alpha-MSH activates immediate defense responses to UV-induced oxidative stress in human melanocytes. *Pigment Cell Melanoma Res* 2009;22:809–18.
- Straker LM, Hall GL, Mountain J, Howie EK, White E, McArdle N, et al. Rationale, design and methods for the 22 year follow-up of the Western Australian Pregnancy Cohort (Raine) Study. *BMC Public Health* 2015;15:663.
- Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Magnusson KP, et al. Genetic determinants of hair, eye and skin pigmentation in Europeans. *Nat Genet* 2007;39:1443–52.
- Westra HJ, Peters MJ, Esko T, Yaghootkar H, Schurmann C, Kettunen J, et al. Systematic identification of trans eQTLs as putative drivers of known disease associations. *Nat Genet* 2013;45:1238–43.
- Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G, et al. Effects of HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000;66:1246–58.
- Zhang M, Song F, Liang L, Nan H, Zhang J, Liu H, et al. Genome-wide association studies identify several new loci associated with pigmentation traits and skin cancer risk in European Americans. *Hum Mol Genet* 2013;22:2948–59.
- Zhu G, Duffy DL, Eldridge A, Grace M, Mayne C, O’Gorman L, et al. A major quantitative-trait locus for mole density is linked to the familial melanoma gene CDKN2A: a maximum-likelihood combined linkage and association analysis in twins and their sibs. *Am J Hum Genet* 1999;65:483–92.
- Zhu G, Montgomery GW, James MR, Trent JM, Hayward NK, Martin NG, et al. A genome-wide scan for naevus count: linkage to CDKN2A and to other chromosome regions. *Eur J Hum Genet* 2007;15:94–102.