Quantitative Measures of the Effect of the Melanocortin 1 Receptor on Human Pigmentary Status[1]

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Variation in human hair and skin color is the most striking visible aspect of human genetic variation. The only gene known to exert an effect on pigmentary within the normal population is the melanocortin-1 receptor (MC1R). Previous studies have used a Mendelian framework to relate MC1R genotype to phenotype, by measuring pigmentary status using categorical scales. Such approaches are inadequate. We report results using direct measures of hair color using objective colorimetric dimensions and HPLC determined hair melanins. We have linked MC1R genotype with chemical measures of melanin quantity and type and objective phenotype measures of color. MC1R genotype was predictive of hair melanin expressed as the ratio of the $\log_e$ of eumelanin to pheomelanin ratio, with a dosage effect evident: MC1R homozygote mean, 1.46; heterozygote, 4.44; and wild type, 5.81 ($p < 0.001$). Approximately 67% of the variance in this model could be accounted for in terms of MC1R genotype. There was also a relation between MC1R status and hair color, most prominently for the b^* axis ($p < 0.001$), but also for the a^* and L^* scales ($L^*a^*b^*$, CIE). We show for one of the most polymorphic human traits that it is possible to demonstrate meaningful relations between various physical characteristics: DNA sequence diversity, hair-wavelength-specific reflectance patterns, and chemical melanin assays.

Key words: pigment/ultraviolet radiation/skin color/skin type/red hair.
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The goal of genetics or, to give it its historical name, experimental evolution, is to explain biologic diversity: to account for variation in form. Perhaps the most striking visible variation in man worldwide is that of skin and hair color. The factors leading to such diversity are of great scientific interest. Whereas for most noncutaneous traits so far examined variability within populations (or continents) greatly exceeds that between populations, for skin and hair color this is not the case (reviewed in Bodmer and Cavalli-Sforza, 1976; Relethford, 2003). Explaining the roles of selection and neutral evolutionary change on skin evolution therefore remains a major challenge (Harding *et al*, 2000; Rees, 2000, 2002, 2003).

Despite many introductory remarks in genetics textbooks about the importance of genes in determining hair and skin color, it is only recently, following the identification of the melanocortin 1 receptor (MC1R) as a control point in the production of pheomelanin and eumelanin, that molecular insight into such diversity within the normal population has been obtained (Ha and Rees, 2002; Rees, 2003).

Eight years ago we showed that sequence variation at the MC1R was associated with red hair and pale skin (Valverde *et al*, 1995; Rees, 2003). Subsequent studies have revealed the MC1R, a 317-amino-acid, G-coupled receptor, to be highly polymorphic, with many sequence variants being associated with red hair, pale skin, a propensity to burn rather than tan, and an elevated risk for melanoma and nonmelanoma skin cancer (Box *et al*, 1997, 2001a, b; Smith *et al*, 1998; Healy *et al*, 2000, 2001; Palmer *et al*, 2000; Bastiaens *et al*, 2001; Kennedy *et al*, 2001). Because sequence diversity at the MC1R is high, and because the prevalence of sequence changes with functional consequences is high, terms such as wild type (WT) and heterozygote (HT) may be misunderstood. For present purposes we use the terms heterozygote or homozygote (HM) to refer to persons with sequence changes on one or two MC1R alleles, respectively, that are thought to show impairment of MC1R function, based on either biochemical or phenotype assays. For alleles without such changes, when the agreed consensus sequence is present (Harding *et al*, 2000), the sequence is referred to as wild type, or when there are sequence changes present which, in the light of present evidence, are not known to be of functional significance, pseudo-wild-type.

Abbreviations: HM, homozygote; HT, heterozygote; LEPR, $\log_e$ eumelanin/pheomelanin ratio; MC1R, melanocortin 1 receptor; WT, wild type.

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An added complication is that sequence changes at the MC1R, even when they are thought to be of functional significance, are not biochemically equivalent (Schioth *et al*, 1999; Healy *et al*, 2001). Many of the reported changes, causally associated with a particular phenotype, are not complete loss-of-function mutations but rather show diminished function of signaling to varying degrees; that is, they show quantitative diminution of function rather than absent signaling activity (non-sense mutations that would often be expected to show complete loss of function have not been examined in the appropriate assays for the MC1R).

Although the number of partial loss-of-function mutations at the MC1R is large, three are relatively common in Northern European populations: the R151C, the R160W, and the D294H (Box *et al*, 1997; Smith *et al*, 1998; Schioth *et al*, 1999; Flanagan *et al*, 2000; Healy *et al*, 2001). These sequence variants are not however, functionally equivalent (Healy *et al*, 2001).

One additional particular variant, the V60L, is worthy of special note, because it is common with an allele frequency of 0.15 in some European populations. Functional assays show an impairment of function compared with WT but less than that seen with the R151C, R160W, or D294H alleles (Schioth *et al*, 1999). Early association studies were inconclusive about its role, but more recent studies have successfully modeled it either as a low-penetrance diminished-function allele or as a risk factor with a lower risk ratio than some of the other alleles such as the R151C, the R160W, and the D294H (reviewed in Flanagan *et al*, 2000; Palmer *et al*, 2000; Box *et al*, 2001b; Rees, 2003). Both these interpretations are compatible with the idea that the V60L change impairs the function of the receptor to a limited degree. Analyses for this article were performed with and without the assumption that the V60L allele shows impaired function, although any differences detected were small.

Using the above definitions there is a clear HT or dosage effect on skin type, freckling, and cancer risk; that is, HT show a different phenotype from either HM or WT or pseudo-WT individuals (Palmer *et al*, 2000; Flanagan *et al*, 2000; Healy *et al*, 2000; Box *et al*, 2001b).

A significant limitation of previous studies, including our own, has been the relative lack of attention paid to defining the phenotype accurately (Rees, 2002; Valverde *et al*, 1995). All studies to date have classified hair color crudely—into broad groupings like red or brown. This is unsatisfactory for a number of reasons. Ambient lighting influences color perception, misclassification of a continuous variable will diminish the power of statistical testing and the strength of any relation observed, and results from different populations and authors are almost impossible to compare. Analogous criticisms can be made about the recording of sun sensitivity using the Fitzpatrick or other ordinal scales, which are highly subjective and show limited instrument validity (Fitzpatrick, 1988; Rampen *et al*, 1988).

In this work we describe more appropriate models to link sequence diversity with pigmentary status measured, wherever possible, on a continuous scale. We therefore link (1) genotype with (2) objective measures of hair melanins, assayed using HPLC detection, and (3) hair color based on widely accepted objective measures of color using the CIE international defined scales (Kollias, 1995; Alaluf *et al*, 2002; Takiwaki *et al*, 2002). This approach not only allows assessment of the quantitative contribution of a locus to variation, but also allows estimates of what variation is not accounted for and therefore what remains to be discovered. Where we do refer to subjective hair color or Fitzpatrick skin typing scale it is either to allow comparison with earlier studies or as a simple descriptive term.

Results

Sequencing was unsuccessful in 3 of the 101 cases. Of the remaining 98, 43 were HM, 29 HT, and 26 WT/pseudo-WT (for definitions see introduction and Materials and Methods).

As expected, freckles were associated with genotype with evidence of a dosage effect ($p < 0.0001$) (Table I): Individuals who answered "yes" in response to a history of freckling in response to sunshine had a lower LEPR compared to those without freckles, 2.43 ± 0.38 ($n = 40$) versus 5.65 ± 0.34 ($n = 17$) (LEPR means, SEM) ($p < 0.01$). There appeared to be a relation between eye color and hair eumelanin (Fig 1; Table II) with brown eyes showing higher melanin values than those with blue or green eyes. This association was more marked for eumelanin than either pheomelanin or LEPR (Table II) with an under representation of brown eyes in MC1R HM of borderline formal significance ($p = 0.049$, Fisher Freeman-Halton test) (Table III). Caution,

Table I. Relationship between MC1R genotype and freckles[a]

	Freckles	No freckles
HM	23 (96)	1 (4)
HT	10 (62)	6 (38)
WT	7 (41)	10 (59)

[a]The presence of freckles was classified categorically. Data are reported as number (%).

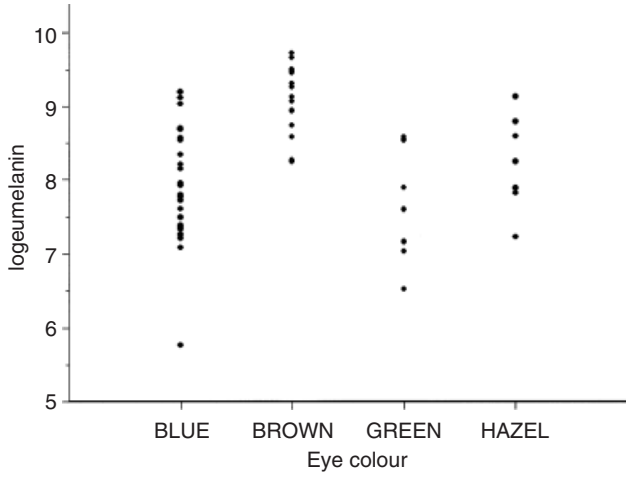

Figure 1
Eye color and hair $\log_e$ eumelanin (ng/mg). The relationship between subjective eye color classification and HPLC quantification of hair $\log_e$ eumelanin content expressed in nanograms per milligram.

Table II. The relationship between eye color and HPLC hair log_e melanins (ng/mg)[a]

Eye color	Mean hair log_e	SEM	SD	n
	Eumelanin			
Blue	7.91	0.14	0.74	29
Brown	9.09	0.12	0.47	15
Green	7.62	0.29	0.78	7
Hazel	8.32	0.23	0.64	8
	Pheomelanin			
Blue	5.38	0.32	1.66	29
Brown	3.26	0.24	0.88	15
Green	6.19	0.94	2.1	7
Hazel	4.78	0.72	1.92	8

[a]Cross-tabulation of eye color and HPLC-derived hair log_e eumelanin and log_e pheomelanin products expressed in ng/mg.

Table III. Relationship between MC1R genotype and eye color[a]

	Eye color			
Observed	Blue	Brown	Green	Hazel
HM	16	1[b]	3	3
HT	5	6	2	3
WT	7	7	1	2

[a]Contingency table of relationship between MC1R genotype and eye color.
[b]$p = 0.049$, Fisher-Freeman-Halton test.

however, is warranted because these were not preplanned comparisons.

LEPR and the hair color variables a* and b* were obviously related, with R^2 values of 0.51 and 0.47, respectively, with a lower R^2 value for L* of 0.22 (all $p < 0.001$) (Fig 2*a–c*). MC1R genotype was also predictive of the LEPR with a clear dosage effect: mean HM, 1.46; mean HT, 4.44 and mean WT, 5.81 ($p < 0.001$) (Fig 3; Table IV). Approximately 67% of the variance in this model could be accounted for in terms of MC1R genotype. If the V60L was included as a loss-of-function allele differences among the three genotypes were still evident but the respective means were HM, 1.91; HT, 4.62; and WT, 6.10; with an R^2 of 0.55 ($p < 0.001$). Other analyses were not markedly altered by inclusion of the V60L allele as a diminished-function allele (data not shown).

We then examined the relation between MC1R genotype and hair color measures assessed colorimetrically. Again, large effects are seen, most prominently for the b* axis (Fig 4), although relations are present with the L* and a* scales with a stepwise progression of the mean (Table V). ANOVA studies showed significant differences between all MC1R genotypes for the b* scores; for the a* scores formal significance between HM and WT and, if two outliers were excluded, also between HM and HT; for L* only HM and WT were significantly different, but a dose response was seen between all the genotypes (Table V).

No obvious trends were evident for the relation between genotype and forehead and lower back L*a*b* scores (data not shown). The relation among skin type (using a modified Fitzpatrick scale), LEPR, and genotype is shown (Fig 5). There is a clustering of values with low skin type and low LEPR who are HM. There is a relation between genotype and skin type assessed using an ordinal scale (median HM, 1.0; median HT, 2.0; median WT, 2.25; $p < 0.001$, Kruskal–Wallis ANOVA) but with the difference between HM and HT more marked than that between HT and WT.

Discussion

The aim of our study was to improve on previous attempts to study the relation between human pigmentation and MC1R genotype. We wanted to use phenotypic assessment measures that are capable of being used by different investigators working on diverse populations in different parts of the world. Only with such standardization of protocols and methods can we hope to understand the evolutionary genetics of human pigmentation. In this article we have therefore studied the relation among genotype, hair melanins assessed using standard HPLC methods, and hair

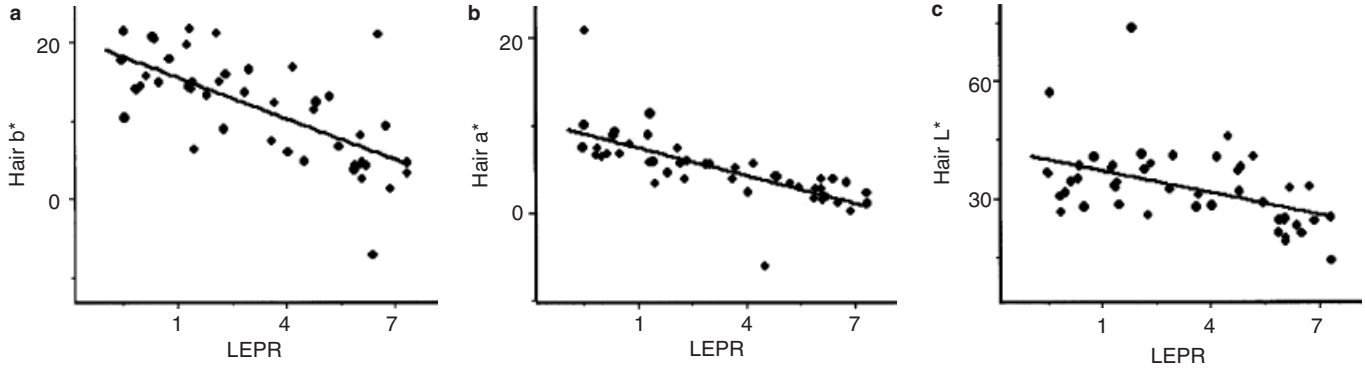

Figure 2
Hair melanins and tristimulus L*, a*, and b* scores. The relationship between hair LEPR determined using HPLC methods and triplicate chromameter readings of parietal scalp hair.

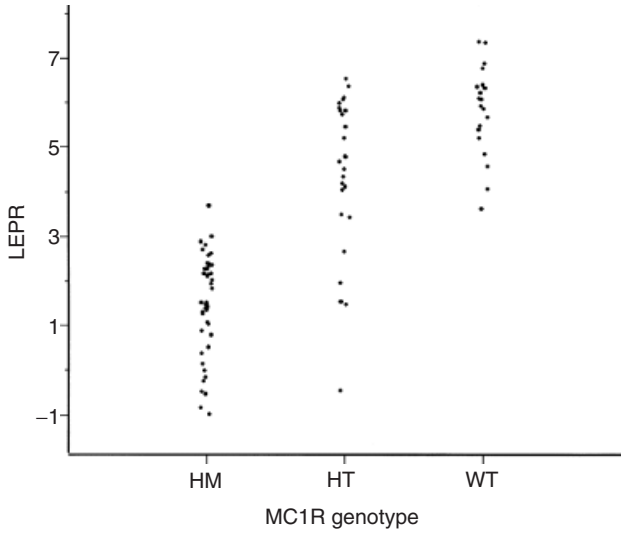

Figure 3
MC1R genotype and hair melanins. The relationship between MC1R genotype and HPLC-determined hair LEPR.

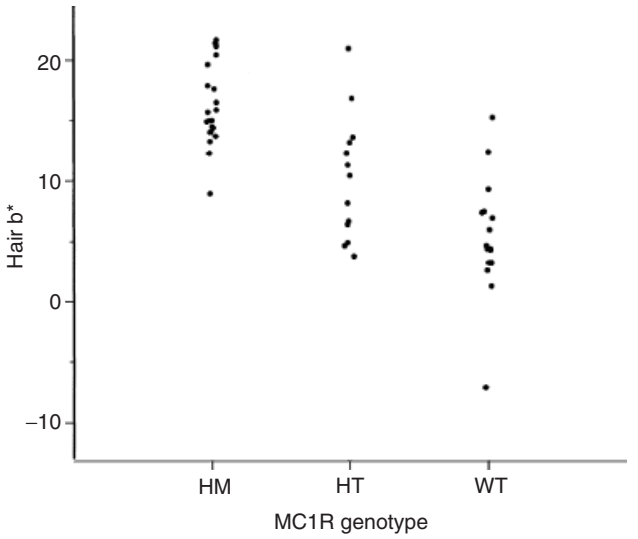

Figure 4
MC1R genotype and hair tristimulus b* score. The relationship between MC1R genotype and the mean of triplicate b* score tristimulus readings from parietal scalp hair. Positive b* values indicate greater shades of yellow.

color assessed using objective measures traceable to physical standards.

Our results suggest that it is possible to move beyond a crude classification of hair color by subjective category: genotype can be related on the one hand to hair melanin and on the other to hair color, measured objectively, without any need for human judgment of color. At present, such results are the only example where it is possible to relate genetic variation in the normal population to a visible human phenotype: our present work sets limits to the strength of this relation.

We observed a clear relation between genotype and hair L*a*b* color parameters, and between genotype and hair melanin, with evidence of a dose response. Much of the variability can be accounted for in terms of the MC1R genotype factor: that which remains must be due to other factors, principally we would assume other genetic loci. There are, however, caveats that need consideration. First, we enriched for reds with the sample chosen not being random. The proportion of variability "explained" is, as always, sample dependent, and for a random population the figure would be lower. Conversely, because there is a tendency for hair color to change with age and in particular for melanin to diminish with age the effect of genotype on color and melanin may have been underestimated had all those studied been young adults. In reality examination of the raw data with age showed little evidence for this, although some ascertainment bias could have contributed to this (if they had not been red they were less likely to be included).

The predictive power of genotype for hair color was greatest for the b* score (yellow-blue) followed by a* (red-green) and L* (light-dark), with the strongest relation between hair melanin and colorimetry seen with the a* and b* axes. We have not explored including all these terms in the regression simultaneously but it is possible that a better fit may be obtained: such a study would be more meaningful if carried out on a random population.

By contrast the essentially negative findings between genotype and skin color at two body sites (forehead and back) are worthy of attention. We were unable to demonstrate a statistically significant association between lower back skin color and genotype in contrast to previous work showing a correlation between buttock skin color and MC1R status (Flanagan *et al*, 2001; Ha *et al*, 2003). This may reflect chance or a real difference between sun-exposed and sun-protected sites. By contrast there were significant differences between skin type and genotype as has been found previously (Healy *et al*, 2000). One interpretation of these findings is that while the relation between hair color and genotype is relatively large, that between skin color and genotype may be less robust, perhaps influenced by

Table IV. ANOVA comparisons for relation between MC1R genotype and hair LEPR[a]

Genotype	Mean LEPR	n	Pairwise estimate		SEM	p value
HM	1.46	42	WT–HM	4.35	0.36	<0.001
HT	4.44	27	HT–HM	2.98	0.33	<0.001
WT	5.81	20	WT–HT	1.37	0.39	<0.001

[a]Summary of ANOVA statistics for MC1R genotype and HPLC-derived hair LEPR.

Table V. ANOVA comparisons for relationship between MC1R genotype and hair chromameter readings[a]

	b*			a*			L*		
Mean (n = 50)									
HM	16.04			6.77			36.91		
HT	10.19			4.01			33.13		
WT	5.34			2.61			29.96		
	Hair b*			**Hair a***			**Hair L***		
	Pairwise estimate	**SEM**	**p value**	**Pairwise estimate**	**SEM**	**p value**	**Pairwise estimate**	**SEM**	**p value**
HT–WT	4.85	1.63	<0.05	1.4	1.22	>0.05	6.17	3.53	>0.05
HM–HT	5.85	1.54	<0.05	2.76	1.15	>0.05[b]	3.78	3.34	>0.05
HM–WT	10.7	1.45	<0.05	4.16	1.08	<0.05	9.95	3.14	<0.05

[a]Summary of ANOVA comparisons for MC1R genotype and hair chromameter readings in 50 individuals. Pairwise estimates, SEM, and p values are shown for HT–WT, HM–HT, and HM–WT comparisons for each hair L*a*b* reading.
[b]$p < 0.05$ if two outliers excluded.

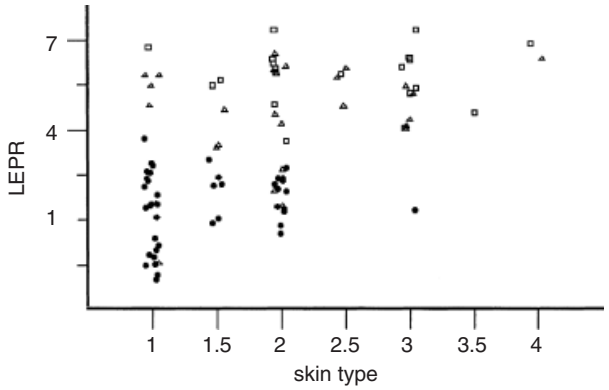

Figure 5
Modified Fitzpatrick skin type classification and MC1R genotype by hair melanin. The relationship between skin type, MC1R genotype and HPLC-determined hair LEPR. (Closed circles, HM; open triangles, HT; open squares, WT or pseudo-WT.)

ultraviolet radiation exposure habits. Again, the contrast with skin type, which did show a relation with genotype, is worth making, because our data support the view that skin type is more than just an instantaneous proxy measure for skin color (Rees, 2002). Finally, although the association between eye color and MC1R status is of borderline significance, it supports recent work by Sturm *et al* (2003).

In conclusion, we show for one of the most diverse human characteristics with a large genetic contribution that it is possible to show meaningful relations between various physical characteristics without the need for subjective scoring: DNA sequence diversity, hair wavelength reflectance patterns, and melanin measures. Future genotype/phenotype studies of human pigmentation, in particular, studies of the evolution of human diversity, would be better to use methods similar to the ones described.

Materials and Methods

Subjects A total of 101 healthy volunteers (59 women and 42 men) from Northern Britain were recruited for sequencing of the MC1R and hair melanin assays. A convenient subset sample 59, not selected because of phenotype, were available for a more detailed phenotype assessment including multiple body site skin colorimetry and hair colorimetry. People with red hair were deliberately overrepresented in recruitment to lend power to the analyzes so that of the 101, 50 were *subjectively* classed as red. For the initial screening a standard L'Oreal hair color chart was used to assign phenotype based on recall of hair color when the subjects were aged 21 or at the time of examination if they were not yet 21 (n = 6). The age range of subjects was 6 to 72 y with a median of 35 y. All volunteers gave informed consent under the statutory responsibility of the appropriate ethics committee and studies conformed with the principles of the Declaration of Helsinki principles (2002) in so far as they apply to nontherapeutic research (http://www.wma.net/e/policy/b3.htm).

Objective phenotype assessment Of 59 volunteers triplicate readings from hair overlying the left parietal region were taken with a tristimulus colorimeter (Minolta chromameter CR300, Osaka, Japan) in 50 (in 9 the hair was dyed or too short). Color was represented using the L*a*b* system (Commission Internationale de l'Eclairage, CIE (http://members.eunet.at/cie/) in which color is represented as summary values in three dimensions designed to be commensurable with human color perception: L*, representing lightness, on a scale of 0 to 100 where 0 is black and 100 is light; a*, representing red-green, on a scale from +60 to −60, where positive values indicate increasing shades of red; and b*, representing a yellow-blue, on a scale from +60 to −60, with positive values representing increasing shades of yellow. This method has been widely used in the study of human skin color and phototype (Kollias, 1995; Alaluf *et al*, 2002; Takiwaki *et al*, 2002), although it has not been recently used in genetic studies of hair color (Reed, 1952). Skin color was also measured on the forehead and lower back in triplicate in 56 subjects. To allow comparison with earlier studies, skin typing using a modified Fitzpatrick scale as previously described was carried out (Flanagan *et al*, 2000; Healy *et al*, 2000).

Freckling tendency classed as a categorical variable in response to the question "do you tend to get freckles when you go in the sun?" was available for 57 subjects, because 2 of 59 subjects were unable to respond owing to sun avoidance. Eye color was also assessed in 59 subjects as blue, green, brown, or hazel. Of the 98 of 101 subjects for whom sequencing results were available, hair melanins were determined in 89 persons, because 9 individuals had dyed hair or hair that was too short to obtain an adequate sample from.

HPLC was used to quantify the amount of eumelanin and pheomelanin according to the methods of Ito and Fujita (1985) and Ito and Wakamatsu (1994), including a recent modification to diminish the effect of non-melanin-derived background 3-amino-4-hydroxyphenylalanine (Wakamatsu and Ito, 2002; Wakamatsu *et al*, 2002). Eumelanin was obtained by multiplying the amount of pyrrole-2,3,5-tricarboxylic acid, a specific marker of eumelanin, by a conversion factor of 160. Pheomelanin was obtained by multiplying the amount of 4-amino-3-hydroxyphenylalanine, a specific marker of pheomelanin, by a conversion factor of 9 (Ito and Fujita, 1985; Ito and Wakamatsu, 1994). MC1R status was determined by automated sequencing of DNA extracted from whole blood according to previously published methods (Flanagan *et al*, 2001).

Statistical analysis In initial analyses R142H, R151C, R160W, D294H, and frameshifts were classed as diminished function alleles, with all other alleles being classed as WT or pseudo-WT. A subsequent analysis was made including the V60L change as a diminished function allele. Because the distribution of the raw melanin data was skewed, $\log_e$ transformations of the melanin data were used in the subsequent analyses. In keeping with previous studies of the MC1R signaling pathway (Wakamatsu *et al*, 2002), the eumelanin-to-pheomelanin ratio is used as a summary of receptor pathway activity. Log_e eumelanin-to-pheomelanin ratio (LEPR) therefore refers to the ratio of the $\log_e$ of eumelanin to pheomelanin. Statistical testing and graphing were carried out using S-Plus 6.1 for Windows (Insightful Inc., Seattle, WA; http://www.insightful.com/), except for the analysis of the $r \times c$ contingency tables for which StatsDirect (http://www.statsdirect.com/, Cambridge, UK) using the Fisher-Freeman-Halton Test was used.

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