Supplementary material

Methods

Statistical analysis

Mediation analysis

To estimate the independent contribution of *MC1R* variants on CM development, we performed a mediation analysis.[1,2] Thus, we decomposed the overall risk estimate for CM associated with *MC1R* into a direct effect due to the non-pigmentary pathway and an indirect effect due to the pigmentary pathway. We estimated the direct effect of *MC1R* (any variant and the nine single common variants vs wild-type (WT)) on CM in the presence and in the absence of the Red-Hair-Color (RHC) phenotype (controlled direct effect: CDE). The nine most common *MC1R* variants were V60L, D84E, V92M, R142H, R151C, I155T, R160W, R163Q and D294H and were tested with the dominant model, which was found in our previous study[3] as the one with the lowest Akaike's Information Criterion for almost all the studies and variants. Following our previous publication,[4] RHC phenotype was primarily defined as the presence of at least one of the following phenotypic characteristics: red hair, freckles, skin types I and II. We also estimated the natural direct effect (NDE) that, essentially, averages CDE over the population, and finally the indirect effect of *MC1R* mediated by RHC phenotype (natural indirect effect: NIE). The formulas used to calculate each effect were previously described in[1,2] and are as follows. Let Y be the binary outcome, M the intermediate variable, A the exposure and C a set of multiple confounders. The outcome Y can be modelled using logistic regression as:

$$logit\{P(Y = 1|a, m, c)\}=\theta_0 + \theta_1 a + \theta_2 m + \theta_3 am + \theta_4 'c$$

where c is considered a vector and may contain multiple confounders.

A dichotomous mediator M can as well be modelled via a logistic regression as:

$$logit\{P(M=1|a,c)\} = \beta_0 + \beta_1 a + \beta_2' c$$

Provided that the outcome is relatively rare and assumption previously described[2] hold, we can derive CDE, NDE and NIE on the Odds Ratio scale as:

$$log\{OR^{CDE}\} = (\theta_1 + \theta_3 m)(a-a^*)$$

where, for a binary exposure, the two exposure levels being compared would be $a^*=0$ and $a=1$. Thus, the CDE expresses how much the outcome would change on average if the mediator were kept at level m uniformly in the population, but the exposure were changed from level $a^*=0$ to level $a=1$.

$$log\{OR^{NDE}\} = \{\theta_1 + \theta_3(\beta_0 + \beta_1 a^* + \beta_2' c + \theta_2\sigma^2)\}(a-a^*) + 0.5\theta_3^2\sigma^2(a-a^*)$$

where σ^2 is the variance of the error term in the regression for the mediator M. Thus, the NDE expresses how much the outcome would change if the exposure were set at level a=1 versus level a*=0 but for each individual the mediator were kept at the level it would have taken in the absence of the exposure.

$$log\{OR^{NIE}\} = (\theta_2\beta_1 + \theta_3\beta_1 a)(a-a^*)$$

Thus, the NIE expresses how much the outcome would change on average if the exposure were controlled at level a=1, but the mediator were changed from the level it would take if a*=0 to the level it would take if a=1. As it can be seen, when the interaction term θ_3 equals zero, then the natural direct and the controlled direct effects coincide. By following[5], it is useful to report both of them and when the exposure interacts with the mediator to cause the outcome, the estimation of the CDE depends on the value of the mediator itself, whereas the NDE provides a single summary of the direct effect in the study population.

Sensitivity analyses were performed by using different definitions of RHC phenotype, calculated as 1) a score obtained by Multiple Correspondence Analysis,[4] and 2) extreme categories of RHC

phenotype: RHC subjects were defined as subjects with skin type I and at least one characteristics between red hair and freckles (N=265); non-RHC subjects were defined as subjects with skin type IV and brown/black hair and no freckles (N=317). The results of the sensitivity analyses basically agreed with those obtained with the primary definition of RHC phenotype and we therefore present only the results using the primary definition, which is simpler to interpret and replicate and is more powerful than extreme RHC definition. Mediation analysis was separately applied to each of the seven studies and Odds Ratios (OR) with 95% Confidence Intervals (CI) were obtained for Total Effect (TE), NDE, NIE, and CDE using unconditional logistic regression models including the following covariates, when available: age, sex, intermittent and chronic sun exposure, lifetime and childhood sunburns, family history of melanoma, common naevi count and presence of atypical naevi.

Following the two-stage analysis approach, we pooled study-specific ORs with a random-effects model. Intra-study correlation between the study-specific estimates was taken into account with the multivariate approach previously described.[6] We calculated the I-Square to assess the percentage of total variation across studies that is attributable to heterogeneity rather than to chance. Sensitivity analysis and meta-regression were performed to investigate possible between-study heterogeneity. Publication bias was graphically represented through the funnel plot and assessed both by Egger's test and Macaskill's method.[7]

Model comparison

We tested the prediction ability to identify CM participants by adding *MC1R* variants to a clinical base prediction model. Variables included in the base model had been included in most of the CM risk prediction models previously reviewed[8]: age, sex, sunburn, number of common naevi, and

RHC phenotype. These covariates were available in a subset of 4,390 (68%) participants from six of the seven studies. Common naevi were dichotomized basing on their median in M-SKIP controls. Family history was not included because all cases in M-SKIP are sporadic. Information on atypical naevi was not included in the main analysis because it was available only in four studies; however it was included in sensitivity analyses. We used unconditional logistic regression to estimate the risk of CM according to the base clinical risk model and to the model including *MC1R* gene, defined as (1) the presence of any *MC1R* variants versus WT, (2) the presence of only *r* variants and presence of at least one *R* variant versus WT, and (3) the presence of each of the nine most common *MC1R* variants or rarer variants.. *R* and *r* alleles were defined based on their association with the RHC phenotype for the most common variants[4,9-12] and on likely pathogenicity using the algorithm previously proposed by Davies et al.[13] for the less common variants.

We compared the predictive ability of the model with *MC1R* over the base clinical model by using receiver operating characteristic (ROC) curves, Net Reclassification Improvement (NRI) and Decision Curve Analysis (DCA). First, we calculated leave-one-out cross-validated predicted probabilities; the correspondent area under the ROC curve (AUC) with 95% CI were estimated for the two models and compared with the De Long test.[14] Looking at the predictive probabilities of *MC1R* compared to the base clinical model, the category-free NRI[15] essentially quantified overall improvement in model sensitivity and specificity. Finally, DCA was useful to graphically evaluate the net benefit for the models with and without inclusion of MC1R variants: the net benefit was defined as the difference between the proportion of patients who are true positive and the proportion of patients who are false positive, the latter weighted by the relative harm of a false–positive and a false–negative result.[16-18] Stratified analysis by RHC phenotype were performed.

Two-sided tests p-values <0.05 were considered statistically significant. The analysis was carried out by using the software SAS (version 9.2) and Stata (version 11.2).

References

1. Vanderweele TJ, Vansteelandt S. Odds ratios for mediation analysis for a dichotomous outcome. *Am J Epidemiol*. 2010;172(12):1339-1348.

2. Valeri L, Vanderweele TJ. Mediation analysis allowing for exposure-mediator interactions and causal interpretation: Theoretical assumptions and implementation with SAS and SPSS macros. *Psychol Methods*. 2013;18(2):137-150.

3. Pasquali E, Garcia-Borron JC, Fargnoli MC, et al. MC1R variants increased the risk of sporadic cutaneous melanoma in darker-pigmented caucasians: A pooled-analysis from the M-SKIP project. *Int J Cancer*. 2015;136(3):618-631.

4. Tagliabue E, Gandini S, Garcia-Borron JC, et al. Association of melanocortin-1 receptor variants with pigmentary traits in humans: A pooled analysis from the M-skip project. *J Invest Dermatol*. 2016;136(9):1914-1917.

5. Petersen ML, Sinisi SE, van der Laan MJ. Estimation of direct causal effects. *Epidemiology*. 2006;17(3):276-284.

6. van Houwelingen HC, Arends LR, Stijnen T. Advanced methods in meta-analysis: Multivariate approach and meta-regression. *Stat Med*. 2002;21(4):589-624.

7. Macaskill P, Walter SD, Irwig L. A comparison of methods to detect publication bias in meta-analysis. *Stat Med*. 2001;20(4):641-654.

8. Vuong K, McGeechan K, Armstrong BK, Cust AE. Risk prediction models for incident primary cutaneous melanoma: A systematic review. *JAMA Dermatol*. 2014;150(4):434-444.

9. Garcia-Borron JC, Sanchez-Laorden BL, Jimenez-Cervantes C. Melanocortin-1 receptor structure and functional regulation. *Pigment Cell Res*. 2005;18(6):393-410.

10. Duffy DL, Box NF, Chen W, et al. Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. *Hum Mol Genet*. 2004;13(4):447-461.

11. Box NF, Wyeth JR, O'Gorman LE, Martin NG, Sturm RA. Characterization of melanocyte stimulating hormone receptor variant alleles in twins with red hair. *Hum Mol Genet*. 1997;6(11):1891-1897.

12. Sturm RA, Duffy DL, Box NF, et al. Genetic association and cellular function of MC1R variant alleles in human pigmentation. *Ann N Y Acad Sci*. 2003;994:348-358.

13. Davies JR, Randerson-Moor J, Kukalizch K, et al. Inherited variants in the MC1R gene and survival from cutaneous melanoma: A BioGenoMEL study. *Pigment Cell Melanoma Res*. 2012;25(3):384-394.

14. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: A nonparametric approach. *Biometrics*. 1988;44(3):837-845.

15. Pencina MJ, Steyerberg EW, D'Agostino RB S. Net reclassification index at event rate: Properties and relationships. *Stat Med*. 2016.

16. Vickers AJ, Elkin EB. Decision curve analysis: A novel method for evaluating prediction models. *Med Decis Making*. 2006;26(6):565-574.

17. Steyerberg EW, Pencina MJ, Lingsma HF, Kattan MW, Vickers AJ, Van Calster B. Assessing the incremental value of diagnostic and prognostic markers: A review and illustration. *Eur J Clin Invest*. 2012;42(2):216-228.

18. Baker SG, Schuit E, Steyerberg EW, et al. How to interpret a small increase in AUC with an additional risk prediction marker: Decision analysis comes through. *Stat Med*. 2014;33(22):3946-3959.

19. Sturm RA, Duffy DL, Box NF, et al. The role of melanocortin-1 receptor polymorphism in skin cancer risk phenotypes. *Pigment Cell Res*. 2003;16(3):266-272.

Table S1. Natural Direct Effect (NDE), Natural Indirect Effect (NIE), Total Effect (TE) and Control Direct Effect (CDE) of the nine most common MC1R variants on melanoma risk according to Red Hair Colour (RHC*) phenotype.

MC1R variant (R/r)	Allele frequency in controls (%)	N studies	NDE SOR (95%CI)	NIE SOR (95%CI)	TE SOR (95%CI)	CDE non-RHC phenotype SOR (95%CI)	CDE RHC phenotype SOR (95%CI)
V60L (r)	12.7	6	1.30 (0.96-1.77)	1.05 (0.97-1.13)	1.32 (0.97-1.80)	**1.70 (1.07-2.70)**	1.27 (0.80-2.00)
D84E (R)	1.1	5	2.63 (0.70-9.82)	1.51 (0.43-5.28)	**4.52 (1.19-17.11)**	1.77 (0.33-9.40)	2.55 (0.72-9.11)
V92M (r)	8.1	7	1.32 (0.92-1.91)	1.03 (0.89-1.18)	1.38 (0.97-1.96)	**2.16 (1.06-4.44)**	1.04 (0.68-1.61)
R142H (R)	0.8	4	**3.55 (1.21-10.47)**	1.15 (0.59-2.24)	**3.77 (1.19-11.93)**	2.50 (0.42-14.81)	3.14 (0.64-15.45)
R151C (R)	6.4	5	**2.52 (1.57-4.07)**	**1.18 (1.00-1.39)**	**2.65 (1.84-3.83)**	**3.24 (1.24-8.42)**	**2.30 (1.56-3.39)**
I155T (R)	0.6	4	1.68 (0.55-5.16)	1.14 (0.56-2.32)	1.97 (0.67-5.80)	1.13 (0.21-6.11)	1.96 (0.45-8.53)
R160W (R)	7.0	6	**2.22 (1.33-3.71)**	0.94 (0.67-1.31)	**2.06 (1.39-3.05)**	**4.35 (1.69-11.23)**	1.42 (0.88-2.29)
R163Q (r)	4.6	6	1.26 (0.78-2.06)	1.10 (0.92-1.32)	1.44 (0.88-2.35)	1.87 (0.68-5.12)	1.28 (0.69-2.34)
D294H (R)	1.5	5	**3.45 (1.26-9.45)**	0.81 (0.34-1.95)	**2.94 (1.44-5.98)**	3.75 (0.98-14.32)	1.85 (0.75-4.52)

Notes: Control Direct Effect (CDE) estimates the direct effect of *MC1R* on melanoma in the presence and in the absence of the Red-Hair-Color (RHC) phenotype; Natural Direct Effect (NDE) essentially averages CDE over the population; Natural Indirect Effect (NIE) estimates the indirect effect of *MC1R* mediated by RHC phenotype; Total Effect (TE) is the overall melanoma risk estimate for *MC1R* variant carriers and in each study it is the product of NDE and NIE.

"R" and "r" alleles were respectively defined basing on their stronger or weaker association with the RHC phenotype for the most common variants[4,9-11,19] and on likely pathogenicity using the algorithm previously proposed by Davies et al.[13] for the less common variants.

Significant results are in bold.

Abbreviations: CI=Confidence Intervals; SOR=Summary Odds Ratio

* Defined as the presence of red hair, freckles or skin type I/II.

Table S2. Odds Ratios with 95% Confidence Intervals for melanoma risk according to a base clinical model and the same model with inclusion of MC1R variants

	ALL PARTICIPANTS (N=4390)		RHC PARTICIPANTS (N=2654)		NON-RHC PARTICIPANTS (N=1736)	
	Base model	**Base model+MC1R**	**Base model**	**Base model+MC1R**	**Base model**	**Base model+MC1R**
Age[a]	0.97 (0.94-1.00)	**0.97 (0.94-0.99)**	**0.96 (0.92-0.99)**	**0.95 (0.92-0.99)**	0.99 (0.94-1.04)	0.99 (0.94-1.04)
Sex						
Male	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Female	1.01 (0.89-1.16)	1.05 (0.91-1.20)	0.92 (0.77-1.09)	0.92 (0.77-1.10)	1.18 (0.96-1.45)	**1.25 (1.02-1.55)**
Sunburn						
None	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Any	1.15 (0.98-1.35)	1.08 (0.92-1.27)	1.16 (0.94-1.43)	1.09 (0.88-1.35)	1.13 (0.88-1.47)	1.07 (0.82-1.39)
Common naevi						
≤30	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
>30	**3.37 (2.90-3.92)**	**3.43 (2.94-4.00)**	**3.46 (2.86-4.18)**	**3.60 (2.96-4.38)**	**3.25 (2.53-4.16)**	**3.24 (2.52-4.16)**
Phenotype						
Non-RHC	1.00 (reference)	1.00 (reference)	-	-	-	-
RHC	**1.64 (1.43-1.88)**	**1.33 (1.16-1.54)**	-	-	-	-
MC1R						
None	-	1.00 (reference)	-	1.00 (reference)	-	1.00 (reference)
V60L	-	**1.24 (1.06-1.46)**	-	1.14 (0.93-1.40)	-	**1.45 (1.11-1.89)**
D84E	-	1.35 (0.90-2.03)	-	1.28 (0.82-2.00)	-	1.79 (0.67-4.77)
V92M	-	1.16 (0.97-1.40)	-	1.01 (0.79-1.27)	-	**1.43 (1.07-1.92)**
R142H	-	**1.90 (1.12-3.21)**	-	1.65 (0.90-3.01)	-	2.89 (0.99-8.42)
R151C	-	**2.09 (1.75-2.51)**	-	**2.11 (1.70-2.62)**	-	**2.09 (1.48-2.94)**
I155T	-	**2.11 (1.22-3.64)**	-	**2.79 (1.40-5.55)**	-	1.11 (0.42-2.95)
R160W	-	**1.63 (1.35-1.97)**	-	**1.44 (1.15-1.80)**	-	**2.10 (1.42-2.94)**
R163Q	-	1.13 (0.90-1.42)	-	1.12 (0.83-1.52)	-	1.13 (0.79-1.64)
D294H	-	**2.20 (1.56-3.10)**	-	**1.91 (1.28-2.86)**	-	**3.13 (1.60-6.13)**
Other rare variants	-	**2.71 (1.89-3.87)**	-	**3.41 (2.22-5.24)**	-	1.35 (0.65-2.78)

Notes: ORs with 95%CI above unit are in bold. All models are adjusted for variables included in the table + study center.

[a] per 5 years increase.

Abbreviations: RHC, Red Hair Color.

Table S3. Statistical measures to evaluate the incremental value of *MC1R* to a base clinical risk model

Risk model	AUC (95%CI)	Change in AUC from base model (%)	p-value (change AUC)	NRI (95%CI) (%)
ALL SUBJECTS (N=4390)				
Base[a]	0.706 (0.691-0.721)	-	-	-
Base + *MC1R* (any variant)	0.713 (0.698-0.728)	0.7	0.002	24 (20-30)
Base + *MC1R* (r or R variants)	0.721 (0.707-0.736)	1.5	<0.0001	37 (32-43)
Base+ *MC1R* (single variants)	0.726 (0.711-0.740)	1.9	<0.0001	34 (28-39)
RHC SUBJECTS (N=2654)				
Base[b]	0.695 (0.675-0.715)	-	-	-
Base + *MC1R* (any variant)	0.700 (0.680-0.720)	0.5	0.03	15 (9-22)
Base + *MC1R* (r or R variants)	0.710 (0.690-0.729)	1.5	0.0005	33 (25-40)
Base+ *MC1R* (single variants)	0.720 (0.700-0.739)	2.5	<0.0001	33 (26-40)
NON-RHC SUBJECTS (N=1736)				
Base[b]	0.678 (0.653-0.703)	-	-	-
Base + *MC1R* (any variant)	0.696 (0.672-0.721)	1.8	0.0008	28 (19-37)
Base + *MC1R* (r or R variants)	0.704 (0.680-0.729)	2.6	<0.0001	12 (3-22)
Base+ *MC1R* (single variants)	0.704 (0.679-0.728)	2.6	<0.0001	24 (14-33)

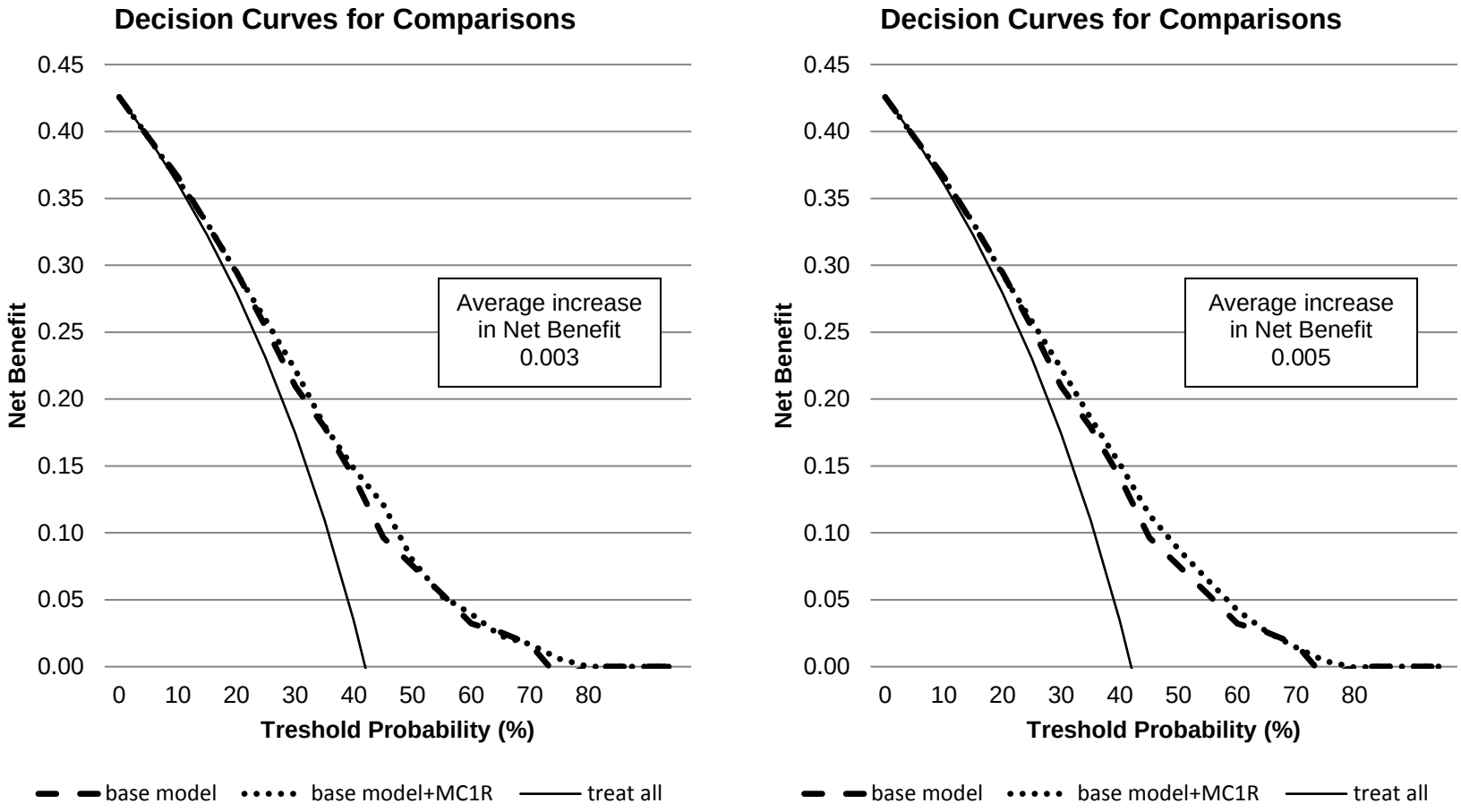
Notes: "R" and "r" alleles were respectively defined basing on their stronger or weaker association with the RHC phenotype for the most common variants[4,9-11,19] and on likely pathogenicity using the algorithm previously proposed by Davies et al.[13] for the less common variants.

[a] Included the following variables: study center, age, sex, sunburn (none/any), common naevi (≤30/30+), phenotype (non-RHC/RHC)

[b] Included the following variables: study center, age, sex, sunburn (none/any), common naevi (≤30/30+)

Figure S1. Decision curve analysis of the effect of prediction models for cutaneous melanoma for non-RHC subjects. Model with *MC1R* variants is plotted against model without *MC1R* variants and treat all. *MC1R* was defined in model represented in a) as presence or absence of any *MC1R* variant and in model represented in b) as no *MC1R* variant, only "r" variants, and ≥ 1"R" variants. Average increase in net benefit for the model with *MC1R* compared to the base model is reported.

(a) **(b)**



Notes: "R" and "r" alleles were respectively defined basing on their stronger or weaker association with the RHC phenotype for the most common variants[4,9-11,19] and on likely pathogenicity using the algorithm previously proposed by Davies et al.[13] for the less common variants.